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SURFACE DESIGN TECHNIQUES AND EMBELLISHMENTS FOR SUSTAINABLE LEATHER PRODUCTS

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India has the largest number of livestock in the world.Since several years particularly from 80s, manufacturing of Leathers, Leather products and Footwear have become a major contribution to the world economy. In today's era leatherindustry is undergoing a complete change through modern leather processing techniques,where sustainability has crawled in every field and aspect of life. As known, the natural animal skin or hide undergoes various chemical processes and mechanical operations to be converted into fine leather. Being made from the natural material and also due to the influence of mechanical, chemical and physical treatments a remarkable portion of the materials gets defected during the conversion. These defected materials are rated as poor selections and rejections and are disposed as wastes or sold for very lower prices. Up gradation of these defected and waste leatherscan help in reducing the waste and resulting in the efficient utilization of unused leather. In this context, the researchers present this work which deals with the up-gradation of leathers that can be achieved through different design techniques, decorations and surface finishing's on these faulted leathers. Surface design techniques and embellishments like appliqué, crochet, knitting, typography and pyrography including aesthetic surface finishing like printed, embossing,painting,mosaic and few moretechniques which can hide these defects onthe lower selections and rejected leathers. These upgraded materials are then applied for designing of leather products and life style fashion accessories with the standardization of quality and providing value addition to final leather products to ensure sustainability in leather product development and marketing them as commercially affordable products.

The research work also give suggestions and design concepts to combine the leather with other eco-friendly fabrics like jute, denim and canvas for making creative lifestyle products with an objective to reuse leather with natural materials for providing a sustainable environment.

Keywords: Surface Design, Leather Embellishments, Typography, Pyrography, Mosaic, Leather Combinations, Sustainable Products

Introduction

The Indian leather industry has a huge domestic market and also great potential for exports; it holds a noticeable position in the Indian economy .Finished leather products are known for their royalty and neatness. The issue of sustainability has gained considerable attention among leather industry customers, consumers and the community at large. As the world is becoming globally sustainable and eco – friendly leather industry has also become leather conscious.

This paper acknowledges about different ways how leather products can be sustainably designed, it focuses on different designs techniques, value addition and surface embellishments on leather.

Craft techniques like leather mosaic, crochets, knitting, appliqué, reverse appliqué and leather combinings can give leather a new life .Surface development techniques and value addition helps in upgrading the rejected leathers and reusing leather scraps.

Many designers like Victoria Ledig and Jorge Penades are becoming sustainably conscious .Currently they are ideals for the leather sector thus alarming and awaking them about the potential of durable material like leather.

Literature Review

The leather industry holds prominent place in the Indian economy, it is known for its consistency in high export earnings and is among top 10 foreign exchange earners for the country, with an annual turnover of US$7.5 billion .Bestowed with an affluence of raw materials this industry has added strengths of skilled man power, innovative technology, increasing industry compliances international environment standards and the dedicated support of allied industries.

The leather manufacturing process is divided into three sub – process -: preparatory stages , tanning and crusting producing finished and value added products .Hides and skins are downgraded due to various ant mortem and post mortem defects , including poor animal husbandry , disease and parasites, bad slaughtering and flaying techniques , during collection and transportation . Estimate from the tanneries have put the percentage of rejected skin at certain time of the year as high as 50 to 60 percent .The amount of leather scraps released from this sector sometimes doubts the sustainability of leather, thus to balance the sustainability of this sector rejected skins can be upgraded by value addition techniques or silica gel can be used to make a new pelt out of them .Leather scraps too can be recycled and repurposed for a long

Research Gap

As we move towards a post – industrial world, ideas and intellect will lead as to a new renaissance in design where the beauty of craft and skill of labour are equally important. A durable material like leather can hold a second life, where the value added leathers and leather scraps can be fused with modern materials and products that can evoke learned heritage and quiet sobriety. Everyday modern classics adopt a timeless and amart style using components such as wood , resin ,ceramic ,smoked glass and textiles .These designs are sharpened up through a considered and graphic   balance of material and form , featuring simple color combinations .

Research Objectives

Sustainability is becoming an increasingly important issue in the leather industry. Present innovations in leather production must bring economic interest, economical soundness and consumer benefit into line. Achieving this balance and turning into valuable asset needs expert skill and labour. The main objectives focused in this paper are -:

- ➤ Repurposing leather scraps.
- ➤ Upgrading of the rejected skins.
- ➤ Using techniques like leather mosaic, crochet, leather combining and pyrography to reuse leather scraps.
- ➤ Recycling leather scraps.

Research Methodology & Implementation

Leather is a dead animal skin perhaps this factor makes it more fascinating, beautiful, precious and grotesque at the same time .Leather products now have entered in global fashion world and lifestyle of every individual .Leather scraps and leather from old bags can be used in various creative ways ways as a embellishments on another products and can be repurposed to make essential items . Lifetime use of leather not only proves its durability but sustainability too. This research paper focuses on examples how designers from various techniques reuse and repurpose leather scraps.

1) PRECIOUS SKIN - VICTORIA LEDIG

Precious skin is a unique collection of handbag made from unusual cow body parts considered as waste . Victoria Ledig is a petite , attractive and immaculately presented , from her precarious heels to her perfect black bob   discovered a primal relationship between waste leather and accessories .In her dedication to her graduation project at Dutch design academy at Eindhoven , she was fascinated with leather as a material while she was doing her internship in ECCO Leathers , in the design department of a tannery in dungeon in the Netherlands where she got acknowledged about the wastage of cow parts , she wanted to use leather in her final project . Victoria in her final project portrays the true meaning or her application of precious skin it was once living skin of animal not a flat sterilize material it is wrinkled, gifted with lots of beauty in real skin of cow.

CONCEPT DEVELOPMENT

The new collection of bag aim to reconnect people with the material's origin and natural beauty. This project started as a material challenge and an experiment by the designer in her final project .She started with the keen observation at the ECCO tannery that the only skin from the torso of the cow is used in the leather industry visit to slaughter house she saw how the left out animal skin is wasted. Satisfied with the process, Victoria procured skin from cow's ear, face, tail and hooves that are discarded from the leather production and used less valued things like dog food .The fresh skins in the start a bit like curiosity. She started with the tanning of various parts into the tanning drums and ended up with beautiful vegetable tanned leather pieces. She made desirable and functional items from these pieces of leather. The dried leather was beautifully converted into a collection of bags its simply portrays the leather of all the imperfections and texture in the different skins. The work is not accommodation but a

awareness to the consumers through her accessories made by the pieces that are normally discarded by the industry.

1) LEATHER MOSAIC

A mosaic is a piece of art or wood made from the assemblage of small pieces of colored glass , stone or other materials .It is often used in decorative art or interior decoration , made from small , flat , roughly square , pieces of stone or glass of different colors , known as tesserae. Mosaics have a long history they were started by Mesopotamians. Pebble mosaics were made in Tiryns in Greece.

Leather mosaics are a piece of art made from the assemblage of small pieces of leather. Egyptian used leather mosaics in their furniture due to the scarcity of wood. Wood was considered as a royal material in Egypt but due to the hot weather of Egypt wood was imported from other states , leather was the only material that could competitive the royalty of wood thus ottoman leather mosaic chairs was a royal furniture in Egyptian kingdom .

Leather mosaics can be made by applying different methodologies and techniques on the waste leather scraps as follows –:

   a) Appliqué – pieces from leather scraps are cut and sewn onto the category product.
   b) Reverse appliqué – A sewing craft in which the outline is cut from the top layer of the leather and the raw edges of leather are turned under and and stitched to expose one or more layers of leather underneath.

Most of the times pieces from leather scraps or rejected leather are cut in a desired shape and sewn together.

2) PYROGRAPHY –

It is the art of decorating wood or other material like leather with burn marks resulting from the controlled application of a heated object such as a poker which gives different tones of shades..It can be

performed using any heated metal implement.the equipment used has to be heated for five minutes. Solid point burners are equivalent to soldering iron.

3) CROCHET -:

Crochet is a process of creating fabric by interlocking loops of yarn, thread or strands of other materials using a crochet hook. The word crochet means small hook. Originated from Europe it is a species of knitting. Products like sweaters, bags, clutches and many more accessories can be made by crocheting. The most important factor for this technique is flexibility and felting which is provided by leather too due to which leather crochets are currently in trend.

4) KNITTING

It is a method of manipulating yarns. Knitting creates multiple loops of yarns.

Needles and cables are used for knitting they can be from 10 to 15 inches. Knitting is a technique for producing a two-dimensional fabric made from a one-dimensional yarn or thread. In weaving, threads are always straight, running parallel either lengthwise or crosswise

5) LEATHER COMBININGS

If natural fabrics are mixed with leather they give a classic to formal look. Mixing leather with natural fabrics like cotton , canvas , velvet and denims not only gives it a different shape , cut texture but royalty also .Cut out from leather scraps can be used to make these mixings.

Leather and canvas

RECYCLING LEATHER SCRAPS

Structural skin is a self produced material made out of the leftovers and off cuts from the leather industry .Spanish designer Jorge Penades came up with a new way to recycle leather, he discovered this during his trip to Japan in 2014, this piece is inspired by boro – a traditional Japanese folk textile .These fabric represents two essential principles of Japanese culture ; motttainai a term that conveys a deep sense of regret concerning waste and Boroboro that celebrates the beauty in something frayed, decayed or repaired. Penades accommodated his new concept through structural skin in which he wanted to come up with a new way to recycle leather after becoming aware of the amount of the leftover material from the car , fashion, shoe and furniture industries.

Born in Spain , Jorge Penades originally studied Interior Design in Barcelona , before graduating with a master's degree in experimental design and conceptual thinking .Through a growing interest in the intangible aspects of the discipline , he sets up an independent Madrid-based office for experimental ideas called officinal Jorge Penades (OJP).Structural skin which is a range of furniture products made out of the innovative use of discarded scraps of leather. Penades has constructed the leather scraps into strong bars which are transformed into pieces of useful furniture like dumb valets and small tables.

Concept development -:

Penades getting fascinated with leather as it is the first human clothing , he collected leather scraps from various leather garments and leather products manufacturing units after this he did the procedure in following steps -:
Penades first turned the leather into strips by feeding sheets of the material through an office-style paper shredder .It is a heavy duty shredder of 48kg.After this animal glue crystals made from animal bone which are readily available in the market used by many of the furniture industries is put in bain marie. So he chooses to use animal bones which are byproducts of the meat industry. These animal glue crystals are are melted in bain marie which is a type of heated bath. .Penades used a liquid filled Bain Marie but traditionally is a wide ,cylindrical, usually container that holds the working fluid and an smaller container that holds the animal glue ,the bain marie is then heated where the inner container is immersed halfway into the working liquid ,the maximum temperature of the glue should not go more than 100 degree Celsius or 212 Fahrenheit .
After this the shredded strips are mixed with the melted glue. The glued steps are then stuffed into iron molds compressed and then left to set .When solidified the top layer of the leather is shaved to reveal marble like pattern created by the strips.To finish the material he used shellac a natural resin that comes from an insect. Shellac is a resin secreted by the female lac bug found on trees in the forest of India and Thailand, processed as dry flakes it is mixed with ethanol to liquidize it which can be used for glaze. The piece is slightly flexible but pretty stiff..Each strip is then fixed together and circular brass plates are added to turn the structures into functional tables.

Results & Discussion

Surface design technique and embellishments on leather products can increase the sustainability of leather industry which is a important issue.

Repurposing and recycling leather scraps increases the sustainability of leather and makes it more ecofriendly, techniques like leather mosaic , crochet , pyrography and leather combining are embellishments with are affordable within cheap cost.

Knowledgeable design research of designers like Victoria Ledig and Jorge Penades proves leather as a material with lots of potential and competitive surface material .Using methods like vegetable tanning and materials like resins which cost low environmental impact make leather more reliable and eco friendly. Many leather industries are working with these designers to make leather a sustainable element.

Conclusion

Leather is a beautiful and antique material to experiment with; being a neutral palette material still it allows the industry to play with different techniques on it due to the flexible collagen fibers in this durable material.

Leather can be value additioned with various finishings. This paper was a very interesting era which gives you a opportunity to learn more about the characteristics of interesting material like leather .Leather forecasting gives a positive welcome to this material in coming years .

Due to various embellishments and value additions on leather it can survive with second life, with this kind of specialty leather has almost entered as a sustainable material

Acknowledgement

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THE EFFECT OF COMBINATION VEGETABLE TANNING MATERIALS AND SULFITED OIL ON THE PHYSICAL PROPERTIES OF JACKET LEATHER

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This research was aimed to determine the combination of vegetable tanning materials and Sulfited oil on the physical properties namely tensile strength, oil content, elongation at break, softness and shrinkage temperature and to determine the factors that affect tensile strength, oil content, elongation, softness and shrinkage temperature leather tanning results. Raw hides/skins were processed up to acidification, pH was set to be 5 then tanned with the variation of vegetable tannin 15%, 17.5% and 20% respectively. Fatliquoring process used sulfited oil with variation of 12%, 15% and 18%. The jacket leather produced were tested using SNI 4593:2011 Kulit Jaket domba/kambing. From this combination of treatment provided 9 (nine) treatment variation, each treatment was preformed triplicate. The results showed that the combination of vegetable tanning materials 17.5% and 12% of sulfited oil was the result of variation in the optimum treatment for the leather to produce 208.29 N/mm^2 tensile strength, oil content of 10.02%, 50.39% elongation and softness 5.13 mm and meets the requirements of SNI: 4593: 2011 with the temperature shrinkage was at 79 ^{o}C. From the analysis of variance showed vegetable tanning material is the dominant factor affecting the tensile strength, oil conten and shringkage temperature. Sulfited oil is a factor that affects the oil content, elongation and softness.

Keywords: tensile strength, leather, jacket

Introduction

The products made from leather is now growing rapidly, along with the development of human civilization. Today, oneof the featured products in Indonesia is the leather jacket. The leatherjacket is consumed both for domestic use and for export. The Statistical data shows that domestic consumption jacket around 240.000 pieces and for export consumption reached at 10.000 jackets (1 jackets require ± 30-40 sqft leather). Finished leathers have an exclusive properties, it is different with the artificial leather. Leather is not only strong, durable but also has a unique porous structure so that it can "breathe". It means that air and water vapor can pass through the tissue so that when worn as clothing /jackets is comfortable (Purnomo, 1985). Generally, the leather jacket is still made using chrome tanning materials.

The objective of tanning process is to create a bond between the fibers, modifying the chemical structure of the fiber skin, increase the hydrothermal stability, increase the physical endurance of the

skin, and resistance to environmental factors such as bacteria, chemicals. The chrome tanning besides have many advantages in the finished product, but also has a disadvantages, especially on the waste produced. The chrome tanning produces 30-40 m^3 wastewaterper tone of raw skin. The chromium content in the wastewater cause environmental problems because chromium is one of hazardous materials. The chromium has properties such as toxic, carcinogenic, and mutagenetic (Kanagaraj, et. all, 2008)

Along with the increasing public awareness of the environment, recently consumers demand eco-friendly products. Leather industry attempted to use a substitute chrome tanning materials. It is necessary to do a research for eco-friendly tanning materials especially for leather jackets, one of eco-friendly tanning materials is vegetable tanning material or in combination with other non-chrome tanning materials.

Vegetable tanning material has been used for tanning leather pads machine, leather soles, leather luggage bags, belts and other leather goods. Because the vegetable tanning materials derived from wood, the leather will have a properties like wood that is solid, rigid and hard, so It would be difficult to manufacture softy leather. The vegetable tanning materials is biodegradable material (can be degraded by microorganisms), or in the other words environmental friendly. Meanwhile, for the manufacture of leather garmen/ jacket required softness, smooth, padded and elongation enough to be able to follow the movement of the body when worn as a jacket or gloves.

The process aims to put the fatliquor oil molecules in space that exists between the fibers of the skin and serves as a lubricant. Oil or fat will change the properties of the skin becomes softer, tough, stretchy, pleasant handle, and smooth surface over its nerf (Purnomo, 2002). Fatliquoring also aims to pave the fibers of the skin so the leather have high tensile strength and elastic when it is curved-bend and can make the leather fibers will not stick to one another and minimize leather against water absorption (Rachmi, 1992). Oil or fat is an important component in the skin that serves to soften the skin or as a skin tissue lubricant in leather tanning process (Sivakumara, et al., 2008). The function of the oil fatliquoring process is to control the difference between the grain shrinkage during the drying process with the corium leather (Etherington and Roberts, 2011). The amount of oil that is used to process leather jacket 10-20%. During fatliquoring process, oil and skin tissue molecules will bind physically stronger than the bond between the oil and emulsifier, so it will make difficult the migration of oil from the leather. Generally, on fatliquoring process using oil such sulfited oil, derived from fish oil, animals and vegetables.Leather tanned with vegetable tanning agent has the properties of solids, stiff and hard but environmentally friendly waste generated, while the leather garment requires a limp nature, soft and stretchy, so It is necessary to do this research.

Materials And Methods

Materials

Raw materials for this research is pickle sheep skin obtained from Yogyakarta, Indonesia. All chemicals used for leather processing were technical grade such as wetting agents, formic acid, sodium chloride, sodium bicarbonate, mimosa powder, white syntan, sulfited oils and anti-fungal. Research tools such as

tanning drum brands Otto Specht No. Series 80304, toggling, tensile strength test tool brand ZwickRoell ZO20 KAP- type TC 4170 series 07 made in Germany.

Methods

Pickle sheep skin was shaved to obtain a skin thickness between 0.5 mm - 0.7 mm and then added wetting agent , adjust the pH using a basic salt to obtain a pH of 5. The next process is tanning on the drum with the addition of variation vegetable tanning materials as much as 15%, 17.5%, 20% and rotated for 3 hours until the shrinkage temperature minimum at 70° C. Furthermore, fatliquoring process is carried out using a variationsulfited oil as much as 12%, 15%, 18% in the tanning drum for 2 hours then fixed by adding formic acid to obtain pH solution at 3.8 to 4.2

Physical testing

Samples for various physical tests from experimental and control lining leather were obtained as per SNI 06-0642-1989 and SNI 06-0643-1989 methods. Specimens were conditioned at temperature of (20$\pm$2) ^{o}C and RH of (65$\pm$2) % for 48 hrs. Tensile strength and percentage elongation (elongation at break) were measured as per standard procedures (SNI 06-1795-1990) and shrinkage

temperature (SNI 06-7122-2005). Quality requirement for the leather jacket based on SNI 06-4593: 2011: Leather Jackets sheep / goat.

Results And Discussion

Skin has the physical properties and chemical composition of different, physical properties include physical strength and structure of the skin (Kanagy, 1971). The physical properties and chemical composition determines the quality of finished leather, according to Djojowidagdo (1987), to determine the quality of the leather, it can be done by physical, chemical and organoleptic testing. The physical properties of the leather is the skin resistance to mechanical influences, humidity and room temperature. Physical strength can be measured quantitatively such as tensile strength, elongation, temperature shrinkage and softness.

Tensile strength

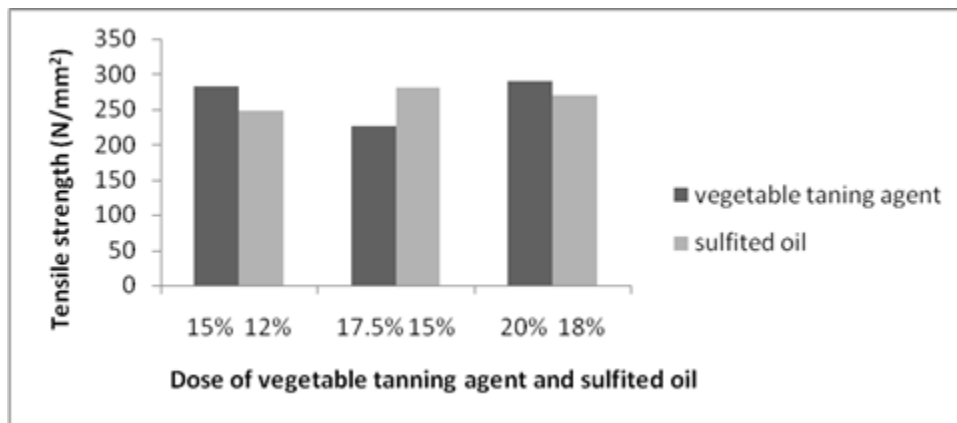Tensile strength test results as presented in Figure 1.

Figure 1. Effect of the percentage of vegetable tanning materials and oil sulfited on the tensile strength

Figure 1 shows that the tensile strength increasing with the addition of tanning agent and oil. This is presumably due to the large percentage of tanning agent increasing molecular size of tanning agent, so that the tanning agent absorbed by the skin and bind to collagen. Furthermore, It increases the bonding fibers into compact structure and resistant to mechanical actions such as traction. Pahlawan, IF and Emiliana K. (2012) states that the tensile strength properties of the leather reflects the stronger bonds between the collagen fibers with tanning agent. Fahidin and Muslich (1999) states that the greater of size molecules tanning agent are getting increasingly absorption of the tanning agent to skin fibers. Vegetable tanning agent will react with collagen and further enhance the bond the fibers of the skin and make the structure of leather is compact.According to Wilson (1941) the high concentration of the tanning agentin the solution increasing the diffusion of tanning agent, therefore the higher the concentration of tanning agent (tannins) increasing amount of tannins that are bound by collagen. Furthermore O'Flaherty (1965) states that the more tannins are bound in the skin causing a tensile strength of leather will be higher.

The tensile strength is influenced also by the process of fatliquoring, because sulfited oil that serves as lubricant will make the fibers of the leather becomes soft, flexible and resistant to the pulling force. Fatliquoring process is a very complex process that depends on many factors and may affect the physical properties of leather such as tensile strength, tear strength, and softness (Sivakumara et al., 2008). At the same time sulfited oil also impact the physical properties of leather, such as tensile strength, waterproof, tear and moisture resistance as well as air and water absorption (Herawati, 1996).

Elongation

Elongation is the length leather when stretched to breaking divided by its original length and expressed as a percent. Tensile strength/ elongation creep show the ability of the leather, the longer the size of the leather at break. Elongation test results are presented in Figure 2.
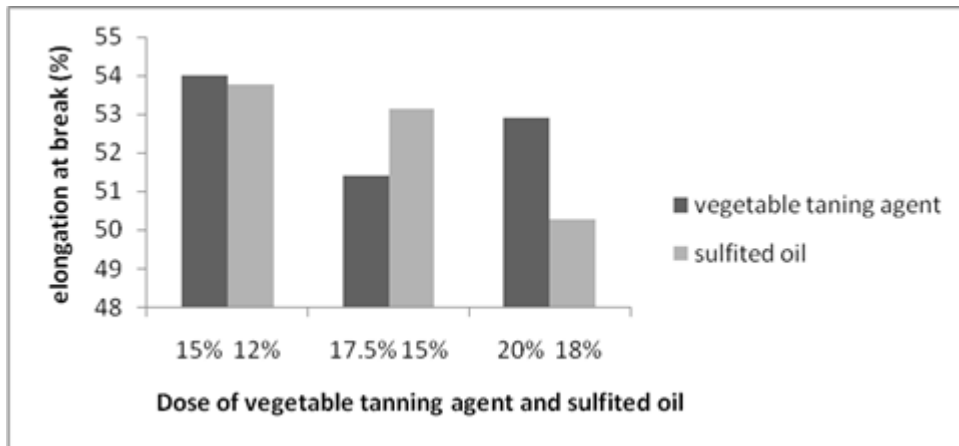
Figure 2.Effect of addition of vegetable tanning materials and sulfited oil on the elongation

Figure 2shows that the elongation tends to decrease with the increasing of the concentration of vegetable tanning agent, this could be caused by the skin tanned with higher concentrations of vegetable tanning agent produces more rigid leather. The more rigid of the leather make the elongation is decreased. According to Purnomo (1985) , vegetable skin tanned has the properties such as rigid, solid and it has a low elongation break. The low elongation on vegetable skin tanned caused by increasing bonding fibers of the skin with vegetable tanning agent, and changes in skin fibers into a compact structure. The compact structure of the skin that inhibits the penetration of oil as a relaxant, causing the skin to become stiff and low elongation.

Figure 2 also shows that the elongation decreased with increasing percentage of sulfited oil, it is apparently due to the increasing concentration of sulfited oil to make an emulsion more viscous, or in other words, oil is not emulsified evenly, consequently sulfited oil is absorbed into the skin so low that the skin becomes less supple or tends to be rigid, rigid skin has a low elongation. Leather elongation level is influenced by factors of lubrication in the final process of tanning. The amount of oil and emulsifier determines the quality of fatliquoring (Mann, 1981). Less precise concentration will cause physical strength of leather decreased (O'Flaherty et al., 1978). If the skin fibers lubricated by oil emulsion only a little, It will produce a low value of elongation at break (Oetojo, 1996).

Softness

The tanning using vegetable tanning agent produce rigid skin. In accordance with the opinion of Purnomo (1985) that the vegetable skin tanned will provide results that are less heat resistance, little stiff, supple, compact, the brown color , and high tensile strength. From the observation it appears that the vegetable skin tanned is still solid, it cause space for sulfited oil to lubricate the skin fiber is decreased, so the leather tends to be low elongation. Softness test results as presented in Figure 3.

Figure 3 shows that softness tends to decrease with increasing of oil sulfited addition, it indicates that the penetration of sulfited oil into the leather is not perfectly during fatliquoring process, so the skin tissue is less lubricated. Thorstensen (1985) states that the use of oils that can affect the physical properties of leather such as tensile strength, the strength of sewing, pleasant handle, softness and the

use of excessive oil will make the leatheris supple, but if the amount is less/oil absorption inappropriately will result in hard leather and can crack when applied to the leather goods.
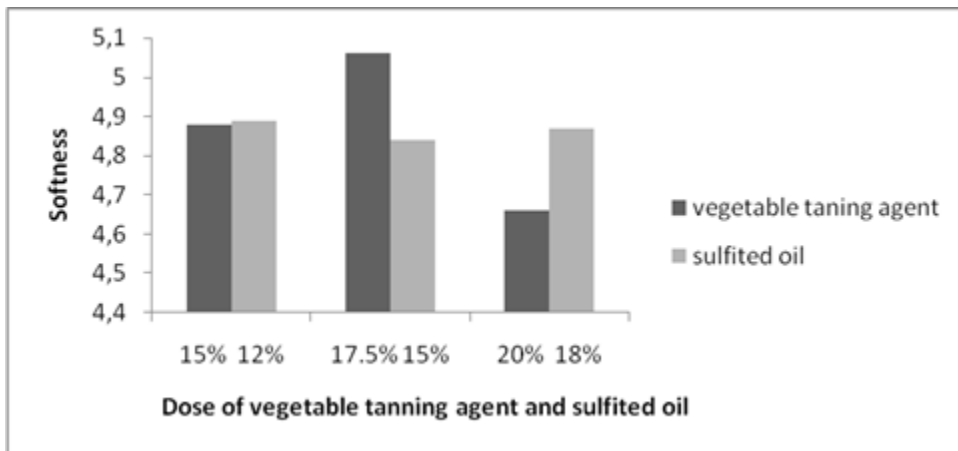

Figure 3. Effect of the addition ofvegetable tanning and sulfited oil on the softness

Shrinkage Temperature

Shrinkage temperature test results as presented in Figure 4. Figure 4 shows that the shrinkage temperature increases with the addition of vegetable tanning agent percentage. This indicates that the penetration of tanning agent and binds to the skin form bonding fibers more compact, thus increasing the heat resistance.The shrinkage temperature is the temperature at which shrinkage occurs collagen structure. Shrinkage occurs because polypeptide chain folded due to the broken strength fibers woven by extreme conditions (eg heating) (Sarkar, 1995). The shrinkage temperature is the temperature that can cause damage and likely to cause deterioration in the connective power of substances contained in the protein (Ayufita, 2007). In leather, vegetable tannin molecules form multiple hydrogen bonds with collagen and make tanning polyphenol bonding matrix. The shrinkage temperature (Ts) of vegetable tanned leather in the range of 70-85°C (Covington, 2009).

Figure 4also shows that shrinkage temperature increases due to rising of sulfited oil addition, it indicates that the oil can lubricate the fibers skin that inhibit the propagation of heat, the more oil will lubricate the skin so the longer the propagation of heat, consequently the shrinkage temperature also increased. Thorstensen (1985) states that the use of oils that can affect the physical properties of the skin tanned.
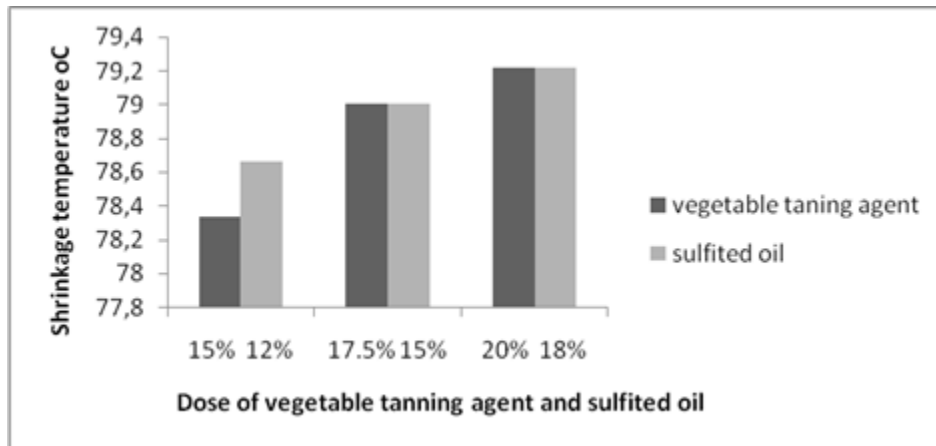
Figure 4.Effect of the addition of vegetable tanning and sulfited oil on theshrinkage temperature

Conclusion

The combination of 17.5%vegetable tanning agent and 12% sulfited oil is the result of variations in the optimum treatment for the skin to generate tensile strength 208.29 N /mm^2, the oil content of 10.02%, 50.39% elongation and softness 5.13 mm and meet the requirements of SNI 06-4593-2011 Leather jacket lamb/goat with a shrinkage temperature of 79°C .

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PHYSICOCHEMICAL PROPERTIES OF COLLAGEN ISOLATED FROM LUMPFISH SKIN IN COMPARISON WITH PIGSKIN COLLAGEN

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As the main component of leather, collagen has been extensively utilized in food and cosmetic industries due to its excellent biocompatibility and safety. In this paper, lumpfish skin was used as raw material to extract acid soluble collagen (ASC) and pepsin soluble collagen (PSC) with triple helical structure by two different extraction approaches. For comparison, collagen (PPSC) was extracted from pig skin using a pepsin digestion method. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern revealed that ASC and PSC were typical type I collagen, consisting of α_1, α_2 and β-chains. Fourier transform infrared (FTIR) spectra of ASC and PSC were observed and suggesting that pepsin hydrolysis did not affect the secondary structure of collagen, especially triple-helical structure. For ASC and PSC, the isoelectric points (pIs) were recorded at pH 5.33 and 4.71 respectively, which supposed to be correlated with acidic and basic amino acids residues. the pIs of PSC and PPSC were lower than that of ASC, this might be attributed to pepsin cleaved the telopeptide region, resulting to difference in amino acid composition in α-chains. The denaturation temperatures (T_d) from the thermal denaturation curve were calculated to be 17.9 ^{o}C and 17.5 ^{o}C respectively. The T_d of ASC, PSC were significantly lower than that of PPSC (37.9 ^{o}C). The results further support that the thermal stability of collagen is correlated with the environmental and body temperature of fish. This research suggests that the outer skin of lumpfish could serve as an alternative raw material of collagen.
Keywords: Physicochemical properties, Lumpfish skin collagen, Pigskin collagen

1. Introduction
The outer skin of mammal is the main raw material of leather industry, such as, bovine hide, pig skin, sheep skin, etc. Furthermore, a variety of fish skins is also used to produce leather because of its structural characters of layer. Main fish skin products are belts, accessories and art crafts welcomed by the consumers. However, since the area of fish skin is small, fish skin leather only accounts for less than 0.1% of the total leather. Collagen is the most abundant and widespread structural protein in vertebrates, constituting about 30% of the total protein of most organisms (Woo et al. 2008). Collagen is a predominant component of extra cellular matrix (ECM), widely distributing in skins, bones, tendons, etc. To date, at least 29 different types of collagen have been identified in vertebrates, and named as

type I-XXIX (Sinthusamran et al. 2013). Among them, type I collagen is the most common one, accounting for more than 90% of the total collagen. Collagen has a wide range of applications in biomedical, cosmetic, food and pharmaceutical fields Wang et al. (2007), due to its biodegradability, low immunogenicity, haemostaticity and biocompatibility properties, which can be used as vitreous implants for the eye, skin substitutes, as carriers for drug delivery, production of wound dressings (Senaratneet et al. 2006; Singh et al. 2011).

Generally, collagen is isolated from the skins and bones of the livestocks, such as cows and pigs. However, the outbreaks of bovine spongiform encephalopathy (BSE) and foot and mouth disease (FMD) have casted restrictions on the use of collagen from these animals (Huang et al. 2011). In addition, as a result of religious constraint, any pig and cow related products cannot be used in some areas of the world (Kittiphattanabawon et al. 2010). Searching the alternative sources of commercial collagen appears to be highly demanded. In recent years, byproducts of fish have received more and more attention as the substitute for collagen from mammals because of its abundantity. (Kittiphattanabawon et al. 2005). Approximately 50–70% of the original raw material generated as wastes during the fish processing can be used for collagen production (Jongjareonrak et al. 2005). Extraction and characterization of collagens from different fish species have been reported, such as surf smelt skins (Nagai et al. 2010), tilapia, grass carp and silver carp skins (Tang et al. 2015), rohu catla scales (Pati et al. 2010), cultured catfish muscles (Kiew et al. 2013), brownbanded bamboo and blacktip shark cartilages (Kittiphattanabawon et al. 2010). There have been no reports so far on the isolation and characterization of collagen from lumpfish. Lumpfish (lumpus), which is commonly found in the iceberg area of arctic or north Atlantic, belongs to the circular fin species. They live in the 200 meters deep in the ocean, where no pollution is a factor. In addition, the fish contains a lot of unsaturated fatty acid and calcium. More important, their skins are very thick, accounting for about one third of their total body weight, which explains why lumpfishes have rapidly caught scientific attention these days. In this paper, we isolated and characterized collagen from the skin of lumpfish and aims to extend the application of lumpfish.

2. Materials and methods

2.1 Chemicals

Lumpfish was purchased from a local fish market. Pepsin-soluble collagen was extracted from pig skin (PPSC) in our laboratory. Pepsin 1:3000 porcine source, β-mercaptoethanol (β-ME), Acrylamide, Coomassie Brilliant Blue R-250 and N,N,N',N'-tetramethyl ethylene diamine (TEMED) were purchased from Shanghai solarbio Bioscience & Technology Co., Ltd (China). Sodium dodecyl sulphates (SDS), Tris (hydroxylmethyl) aminomethane, Acetic acid, Sodium chloride (NaCl) were obtained from Sinopharm Chemical Reagent Co., Ltd (China). All other chemicals and reagents used were of analytical grade.

2.2 Isolation of collagen

2.2.1 Pretreatment of skin

The fish skin was washed with distilled water, cut into small pieces (5mm×5mm) and treated with 0.1 M NaOH (1:8, w/v) for 24h, to remove non-collagenous proteins. Then the alkali-treated skins were removed fat with 10% n-butyl alcohol (1:8, w/v) for 24 h. The residue was fully washed with distilled water and lyophilized. All of the procedures were carried out at 4 °C.

2.2.2 Extraction of acid-soluble collagen (ASC)

The pretreated fish skin was used to ASC extraction with 0.5 M acetic acid, at a ratio of 1:5 (w/v) for 3 days at 4 °C. After extraction, the mixture was centrifuged at 26,000×g for 15 min. The supernatant was salted out by adding NaCl to a final concentration of 2.3 M and followed by precipitation of collagen by the addition of NaCl, to a final concentration also of 2.3 M at a neutral pH (0.05 M Tris–HCl, pH 7.0). The resultant precipitate was obtained by centrifugation at 15800×g for 15 min and dissolved in 0.5 M acetic acid, then dialyzed against 0.1 M acetic acid and distilled water. After being lyophilized, the collagen was stored at -25 °C until use.

2.2.3 Extraction of pepsin-soluble collagen (PSC)

The pretreated fish skin was completely soaking in 0.5 M acetic acid and subjected to limit hydrolysis with 0.05% (w/v) pepsin for 24 h at 4 °C with gentle stirring. The mixture was centrifuged at 26,000×g for 15 min at 4 °C and the supernatant obtained was dialyzed against 0.02 M sodium phosphate buffer (pH 7.2) for 3 days. Other steps were consistent with those used for ASC as described above.

2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed following the method of (Veeruraj et al. 2015). The lyophilized collagen samples (ASC, PSC and PPSC) were mixed with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 1% SDS ,10% glycerol and 0.02% BPB) in the presence or absence of β-ME (Mercaptoethanol). The mixtures were kept in boiling water for 2 min. 10uL Sample solutions were loaded onto polyacrylamide gels comprising 7% running gel and 5% stacking gel. After electrophoresis, the gel was stained with 0.05% (w/v) Coomassie brilliant blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High-molecular weight markers (11-245 kDa, Solarbio) were used to estimate the molecular weights of proteins.

2.4 Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of ASC, PSC and PPSC were obtained with attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopic analysis, using a FTIR spectrometer (Thermofisher, Nicolet iS5, USA), according to the published method (Matmaroh et al. 2011). Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000 ~ 650 cm^{-1} at room temperature.

2.5 UV absorption spectrum

UV absorption spectrum of ASC, PSC and PPSC were measured using a Shimadzu-UV Spectrophotometer. The collagen samples were dissolved in 0.5 M acetic acid to obtain a concentration of 1 mg/ml. The UV spectrum was measured at wavelength between 200 to 400 nm at scan speed of 2 nm/s.

2.6 Zeta potential analysis

Zeta potential analysis was performed based on the method of (Matmaroh et al. 2011). ASC, PSC and PPSC (50mg) were dissolved in 0.5 M acetic acid (100ml), respectively. The mixtures were placed at 4 °C until the samples were completely solubilized. The pH of the collagen solutions were adjusted to 2 ~ 8 using 6.0 M NaOH. Then solutions were subjected to a zeta potential analysis using a model Nano-ZS90 (Malvern Instruments Ltd. UK). The zeta potential for each sample was recorded.

2.7 Determination of denaturation temperature (T_d)

The denaturation temperature (T_d) of ASC, PSC and PPSC solutions were measured by the method of Nagai et al. (2010), using an Ostwald′s type viscometer. The lyophilized collagen sample was dissolved in 0.1 M acetic acid to obtain a concentration of 0.3 mg/ml, and a 10 ml collagen solution was placed in the viscometer. The denaturation curve was obtained by measuring the viscosity of solution at different

temperatures (10 ~ 50 $^{\circ}$C). The sample solution was held for 30 minutes in a water bath at each temperature.

The relative and specific viscosities of collagen solution were calculated as follows:

$$Relative\ viscosity\,(\,\eta_r\,)=\frac{t}{t_0}$$

<div align="right">Eq.1</div>

$$Specific\ viscosity\,(\,\eta_{sp}\,)=\frac{t-t_0}{t_0}=\eta_r-1$$

<div align="right">Eq.2</div>

Where t_0 is flow time of pure 0.1 M acetic acid, and t is flow time of collagen solution. T_d was determined as the temperature at which the change in viscosity was half completed. Each point was carried out three times.

3.Results and discussion

3.1 Electrophoretic characterization

The protein patterns of ASC, PSC from lumpfish skin and PPSC from pig skin, under reducing and non-reducing conditions, were observed in Fig. 1. It is apparent that the major components of all collagens consisted of two different α-chains (α$_1$, α$_2$) and one β chain. The α$_1$ and α$_2$ chains with
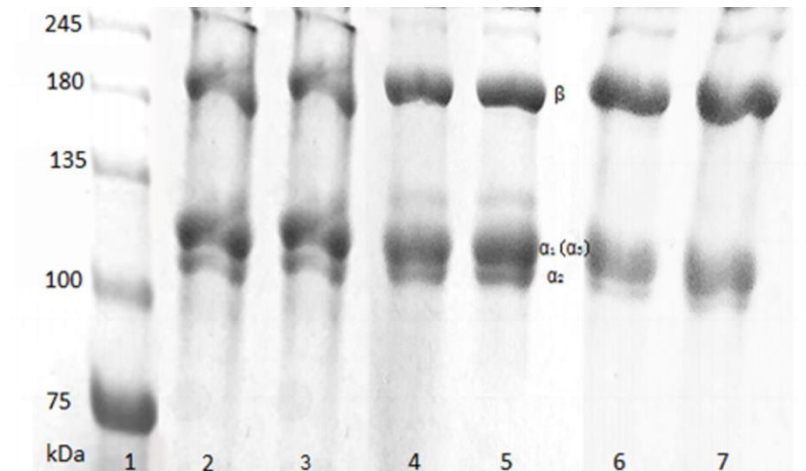
Fig. 1 Protein patterns of ASC, PSC from lumpfish skin and PPSC from pig skin. Lane 1 represents the protein molecular weight marker; Lane 2, 3 represent PPSC in the presence and absence of β-ME; Lane 4, 5 represent PSC in the presence and absence of β-ME; Lane 6, 7 represent ASC in the presence and absence of β-ME.

molecular weight between 100 and 135 kDa, were found at a ratio of approximately 2:1, suggesting the existence of [α$_1$(I)]$_2$α$_2$(I) or α$_1$α$_2$α$_3$ in collagen triple helix (Nalinanon et al. 2011). This result indicated that ASC, PSCextracted from lumpfish skin and PPSC extracted from pig skin were Type I collagen. The electrophoretic patterns of lumpfish skin ASC and PSC were in agreement with the collagens of grass carp (Chen et al. 2015), marine eel fish (Veeruraj et al. 2013), sheephead seabream, black drum (Ogawa et al. 2004). In above protein patterns, β-chain (dimer) andγ-chain (trimer) were also observed in ASC, PSC and PPSC, representing the presence of higher intra- and intermolecular cross-links of isolated collagens with large molecular weight using the acid and pepsin methods. It cannot be stated whether α$_3$-chain exists in the ASC, PSC or PPSC, because α$_3$-chain and α$_1$-chain were able to migrate to the same

mobility, and it cannot be separated from α_1-chain under the electrophoretic conditions employed. It was reported that the heterotrimer ($\alpha_1\alpha_2\alpha_3$) was found as a major component in ASC from the scale of sheep head and black drum (Ogawa et al. 2004). In addition, no differences in the protein patterns of ASC, PSC and PPSC analyzed in the presence and absence of β-ME were observed, which suggesting that no disulphide bonds were present in the ASC, PSC from skin of lumpfish and PPSC from skin of pig (Veeruraj et al. 2015).

3.2 Fourier transform infrared (FTIR) spectra

The FTIR spectra in the range of 4000 ~ 650 cm^{-1} of ASC, PSC and PPSC were shown in Fig. 2 and Table 1. The major peaks identified in the three FTIR spectra have amide A, B, as well as Amide I, II and III. But the spectra of ASC, PSC from lumpfish skin were slightly different with that from land-based animals (such as pig)and some other fish skins, such as Nile perch (Muyonga et al. 2004),

Table 1

FTIR spectra peak positions and assignments for ASC, PSC from the skin of lumpfish and PPSC from the pig skin.

| Region	Peak wavenumber (cm-1)			Assignment
	ASC	PSC	PPSC	
Amide A	3304	3295	3315	NH stretch coupled with hydrogen bond
Amide B	3082	3064	3073	CH$_2$ asymmetrical stretch
—	2928	2919	2946	CH$_2$ asymmetrical stretch
Amide I	1650	1649	1641	C=O stretch/hydrogen bond coupled with
Amide II	1552	1552	1552	COO-
—	1445	1454	1445	NH bend coupled with CN stretch
Amide III	1243	1243	1244	CH$_2$ bend
—	1078	1078	1078	NH bend
				C-O stretch

– No common name for the spectral region
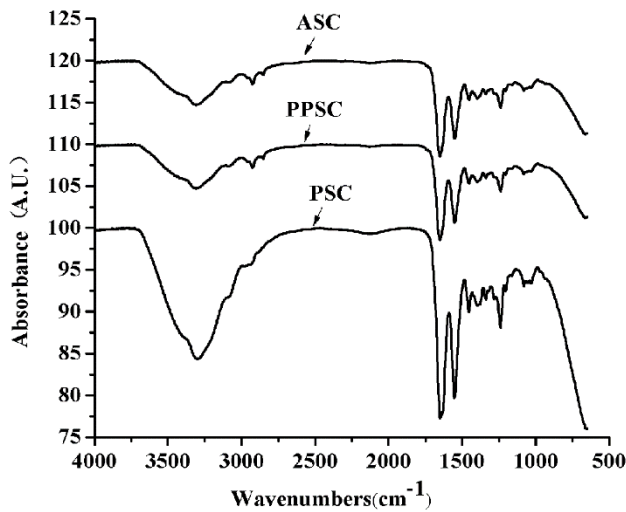
Fig. 2 FTIR spectra of ASC, PSC from lumpfish skin and PPSC from pig skin

Seabass Sinthusamran et al. (2013) and arabesque greenling (Nalinanon et al. 2010), which indicating slight differences in the secondary structure of collagens extracted from different fish skins.

The absorption peaks of amide A occurs in the range of 3400 ~ 3440 cm^{-1}, generally coupled with N-H stretching vibration (Jeong et al. 2013). The amide A band of ASC, PSC and PPSC were found at 3304, 3295 and 3315 cm^{-1}, respectively. When the N-H group is involved in hydrogen bond, the absorption peak will shift to lower frequency, suggesting more N-H group in PSC was involved in hydrogen bond, compared with ASC and PPSC. Amide B peaks of ASC, PSC and PPSC were observed at 3082, 3064 and 3073 cm^{-1}, assigned to the asymmetrical stretch of CH_2.

The characteristic absorption peak of ASC, PSC and PPSC were identified at 1650, 1649 and 1641cm^{-1}, representing C=O stretching vibration or hydrogen bond coupled with COO^-. It was in accordance with that the wave number of characteristic absorption peak in Amide I bond is usually in the range of 1600 ~ 1700cm^{-1}. While amide I band of collagen from scales of grass carp was reported to be 1658 cm^{-1} (Li et al. 2008), which might be associated with the degree of order of collagen molecules and lower inter-molecular cross-links in the skin collagen of lumpfish .

Amide II of all collagen samples were obtained at 1552cm^{-1}, takes responsibility for the N–H bending vibration and the C–N stretching vibration of collagen amide groups. Generally, the shift to the lower wave number showed the existence of hydrogen bonds in each collagen (Ahmad and Benjakul 2010). Amide III bands were found at similar wave number for ASC, PSC and PPSC at 1243, 1243, 1244 cm^{-1}, respectively. It was attributed to N–H bending vibration of amide III. Furthermore, the absorption ratio between amide III and 1454 cm^{-1} band was approximately equal to 1.00 for all collagens. This result indicated the presence of triple helical structure of ASC, PSC and PPSC (Sinthusamran et al. 2013). The fact that the FTIR spectra of ASC, PSC from lumpfish skin and PPSC from pig skin were similar to that of other type I collagens, confirmed that the extracted collagens were typical type I collagen.
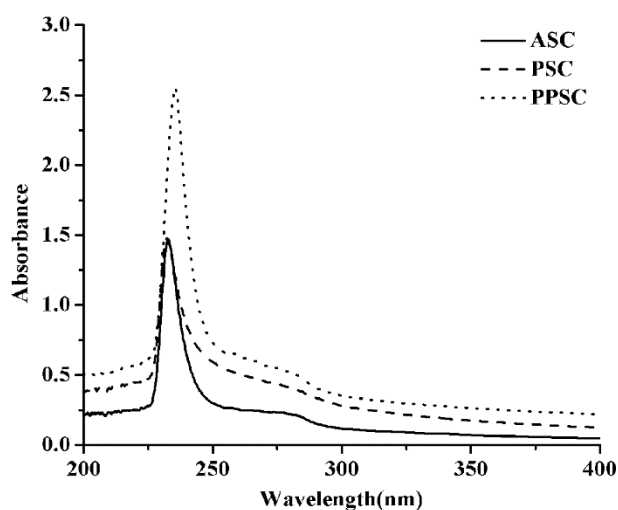
3.3. Ultraviolet spectra

Fig. 3 Ultraviolet spectra of ASC, PSC from the skin of lumpfish and PPSC from pig skin

The UV absorption spectrums of ASC, PSC and PPSC at the wavelength ranges 200–400 nm were showed in Fig. 3. Most proteins have a maximum ultraviolet absorption in the ultraviolet region at 280 nm. However, the absorptions of ASC, PSC and PPSC at 280 nm were very weak, indicating that the amount of aromatic amino acid is less than other proteins in the three collagens. Both ASC and PSC isolated from the skin of lumpfish showed maximum absorption at 232 nm which is similar to some collagens from channel catfish skin (232 nm) (Liu et al. 2007), largefin longbarbel catfish (233 nm) Zhang et al. (2009) and slight lower than pig skin collagen (236 nm). This result suggested that the groups of C=O, —COOH and $CONH_2$ were accessible in polypeptides chains of collagen.

3.4. Zeta potentials
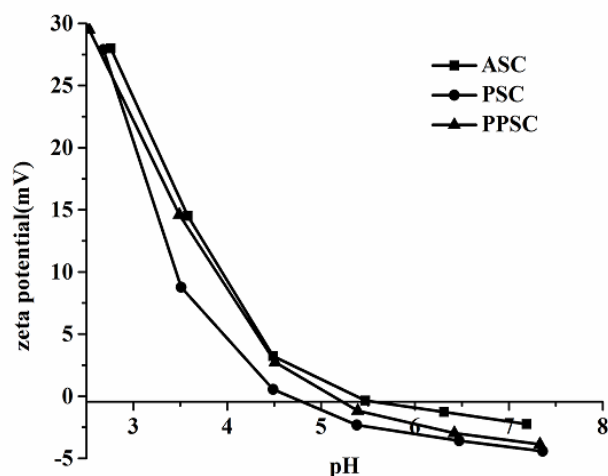
Fig. 4 Zeta potential of ASC, PSC from the skin of lumpfish and PPSC from the skin of pig at different pH

Zeta potentials of ASC, PSC from the skin of lumpfish and PPSC from the skin of pig at different pH were displayed in Fig. 4. At pH 2 ~ 8, the surface charge of all collagens continuously decreased as the pH

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increased. For ASC, PSC and PPSC, zero surface net charge was observed at pH 5.33, 4.71 and 5.12, respectively. When the positive charges on a protein are equal to the negative charges, the net charge of the protein is zero, and the pH in this time is defined as the isoelectric point (pI) (Vojdani 1996; Bonner 2007). Therefore, the pIs of ASC, PSC from lumpfish skin and PPSC from pig skin were estimated to be 5.33, 4.71 and 5.12. In our study, the pIs of PSC and PPSC were lower than that of ASC, this might be attributed to pepsin cleaved the telopeptide region, resulting to difference in amino acid composition in α-chains (Matmaroh et al. 2011).When pH value were below and above the pIs, ASC, PSC and PPSC had a net positive or a negative charge, respectively. Zeta potential results suggested that collagens from different fishes showed different pIs, such as the pIs of ASC and PSC from striped catfish were 4.72 and 5.43 (Singh et al. 2011), the pI of ornate threadfin bream skin collagen was 6.40 (Nalinanon et al. 2011), the pIs of ASC and PSC from grass carp were 6.67 and 6.82 (Chen et al. 2015). Amino acid composition of collagens from different sources are similar, however, due to the difference in acidic and basic amino acids residues and in degree of protonation or deprotonation, the net surface charge at different pHs are different (Benjakul et al. 2010).

3.5. Thermal stability

The thermal denaturation temperature (T_d) curves of ASC, PSC from lumpfish skin and PPSC
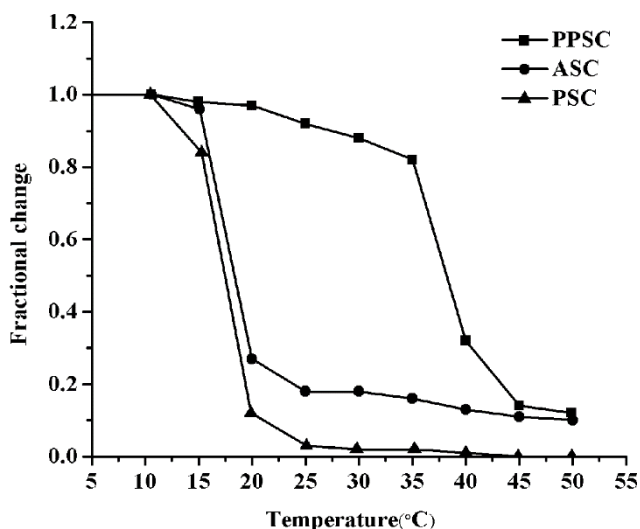

Fig. 5 Thermal denaturation curves of ASC, PSC from the skin of lumpfish and PPSC from pig skin

from pig skin were shown in Fig. 5. According to the thermal denaturation curve, it was calculated that the T_d of ASC, PSC and PPSC were about 17.9^{o}C, 17.5^{o}C, 37.9^{o}C, respectively. The T_d ofASC, PSC from the skin of lumpfish were significantly lower than that of pig skin collagen (PPSC). It is well known that the thermal denaturation temperature (T_d) was possibly correlated with the number of hydroxyproline and proline of collagen (Ikoma et al. 2003). T_d, as well the thermal stability of collagen decreased with the number of hydroxyproline and proline decreased. Besides, this value was much lower than those from temperate and tropical fish species reported as follows: eagle ray (34.1^{o}C), red stingray (33.2^{o}C) and yantai stingray (32.2^{o}C) (Bae et al. 2008); Amur sturgeon (32.52^{o}C) (Wang et al. 2014); Japanese sea-bass (26.5^{o}C), chub mackerel (25.6^{o}C) and bullhead shark (25.0^{o}C) (Nagai and Suzuki

2000); ocellate puffer fish (28.0^{o}C) (Nagai et al. 2002). However, the T_d of ASC and PSC from lumpfish skin were similar to those of cod skin collagen (15 ^{o}C) Rigby (1968) and Alaska pollack skin collagen (16.8 ^{o}C) (Kimura and Ohno 1987). These results further proved that the thermal stability of collagen was correlated with the environmental and body temperatures of fishes (Zhang et al. 2007).

4.Conclusions

Acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) were successfully isolated from the skin of lumpfish. SDS-PAGE and FTIR revealed that ASC, PSC were characterized as type I collagen with triple helical structures, consisting of α-chains (α$_1$, α$_2$) and β chain. Moreover, ASC and PSC showed maximum UV absorption at 232 nm. Due to the cleavage of telopeptides in PSC by pepsin, ASC and PSC have slight differences in physicochemical properties. It was found that the thermostability and the isoelectric point (pI) of ASC were a little higher than those of PSC. Due to lumpfish was deep-sea fish, its physicochemical properties are different from that of temperate and tropical fish species, especially the thermal denaturation temperature (T_d). Rich in collagen and without pollution, lumpfish could serve as an alternative source of collagen.

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THE AGGREGATION BEHAVIOR OF COLLAGEN MOLECULES IN DIFFERENT CONCENTRATIONS OF ACETIC ACID SOLUTION

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A large amount of bovine split wastes were discharged in the leather industry, which contained high-value native collagen. Collagen-based materials were often used in the form of solution or involved in processing of collagen solution. However, collagen molecules have tendency to entangle and aggregate in solution. The physicochemical properties of collagen were correlated to its aggregation behavior in solution. Systematic studies on the aggregation behavior of collagen molecules in different concentrations of acetic acid (AA) was performed to provide information on their utilization. For this purpose, collagen solutions in the presence of 0.1-2.0 M acetic acid (AA) were investigated. Fluorescence measurement of pyrene showed that the critical aggregation concentration (CAC) of collagen increased from 0.518 to 1.581 within the AA concentration ranging from 0.1 to 2.0 M, indicating that aggregated state of collagen molecules was associated with AA concentration. A distinct decrease in the average size of collagen aggregates across the whole AA concentration range was detected by dynamic light scattering, which demonstrated that the disaggregation of collagen aggregates was enhanced with the increase in electrostatic repulsion between collagen chains. Meanwhile, the variations in the intrinsic viscosity and Huggins coefficient depended on the molecular interaction among collagen molecules. Furthermore, the increased fluorescence anisotropy demonstrated that the rigidity of collagen molecules in solution was strengthen through the greater ionization of AA solution. This observation was manifested by the changes in the morphology of collagen molecules observed by atomic force microscopy.
Keywords: collagen molecules; acetic acid; aggregation behavior

1.   Introduction

Collagen, the principle structural protein in connective tissue, is widely used in pharmaceutical formation, food, cosmetic products and the chemical industry due to its low antigenicity, good biocompatibility and controlled biodegradability (Boruah et al. 2010; Khougaz et al. 1996; Potorac et al. 2014; Mehta et al. 2014). These excellent bio-functionalities of collagen are mainly ascribed to its unique right-handed helical conformation, which is supercoiled by three left-handed α-chains, exhibiting a

molecular weight of as high as 300 kDa (Arafat et al. 2015; Prasad et al. 2006; Fathima et al. 2004; Kezwon et al. 2014). Both the long peptide chains and high molecular weight endow collagen with aggregated characters. Critical aggregation concentration (CAC) is one of the most important parameters for characterizing the aggregation behavior of macromolecules (Yan et al. 2010). It is formally defined as the concentration where the formation of an aggregated complex occurs in solution (Ritacco et al. 2003; Jain et al. 2004). Collagen extracted from calf skin was currently chosen as the most widely available potential source of biocompatible material (Kezwon et al. 2016), following which collagen molecules self-associate with an approximate CAC of 0.5 mg/mL in 0.1 M acetic acid (AA) (Wu et al. 2013; Ding et al. 2014; Shi et al. 2001). As a natural polymer, collagen molecules have a tendency to aggregate into macromolecular structures above the CAC, and thus its solution presents remarkable viscoelastic behavior. For instance, the complex viscosity (η^*) values of collagen solutions with concentrations of 5, 7.5, 10, 12.5 and 15 mg/mL were 2.4, 3.6, 31.7, 94 and 406.92 Pa s, respectively, in 0.5 M AA respectively at 0.01 Hz. The poor fluidity of collagen solution was more obvious for increased collagen concentrations. The exponential increase in the viscoelastic property was dependent on the enhancement of entanglement and the more complicated structural collagen aggregates (Lai et al. 2008). In addition, the thermal denaturation of collagen solution was changed with the aggregated state of collagen molecules. Calorimetric measurements showed that the transition temperature of 5 mg/mL collagen solution was 40.7 °C in 0.1 M AA, compared with 39.0 °C for 20 mg/mL collagen in the same experimental conditions (Liu et al. 2010). The physicochemical properties of collagen are closely associated with its aggregation behavior in solution. Furthermore, the polymer/solvent interaction can also be significantly affected by the changes in the solvent characteristics, which can simultaneously modify the properties of the polymer solution (Damas et al. 2008). Traditionally, collagen for biomaterials is mainly prepared in the form of an aqueous solution or is involved in the processing of the solution. It is therefore necessary to investigate the interaction of collage molecules and acidic solvents. AA is a preferred weak acid agent used to dissolve collagen. It not only disperses collagen into a homogeneous solution, but it also maintains the collagen native structure (Chakrapani et al. 2012; Kazanci et al. 2014). AA at concentrations of 0.1–0.5 M is a traditional solvent for dissolving lyophilized collagen with the final release of molecules (Hofman et al. 2011). However, the low CAC value and weak fluidity of collagen solution are the major drawbacks encountered in this AA concentration range, which affects the manufacturing of collagen-based materials and limits some applications, such as electrospinning and injectable products (Qi et al. 2015). Some reports have actively investigated electrospun of collagen using concentrated AA solution as a solvent, since more concentrated AA in water progressively increased the number of ions available for charge repulsion, accompanied by decreased entanglements of the collagen molecules (Nagarajan et al. 2014; Liu et al. 2013). In virtue of the CAC of collagen being determined at 0.5 mg/mL in 0.1 M AA, the aggregation behavior of collagen molecules was changed with increasing AA concentration. In our previous work, collagen solutions (0.5 mg/mL) with various concentrations of AA (0.1–2.0 M) were prepared and the changes in aggregation behavior of the collagen molecules were examined. The collagen molecules tended to form larger aggregates at AA concentrations below 1.0 M and smaller aggregates at AA concentrations above 1.0 M. Moreover, the disaggregation was more pronounced for smaller aggregates (Yang et al. 2016). Hence, the response of collagen molecules to AA concentration was closely correlated with the collagen aggregation behavior. In order to clearly understand the interaction of collagen molecules in different

concentrations of AA solution, it is crucial to investigate the changes in CAC values and the aggregation properties of collagen molecules above/ below the CAC.

In the present work, the CAC of collagen solution with various AA concentrations (0.1–2.0M) was firstly investigated by using an external fluorescence probe pyrene. Then, the aggregation properties above/below the CAC were further studied by fluorescence anisotropy, dynamic light scattering (DLS), intrinsic viscosity and atomic force microscopy (AFM). The obtained results will be helpful for attaining high collagen concentrations with excellent fluidity and act as guidance for its biomedical applications.

2. Materials and methods

2.1. Materials

Pepsin-soluble collagen was extracted from bovine split wastes as described by Zhang et.al with only a slight modification (Zhang et al. 2008). The precipitate was dissolved sufficiently in 0.5 M AA and then dialyzed against 0.1 M AA for 3 d to remove sodium chloride. The obtained collagen solution was eventually lyophilized by a freeze dryer (Labconco Freeze Dryer FreeZone 6 Liter, USA) at -50 °C for 2 d and stored in a desiccator for later use. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed to identify as type I collagen (Tian et al. 2014).

Pyrene was purchased from Sigma Chemicals (Sigma-Aldrich, Munich, Germany) and was used without further purification.

2.2. Preparation of collagen solution

The lyophilized collagen was dissolved in different concentrations of AA solution (0.1, 0.5, 1.0, 1.5 and 2.0 M) to achieve a concentration of 3 mg/mL. All solutions were stirred continually for 24 h at 4 °C and then centrifuged at 8000 ×g for 10 min to remove entrapped air bubbles for the following measurements.

2.3. Fluorescence measurements with pyrene

Collagen solutions covering a concentration range of 0.001–3.0 mg/mL were prepared to determine the value of the CAC in different concentrations of AA solution. Fluorescence emission spectra of pyrene in collagen solutions were measured using a fluorescence spectrophotometer (Cary Eclipse, Agilent, Australia) according to the method of Nakashima et al. with some modification (Nakashima et al. 1994). The hydrophobic probe pyrene was dissolved in methanol to give a concentration of 400 µM. A stock solution (125 µL) was introduced into a 25 mL brown glass volumetric flask and gently evaporated overnight under a nitrogen gas stream. Then, 25 mL of collagen solution was added into the volumetric flask. After which, the mixed solutions were homogenized fully and stored at 4 °C for 24 h in total darkness before the spectroscopic measurements. The samples were excited at 343 nm and the emission spectra for 360–460 nm were examined with a scanning rate of 120 nm/min. The slit openings for excitation and emission were fixed at 5 and 2.5 nm, respectively. All fluorescence measurements were carried out in triplicate.

2.4. DLS measurements

The average aggregation size and size distribution were determined by DLS with a Zeta sizer (Nano-ZS, Malvern, UK). Diluted collagen solutions with a concentration of 1.0 mg/mL were firstly filtered through a 2 µm filter (Millipore, USA) and then transferred to a small volume polystyrene cuvette for the measurements. All measurements were performed in triplicate at 25 °C.

2.5. Intrinsic viscosity measurement

The intrinsic viscosity ($[\eta]$) of the collagen solutions was obtained using Huggins method, employing an

Ubbelohde viscometer at 25 °C in a water bath regulated to 0.01°C. A concentration series (5×10^{-3}–3×10^{-2} g/dL) of collagen solutions in different concentrations of AA solution was prepared by stepwise diluting of a stock solution (3×10^{-1} g/dL) with ultrasonic treatment for 3 min. After that, the solutions were stirred under ice-bath condition for 3 h, to ensure the thorough dissolving of the collagen aggregates. The flow times of collagen samples were tested after a thermal equilibrium time of 30 min. The viscosity measurement was based on the flow rate of collagen solution through the capillary of the viscometer (Fathima et al. 2009). The flow times were measured with a digital stopwatch at least five times and the average was taken (Kandamchira et al. 2013). The [η] of the collagen solutions was estimated by the expressions of Huggins (Arias et al. 1998):

$$\frac{\eta_{sp}}{C} = [\eta] + [\eta]^2 C \quad (1)$$

$$\eta_{sp} = \frac{t - t_0}{t_0} \quad (2)$$

where $\frac{\eta_{sp}}{C}$ is the reduced viscosity, C (g/dL) is the concentration of collagen solution, t is flow time of the collagen solution and t_0 is the flow time of the solvent.

2.6. Fluorescence anisotropy of collagen solutions

The fluorescence anisotropy of the collagen solutions (1 mg/mL) was collected using a fluorescence spectrophotometer as described above, with excitation and emission wavelengths of 278 nm and 300–450 nm, and a scan rate of 120 nm/min. Excitation and emission slit widths were set at 5 and 2.5 nm, respectively. Polarizers were fixed vertically or horizontally as required, and an average of three scans per spectrum was conducted.

The values of fluorescence anisotropy (r) in different concentrations of AA solution were calculated by the following equation:

$$r = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2G I_{VH}} \quad (3)$$

$$G = \frac{I_{HV}}{I_{HH}} \quad (4)$$

where I_{VV} and I_{VH} are the intensities measured with the detection polarizer oriented along the vertical and horizontal directions, respectively, while the incident light is vertically polarized. I_{HV} is the emission intensity observed vertically with horizontally polarized light excitation and I_{HH} is the emission intensity detected in the same horizontal plane as it is excited (Ding et al. 2016; Saha et al. 2015).

2.7. AFM measurements

Collagen samples of 1 mg/mL were dispersed in 0.1 and 2.0 M AA by mild stirring for a short period to obtain a concentration of 40 µg/mL. Droplets of these diluted collagen solutions (15 µL) were immediately evaporated on freshlycleaved sheets of mica and then dried at room temperature for 2 d. The surface morphology of the dried samples was observed by AFM (SHIMADZU SPM 9600, Japan) in dynamic mode at room temperature. Each sample was scanned with scanning rates of 1 Hz and 2 Hz, respectively.

3. Results and discussion

3.1. Determination of CAC of collagen in different concentrations of AA solution

Pyrene, as a probe for the hydrophobicity of the micro-environment, is applicable for monitoring the association of collagen molecules in solution. The accumulation of aggregates in aqueous solution leads to the formation of hydrophobic cores. An incorporation of pyrene into such cores is reflected by the

changes in fluorescence intensity. Therefore, a pyrene fluorescence technique waspreferably used to define the CAC values of collagen in different concentrations of AA solution (Guan et al. 1999; Schneider et al. 2010).

The fluorescence emission spectra of pyrene displayed four main peaks at ~372, ~378, ~383 and ~392 nm in different collagen solutions (data not shown), consistent with the results reported by Ray et al. (Ray et al. 2006). The intensity ratio of the vibrational band 1 (I_1) at 372 nm to vibrational band 3 (I_3) at 383 nm was taken as an index of the polarity of the microenvironment, which was high in polar media and low in hydrophobic environment. Plots of the pyrene I_1/I_3 ratio versus the logarithm of the collagen solution are shown in Fig.1. The concentration where the decrease in the ratio I_1/I_3 reached 50% of the initial value could be regarded as the CAC of collagen (Zhang et al. 2013). According to this method, the CAC values of collagen in different concentartions of AA solution are summarized in Table 1. It can be found that the CAC of collagen in 0.1 M AA was 0.518 mg/mL, consistent with the results reported by Wu et al (Wu et al. 2013). When the AA concentration was increased from 0.1 to 2.0 M, the CAC of collagen increased by 1.063 mg/mL. The aggregated state of collagen molecules was therefore markedly affected by AA concentration.

Collagen self-aggregation in aqueous solution could be explained by its molecular structure. At relatively lower AA concentrations, many aggregates were formed in the solution, due to theweak electrostatic repulsion that cannot inhibit the repolymerization of smaller collagen molecules into larger aggregates. However, at relatively higher AA concentrations, the weakened interaction of the intra- and intermolecular hydrogen bonds was due to the increased repulsion of $-NH_2^+-$ groups in collagen molecules, which resulted in lessaggregates and hydrophobic microdomains among the collagen molecules. Collagen samples with higher CAC values may reflect less aggregates and a higher content of collagen molecules in solution (Yan et al. 2010). Simultaneously, it is well known that hydrophobic interactions remarkably influenced the aggregation process. With increasing AA concentration, the greater ionization in the more polar environment reduced the hydrophobic interactions of the polypeptide chains. Therefore, both the enhanced electrostatic repulsion and weaker hydrophobic interactions might inhibit the aggregation of collagen molecules in solution , resulting in an increased value of CAC (Golabiazar et al. 2014).

3.2. DLS analysis

DLS is a useful method for measuring the size of aggregates. Changes in the distribution and size of collagen aggregates were measured in different concentrations of AA solution. In view of the CAC of collagen in different concentrations of AA solution, collagen solutions with 1.0 mg/mL were represented as the pre-aggregation state (AA ≥ 1.0 M, below CAC) or the post-aggregation state (AA<1.0 M, above CAC) of collagen molecules. As can be seen in Fig. 2, all collagen solutions displayed two distribution regions of collagen aggregates with values of 32–123 nm and 955–3580 nm, respectively, which might be attributed to the collagen aggregates with different sizes. Variations in the intensity of two distribution regions were observed. When the AA concentration was increased to 1.0 M, the intensity of the two distribution regions changed inconspicuously, suggesting that a negligible variation took place for the larger aggregates above the CAC. When further increasing the AA concentration to 2.0 M, the intensity of the first distribution region increased slightly, whilst the second one decreased markedly, indicating that smaller-sized aggregates were more sensitive to AA and could more easily to become loose below the CAC (Liu et al. 2013). The average size of the collagen aggregates in the 0.1, 0.5, 1.0. 1.5

and 2.0 M AA solutions were 2027.1, 1932.9, 1565.2, 1123.7 and 846.5 nm, respectively. The reduction in the size might be caused by the disaggregation of collagen aggregates due to an increase in electrostatic repulsion between collagen chains. Consequently, the results were in good agreement with the results for CAC values in different concentrations of AA solution.

3.3. Intrinsic viscosity measurements

Capillary viscometric studies of dilute solutions showed a marked molecular interaction between the collagen molecules. Fig. 3 displays the plots of reduced viscosity, η_{sp}/C, versus concentration, C, for collagen in different concentrations of AA solution. It can be found that the plot of $\eta_{sp}/C = f(C)$ should be a straight line with the intercept equal to $[\eta]$ and the slope equal to $k_H [\eta]^2$ (Ma et al. 2007). Therefore, the $[\eta]$ and k_H can be calculated graphically and are listed in Table 2. For AA concentrations ranging from 0.1 to 2.0 M, the values of $[\eta]$ decreased from 13.94 to 9.60 g/dL, which reflects the fact that the hydrodynamic volume of collagen molecules decreased with increasing AA concentration (Fathima et al. 2006).

It has been reported that the intrinsic viscosity can be considered as a criteria of hydrodynamic volume of molecules in dilute solution, and is generally dependent on the dimension of the polymer chain and polymer-solvent interaction (Arias et al. 1998). Although the dispersion of collagen in solution does not exclude the possibility of the presence of colloidal collagen particles, the main focus of this experiment is to study the interactions of collagen molecules in different concentrations of AA solution, through reporting the relative changes in $[\eta]$ and k_H, rather than obtaining the true absolute values of intrinsic viscosity (Ma et al. 2007). Collagen aggregates with larger sizes can be regarded as having a relatively higher molecular weight, and the disaggregation of aggregates should lead to a lower molecular weight. The measureable reduction in $[\eta]$ would be equivalent to the decrease of this high molecular weight fraction. Furthermore, the decreased $[\eta]$ was also attributed to the disruption of the structural water molecules involved in the aggregation of the hydrophobic group (Li et al. 2011). Namely, fewer aggregates would be formed as the AA concentration increased, due to the weaker hydrophobic interactions among collagen molecules in solution.This reinforces our earlier view based on the variations of the CAC. To further investigate the relationship between $[\eta]$ and solvent concentration, $[\eta]$ values were plotted against AA concentration (Fig. 4) and a clear negative linear correlation was observed. The following equation describes the dependence of $[\eta]$ on solvent concentration:

$$[\eta] = 14.69 - 2.41 \times C_{AA}$$

where C_{AA} is the AA concentration.

In addition, k_H is a non-dimensional parameter and can be used to describe the thermodynamic rigidity of macromolecules and the thermodynamic affinity between polymer and solvent. It is noteworthy that the usually accepted value of k_H for macromolecules in solvents (0.5–0.8) is applicable to flexible chain polymers (Ma et al. 2007). The large value for the collagen solution might be ascribed to the stiff triple helix chains of collagen, in agreement with the previous reports that the absolute values of k_H for semirigid and rigid chains are larger than those for flexible chains (Li et al. 2011). As shown in Table 2, the values of k_H increased from 0.786 to 1.635, indicating that the collagen molecules with a rigid conformation have a stronger interaction with the aqueous solvent as increasing AA concentration (Schneider et al. 2010).

3.4.Fluorescence anisotropy

To gain further insight into the relationship between collagen chains and solvent, fluorescence

anisotropy measurements were performed. Fluorescence anisotropy of fluorophores is known to be a convenient tool for studying the rotational motion of collagen chains. Direct information on the aggregation behavior of collagen molecules in different concentrations of AA solution was obtained (Wu et al. 2015; Fiejdasz et al. 2013). Therefore, the fluorescence anisotropy values could indicate the state of collagen aggregates by determining the molecular motion.

For polyelectrolytes, which have electrostatic charge repulsion within the polymer chains, the fluorescence anisotropy value is closely associated with the environment surrounding the molecules. The faster the molecule rotates, the lower values of the fluorescence anisotropy that can be expected (Pa et al. 2001). It has been reported that the fluorescence anisotropy of tyrosine residues is close to zero when the tyrosine residues are free to rotate in solution, while the fluorescence anisotropy increased as the tyrosine residues existed in aggregates that could hardly rotate. From Table 3, a slight increase in the fluorescence anisotropy values of the tyrosine residue in collagen was observed when the AA concentration increased from 0.1 to 1.0 M, demonstrating the weak restriction on the rotation of fluorophore molecules above the CAC of collagen. In contrast, the value of fluorescence anisotropy increased significantly as the AA concentration increased above 1.0 M, indicating that the restriction was greater below the CAC of collagen. This result suggests that the enlarged electrostatic repulsion between the collagen chains brought about difficulties in the molecule rotation, and thus the rigidity of collagen molecules was enhanced with increasing AA concentration.

3.5. AFM images

The morphological changes of collagen samples at different AA concentrations were observed by AFM. Above the CAC of collagen (Fig.5A), collagen fibers were intertwined with each other and indicated a disorder morphology, which was due to the collagen molecules have lower $-NH_2^+-$ charge destiny and behave more like flexible polymer. In contrast, below the CAC of collagen (Fig.5B), the fibers have a particular molecular orientation, suggesting that the collagen molecules have higher $-NH_2^+-$ destiny and behave more like rigid rods.Overall, these variations in molecular morphology observed by AFM were in good agreement with the aforementioned fluorescence anisotropy analysis.

4. Conclusions

The influence of AA concentration on the CAC and aggregation properties of collagen were discussed in this study. Increasing AA concentration in collagen solution caused an increase in $-NH_2^+-$ concentration on collagen molecules and enlarged the electrostatic charge repulsion among them. The increased CAC values indicated the higher content of collagen molecules in solution with increasing AA concentration. Furthermore, the response of collagen molecules to AA concentration was different when the collagen concentration was above or below the CAC. These experimental data suggest that increasing AA concentration encouraged greater ionization in collagen solution and that the interaction between collagen molecules changed with the surrounding polar environment. Therefore, the obtained information may be useful in the manufacturing of collagen products with low viscosity.

5. Acknowledgements

6. References

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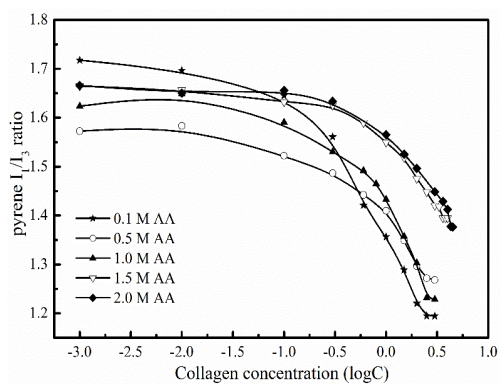
Fig. 1. Plots of the pyrene I_1/I_3 ratio versus the logarithm of the collagen solution
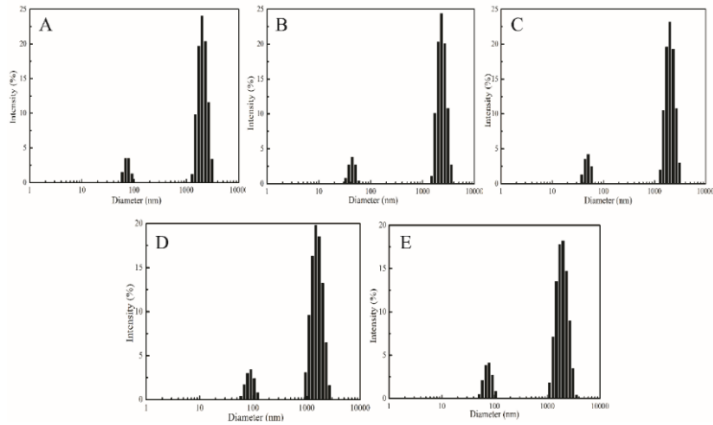
Fig. 2. The size and distribution of collagen aggregates in different concentrations of AA solutions: (A) 0.1 M AA, (B) 0.5 M AA, (C) 1.0 M AA, (D) 1.5 M AA and (E) 2.0 M AA
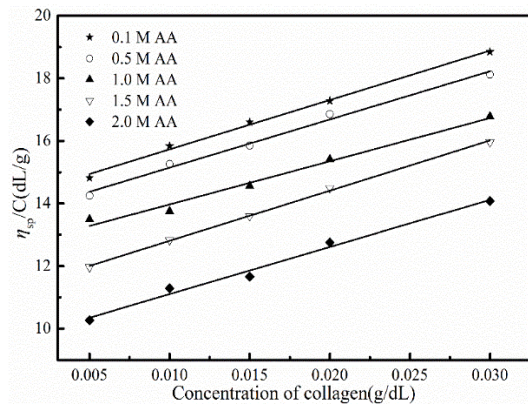
Fig. 3. Plots of reduced viscosity versus collagen concentration in different concentrations of AA solution
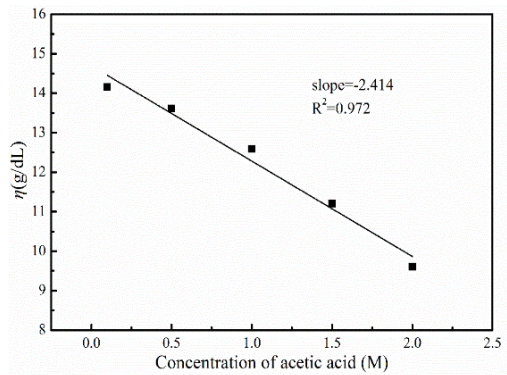
Fig. 4. Correlation curves of [η] against the concentration of AA solution
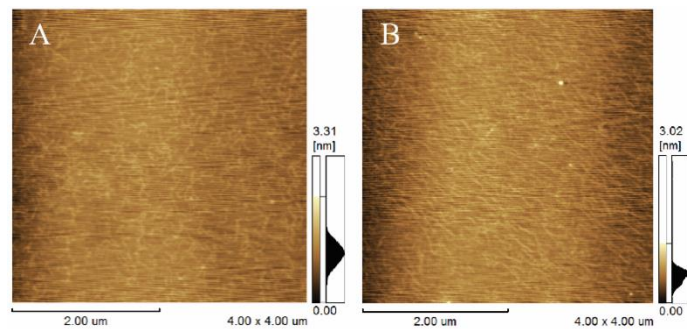
Fig. 5. AFM height images of collagen solution in different concentrations of AA solution (A: 0.1 M AA; B: 2.0 M AA)

Table 1 CAC values of collagen in different concentrations of AA solution

AA concentration (M)	CAC (mg/mL)
0.1	0.518
0.5	0.830
1.0	1.042
1.5	1.286
2.0	1.581

Table 2 Intrinsic viscosity [η] and Huggins coefficients k_H of collagen in different concentrations of AA solution

AA concentration (M)	[η] (g/dL)	k_H
0.1	14.16	0.786
0.5	13.61	0.831
1.0	12.59	0.870
1.5	11.20	1.278
2.0	9.60	1.635

Table 3 Fluorescence anisotropy values of collagen solution in different concentrations of AA solution

AA concentration (M)	Fluorescence anisotropy values of collagen solution
0.1	0.1289±0.0349
0.5	0.1362±0.0158
1.0	0.1401±0.0083
1.5	0.1745±0.0208
2.0	0.1997±0.0530

ENCAPSULATED CHLORHEXIDINE DIGLUCONATE USAGE ON THE DIABETIC FOOTWEAR UPPER LEATHERS

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It is important for a therapeutic shoe to have a wearable quality and its comfortable use is also prominent in terms of providing an increase in user's quality of life. In long-term treatment of diseases, medical products which are to be manufactured with medical leather materials might be a prominent alternative via local effect application as an adjuvant to the treatment. It is aimed in this study to produce micro-particles which contain the active agent chlorhexidine digluconate and application of these micro-particles on upper leather for manufacturing diabetic shoes while providing them a functional quality. Within the scope of the project, micro-particles loaded with drugs were obtained via spraying chlorhexidine digluconate active agent and through spraying with ethyl cellulose polymer and ustulation. In vitro characterisation studies were performed on the acquired microparticles. Additionally, active agent quantitation and in vitro drug delivery studies were also performed. Following the studies, the determined optimum microparticle formulations were applied on the leather, then existence and efficiency of microparticles within the leather was shown in the subsequent studies.

Keywords: Chlorhexidine digluconate, encapsulation, diabetic shoes, upper leather

1. INTRODUCTION

Diabetic foot disease is a serious problem, with a life-time prevalence of 15–25% in the diabetic population. Previous reports highlight that approximately 15% of people with diabetes world-wide will at some stage develop diabetic foot ulceration that could lead to amputation (Boulton 2000). While in the UK up to 100 people/week have a limb amputated as a result of diabetes, it is indicated that up to 80% of these amputations could have been prevented with correct management (Anonymous 2011). Foot ulcers in people with diabetes are multi-factorial and linked to a variety of clinical risk factors, like peripheral neuropathy and vascular insufficiency, as well as biomechanical risk factors, such as increased plantar pressure (Ledoux et al. 2013; Crawford et al. 2007). In shoe plantar pressure assessment is becoming increasingly popular in both research and clinical practice to evaluate the effects of prescribed footwear in diabetic patients who have a foot ulcer or who are at risk for ulceration (Singh et al. 2005).

Diabetic footwear plays an important role for the reduction of plantar pressure in people with diabetes (Panagiotis et al. 2015).

Microencapsulation is one of the most important forms of controlled release of active ingredient. This technology allows heat, temperature or pH sensitive components to be physically enveloped in a protective matrix or wall material in order to protect these ingredients or core materials from adverse reactions, loss or against light, heat and prolonged contact with air. It is also one of the most important forms of controlled release of substances and allows the utilization of some that otherwise would be unfeasible (Nirmala and Dilip 2013; Magdalena et al. 2015). These systems offer some advantages over conventional dosage forms, including improved efficiency, reduced toxicity and improved patient compliance (Grattard et al. 2002).

In the footwear industry, the incorporation of microencapsulated substances into materials or components allows the concept of active shoes to be realized, which contributes to improve the welfare of users, satisfying their needs and expectations (Morace and Ferrarini 2012). Fragrances applied to footwear, both directly and through packaging, cover one of the main consumer demands regarding the solution of bad odours generated during footwear use (Misher 2007). Along the same lines, in shoe packaging, microencapsulation allows the development of active issues with different purposes: trapping undesirable odours or incorporation of antimicrobial agents, to be released over time in order to improve the useful life of the packed shoe or the incorporation of controlled released scents to avoid their degradation and to improve the durability of the aroma (Sanchez-Navarro et al. 2011; Sanchez-Navarro et al. 2012).

It is aimed in this study to produce microparticles which contain the active agent Chlorhexidine digluconate (CHD) and application of these microparticles on upper leather for manufacturing diabetic shoes while providing them a functional quality.

2. MATERIAL AND METHOD
2.1. Material

CHD 20% solutions was purchased from Sigma-Aldrich. Aquacoat ethyl cellulose dispersion (Aquacoat® ECD) was gift from FMC BioPolymer (Philadelphia, PA). All other materials were of analytical grade.

2.2. Method
2.2.1. Preparation of Microparticles

Microparticles were carried out in a spray dryer model SD-Basic (Lab-Plant, Huddersfield, U.K). Aqueous ethyl cellulose dispersion (AquacoatECD) was used as a polymeric system. CHD and Aquacoat ECD were mixed in distilled water. The drug to polymer ratios in the microencapsulating compositions was maintained in 1:1, and 1:2, respectively. The compositions of the formulations in spray-drying are shown in Table 1.

Table 1. The composition of the formulations

Formulation Code	CD: EC*	Pump Speed (mL/dk)	Inlet Temperature (˚C)	Outlet Temperature (˚C)
F1	1:1	10	120	80
F2	1:2	10	120	80

(*): Aquacoat ethyl cellulose

2.2.2. Particle Morphology

The morphology of the microparticles was examined by a scanning electron microscope (SEM, FEI Quanta 250 FEG). The sample was mounted onto an aluminum stub and sputter-coated with gold palladium (Au/Pd) using a vacuum evaporator.

2.2.3. In Vitro Drug Release of the Microparticles

In vitro release studies were performed speed of 100 rpm in PBS at 37ºC. Microparticles were suspended in tubes containing 10 ml of PBS. At the appropriate time intervals, the medium in the corresponding tube was filtered through 0.22 µm filter and released CHD amount determined by validated UPLC method. Sink conditions were maintained in the receptor compartment during *in vitro* release studies. The experiment was carried out five times.

2.2.4. Application of the Microparticles on Upper Leathers

Microparticules were applied on the upper leathers for diabetic footwear in the finishing process by using spraying pistol. Microparticules that are containing antimicrobial material were added into finishing recipe (Table 2) as 20 g per m^2 (Kleban et al. 2002).

Table 2. Basic finishing recipe with microparticles

Material	Quantity	Application
Water	100 part	
Anionic wax	50 part	
Nonionic aliphatic polyurethane binder	25 part	3x Sprey
Microparticles	..12 part	

2.2.5. SEM of the Upper Leathers with the Microparticles

The morphology of the samples was examined by a SEM (HITACHI TM 1000).

2.2.6. In Vitro Drug Release of the Upper Leathers with Microparticles

In vitro release studies were performed speed of 100 rpm in PBS at 37ºC. Upper leathers with 10 cm^2 area were placed in beaker containing 125 ml of PBS. The samples withdrawn directly at appropriate time intervals were analysed by a validated UPLC method as previously described. The experiment was carried out five times.

2.2.7. Microbiologic Studies on Upper Leathers with Microparticles

Agar disc diffusion method was used to examine the effect of drug, microparticles and upper leather with microparticles against to the test microorganisms. Test microorganisms were incubated at 37 ^{o}C for 18 hours in the Muller Hinton Broth (MHB) medium. After incubation, microorganisms were inoculated into petri dishes containing Muller Hinton Agar (MHA) medium as 10^5 CFU/mL. Then, upper leather samples with 12.7 mm diameter were placed into the petri dishes. All petri dishes were incubated at 37 ^{o}C for 24 hours. Finally, inhibition zones were measured for determining the antibacterial activity.

3. RESULTS AND DISCUSSION
3.1. Particle Morphology

The morphology of the spray dried microparticles was examined by SEM. According to the SEM images, microparticle formulations had a spherical shape with a rough surface morphology. The microparticles exhibited irregular shape also. They do not show the presence of free drug on their surfaces. These morphological characteristics point out that the CHD is dispersed all over the microparticles. Figure 1 showed morphology of the microparticle formulations. In other spray-drying studies performed by using aqueous polymeric dispersions, microparticles with similar morphological characteristics were also obtained (Rattes and Oliveria 2007; Arici et al. 2014).
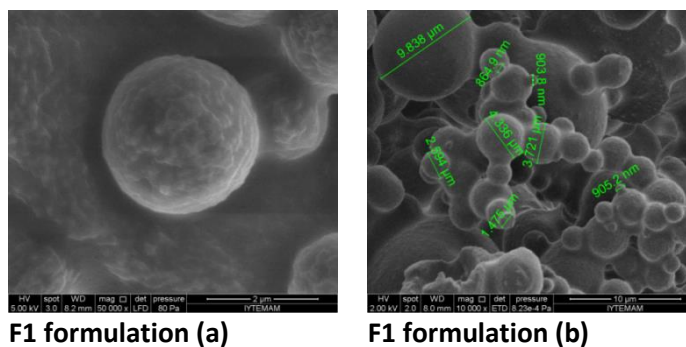
F1 formulation (a)   **F1 formulation (b)**

F2 formulation (a)   **F2 formulation (b)**
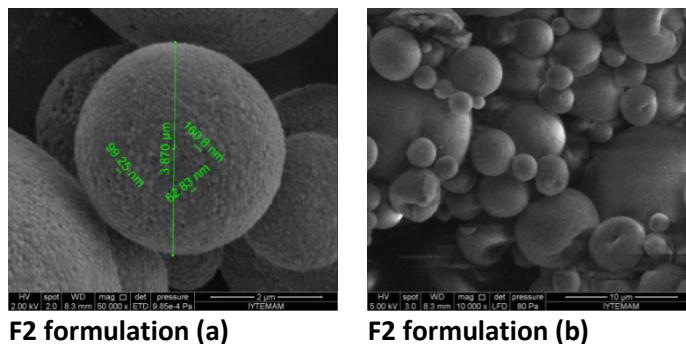
Figure 1. SEM images of microparticle formulations at 50000x magnification (a) and at 10000x magnification (b).

3.2. *In Vitro* Drug Release of the Microparticles

In vitro drug release studies showed that in CHD release from microparticles was very fast which is probably the consequence of very good swelling properties or it could be related with burst effect. Figure 2 showed *in vitro* release of the microparticles. In general, the initial rates of release for many drug delivery systems are high during the first period, most likely due to the release of drug enriched on the sample surfaces (Kenawy et al., 2002). The same behaviour, defined as the initial burst release, is presented in the microparticles. Microparticles prepared by using spray drying method generally have a matrix structure. For this reason, besides drug substance being in the particles, depending on the loading concentration, it can also be on the outer surface of the particles. When the microparticles are exposed to the dissolution media, the drug on the outer surface (non-encapsulated drug) causes a sudden drug release (Ghorab et al., 1990; Saravanan et al., 2003).
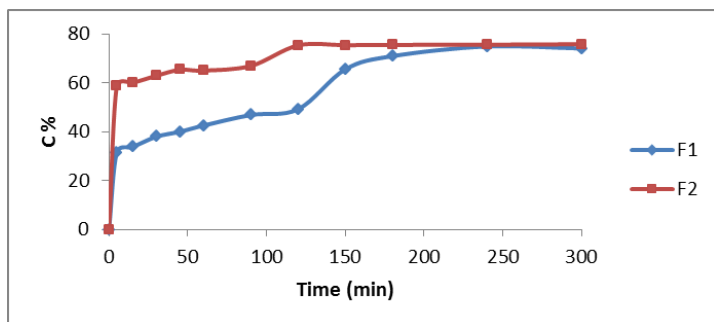
Figure 2. *In vitro* release of microparticles

3.3. SEM of the Upper Leathers with the Microparticles

Upper leather samples that were applied finishing recipes with microparticles containing antibacterial drug and without microparticles was examined by Scanning Electron Microscope. As seen in Figure 3, there were fewer microparticles on the upper leather surface because of the F1 formulation's polymer quantity is half of the F2 formulation.

| without microparticles | F1 formulation | F2 formulation |
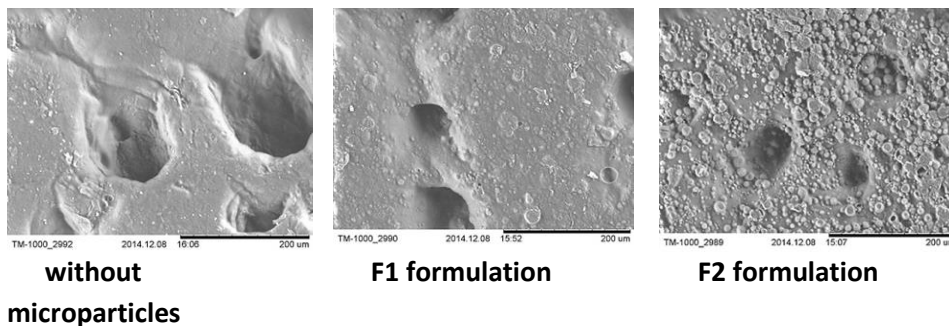
Figure 3. SEM images of the upper leather after finishing process

3.4. *In Vitro* Drug Release of the Upper Leathers with Microparticles

The *in vitro* release results of the leathers in pH 7.4 PBS are presented in Figure 4. As seen in Figure 4, there was a steady release of the CHD into the PBS for all formulations. CHD entrapped deep within the microparticles sustained the release to more than 24h. The drug release ratio of upper leathers with F1 and F2 formulations was synchronised first 8 hours. Comparing the formulations among each other, the drug release ratio of upper leather with F1 formulation was slightly higher than the release of upper leather with F2 formulation at the end of 25 hours.
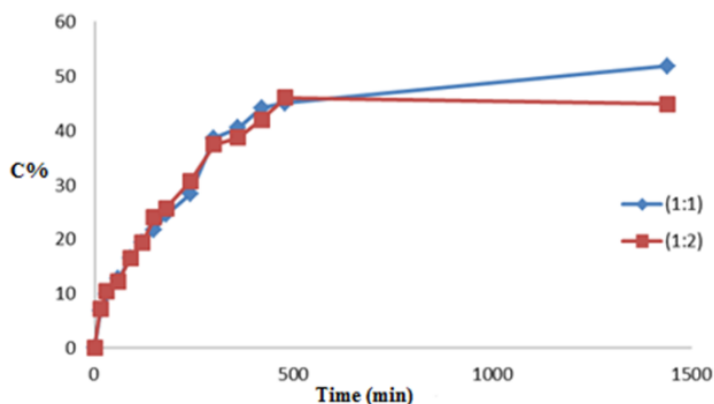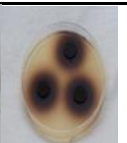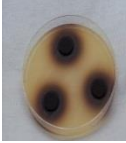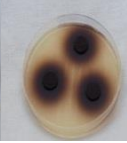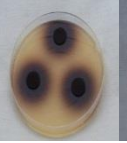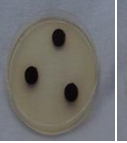
Figure 4. *In vitro* release of upper leathers with microparticles

3.5. Microbiologic Studies on Upper Leathers with Microparticles

It was not seen any clear inhibition zone around the upper leather samples with microparticles in Table 3. On the other hand, slightly inhibition zone was seen on some leather samples examining by microscope. This situation could be interpreted that microparticles don't show antimicrobial property comparing the non-capsulated drug. Effect of applying the microparticles on the upper leather of the diabetic shoes was positive considering that encapsulation is used the permanence of the drug on the material and controlled release.

Table 3. Microbiologic test results of upper leathers with microparticles

Formulation	Staphlylococcus aureus ATCC 6538-P	Escherichia coli ATCC 12228	Pseudomonas aeruginosa ATCC 27853	Candida albicans ATCC 10239	Klebsiella pneumoniae CCM 2318	Enterococcus faecalis ATCC 29212	Staphlylococcus epidermidis
F1							
F2							

Chlorhexidine digluconate diffusion was not occurred on the upper leather surface, because of this inhibition zone was not seen. However, antimicrobial effect can be evaluated with proliferation or without proliferation in the area under the leather samples. This effect is expressed as contact inhibition. It was not seen any proliferation on the contact surface of the upper leathers in Table 3. Also, there was not seen any proliferation surface or edge of the upper leathers.

4. CONCLUSION

In this study, CHD was microencapsulated by the spray drying method using an aqueous EC dispersion, followed by its application on upper leather for manufacturing diabetic shoes. Aquacoat® EC dispersion has proved to be a useful polymer for formulating CHD microparticles using spray drying technology in an aqueous system. Using microparticles produced with an aqueous EC dispersion have the main advantage of being eco-friendly, due to the fact that organic solvents can be avoided. Water was the only solvent used. SEM photographs showed smooth shaped microparticles and good adhesion between the leathers and the microparticles.

In a conclusion, diabetic shoes with microparticles will be an adjuvant therapy to the oral therapy by releasing drug on the applying area for a long time.

5. Acknowledgements

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THERMAL AND ANTIMICROBIAL CHARACTERIZATION OF *OLEA EUROPAEA* LEAF REINFORCED TPU-BASED BIO-COMPOSITES

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In this study, the use of plant leaf obtained after the harvest as a potential reinforcement material in polymer composites was investigated for the production of footwear sole material. For this purpose, olive (*Olea europaea*) leaf was used as the reinforcement material for thermoplastic polyurethane (TPU) based composites. Alkali and silane treatments were applied for modifying the surface of olive leaf to increase the compatibility between the filler and polymer matrix. The preparation of the composites with different filler loadings (10, 20 and 30 wt%) was performed via hot melt extrusion. The bio-composites were characterized using Fourier Transform Infrared (FT-IR) Spectroscopy, Differential Scanning Calorimeter (DSC), Thermogravimetric Analysis (TGA) and Scanning Electron Microscopy (SEM) analyses as well as antimicrobial studies. The antimicrobial activity was tested with Gram-positive and Gram-negative bacterial strains for the prepared bio-composites. The FT-IR results showed that the olive leaf was incorporated into the polyurethane matrix successfully and partial structural modifications were occurred as a result of the alkali treatments. Although the thermal resistance of composite materials at low temperatures was found slightly lower than the TPU, higher thermal resistance values were obtained at higher temperatures. The obtained bio-composite materials were found to be a good candidate to use as bio based antimicrobial footwear sole material.

Keywords: Bio-composites, olive leaf, TPU, DSC, FT-IR, SEM, antimicrobial activity

1. Introduction

In the footwear industry, there are many requirements to ensure the foot comfort such as; wear hygiene, durability of the shoe materials and aesthetic properties of all types of footwear. The shoe sole is one of the most important part of footwear produced from polymer, rubber, leather and neolith (Bonham et al., 1980). All these materials have many different characteristics with their positive and negative aspects. For instance, leather soles are healthy, lightweight and have high air permeability as well as its disadvantages like low abrasion resistance, low sliding strength, high water absorption and high prices (Dikmelik 1994; Cavunt 2005). Polymeric sole materials such as thermoplastic polyurethane (TPU), polyvinyl chloride (PVC), ethylene vinyl acetate (EVA) have many features that can be considered advantageous for footwear soles but still all the features desired are not in one material (Hanhi, 2007;

Maries et al., 2008; Intertek 2010; Intertek 2011; Muehren and Westerdale 2012). Thermoplastic polyurethane soles have advantages like, good abrasion resistance, high flexibility, superior toughness and durability and ease of processing. All these characteristics makes TPU desirable in footwear sole industry (Tayfun et al., 2014).

In recent years, the composite sole production studies have gained importance due to the higher mechanical and other strength properties and decreasing production costs. In particular, in the past few decades there are many researches about natural fiber reinforced polymer composites and various studies have been carried out on the adaptation of these studies to shoe sole production such as bambu fibers reinforcement for the rubber shoe sole production (Toda et al., 2007), wastes of shoe production utilized in rubber sole (Ferreira et al., 2010) and etc.

For this purpose, olive leaves as a potential reinforcement material in polymer composites for the production of footwear sole material was investigated. The olive tree has natural antioxidant contents because of its phenolic compounds (Bayçın et al., 2007). In addition, plenty of olive trees in the Aegean region, conservation of the leaves of olive plant all season, long life features and positive effects on human health allow this plant to use in many areas. Due to these features, olive leaves were used to strengthen the TPU matrix. Surface treatments were applied for modifying the surface of natural fibers to increase the compatibility between the fibers and polymer matrix. The preparation of the composites with different filler loadings (10, 20 and 30 wt%) was performed with extrusion process. The bio-composites were characterized using Fourier Transform Infrared (FT-IR) Spectroscopy, Differential Scanning Calorimeter (DSC), Thermogravimetric Analysis (TGA) and Scanning Electron Microscopy (SEM) analyses as well as antimicrobial studies. The antimicrobial activity was tested with Gram-positive and Gram-negative bacterial strains for the prepared bio-composites.

2.   Materials and Methods

In this study, the olive leaves were used as the reinforcement materials for the thermoplastic polyurethane (TPU) based composites. The olive leaves were supplied from the region of Kemalpaşa, Izmir.

The commercial thermoplastic polyurethane based on polyester (Termopan TPU 65) was used as a matrix component of bio-composites. It does not contain any phthalate and has an antistatic property. It has a density and hardness of $1.19 g/cm^3$ and 67 (Shore A) respectively. Analytical grade sodium hydroxide (NaOH) was purchased from Sigma Aldrich and used for alkaline treatment and 3-(Triethoxysilyl)propilamine (APTES) silane was obtained from Merck, Germany as a coupling agent.

Surface treatments of Bio-fillers

The olive leaves were milled to particle size of 200 μm using laboratory grinder. The olive leaves were treated with 2 wt% solution of NaOH for 120 minutes under continuous mixing. Then the bio-fillers were washed with distilled water until the neutralization of all NaOH and the pH was adjusted to 6-7 using

0.05% acetic acid (CH_3COOH). Later the bio-fillers were dried at 80°C oven for 24 hours (Tayfun et al., 2014; Tayfun et al., 2015). For silane treatment, firstly 0,5 % silane/distilled water solution prepared with 1h mixing process. Then, bio-particles were left in this solution and pH was reduced to 3.5-4 with 1M acetic acid solution. Bio-particles and coupling agent mixture waited during 24h and then washed.

Preparation of composites

The preparation of the composites with different filler loadings (10, 20 and 30 wt%) was performed via hot melt extrusion.

Characterization of bio-composites

Fourier transformed infrared (FT-IR) spectroscopy measurements were performed for TPU, natural fillers and composites by using IR spectrum in ATR mode (Perkin Elmer Spectrum 100) at a resolution of 2 cm^{-1} with 100 scans between 4000-600 cm^{-1} wavelengths.

Thermo gravimetric analysis (TGA) was performed on raw materials and composites using Perkin Elmer Diomand TG/DTA apparatus applying a heat range of 10°C/min up to 600°C.

Differential Scanning Calorimetry (DSC) analyses were carried out using Shimadzu-DSC 60 Plus apparatus. The samples were kept in aluminum vessels and the heat exchange curves with 10 ml/min flow rate of N_2 at 10°C/min temperature rise applying a heat range of 20°C/min up to 200°C.

Determination of antibacterial activity by agar diffusion method

The agar diffusion method was used to evaluate the antimicrobial activity of the bio-composites against *Escherichia coli, Staphylococcus aureus*, *Klebsiella pneumoniae* and *Bacillus cereus*. The composite samples were sterilized both with UV light and in 75% (v/v) ethanol solution for 1 or 2 minutes. Then the samples were placed in separate positions on *Escherichia coli, Staphylococcus aureus*, *Klebsiella pneumoniae* and *Bacillus cereus* cultured nutrient agar plates in aseptic conditions as duplicates. Afterwards, three or four drops of sterile agar were poured onto the film samples to provide the fixing of composites between agar layers. The inoculated agar plates were incubated at 37 ºC for 24h and zones of inhibition were measured in millimeters.

3.   Results and Discussion

FTIR spectra of the TPU matrix, bio-fillers and the composites with different additive loadings are given in Figure 1-4. The characteristic bands of polyurethane matrix appeared at 3333 cm^{-1}, 2956 cm^{-1} and 1726 cm^{-1} due to the stretching vibrations of N-H of urethane group, C-H of alkyl chains and C=O groups, respectively. The vegetable based additives used in the study have complex structures that are usually composed of the mixture of cellulose, lignin, hemicellulose and glucose. They showed similar IR absorption characteristics regarding the phenolic –OH groups, -CH and carbonyl (C=O) group vibrations

observed at 3550-3230 cm^{-1}, 3000-2800 cm^{-1} and 1734-1625 cm^{-1}, respectively. In addition C=C aromatic ring vibrations was observed at 1625-1590 cm^{-1}.

The IR spectra of composites showed both the characteristics of the polyurethane and vegetable additives where the maximum absorbance regions of the vegetable additives at 3000-3600, 1600-1700, 900-1030 cm^{-1} increased the intensity of the spectrum of TPU at the same regions. It was also observed that the increase in the loading ratio of additives also resulted in decreased intensities.

The FTIR spectra of the composites containing alkaline treated additives are shown in Figure 4 and 5. The alkaline treatment leads to partial hydrolysis of the structure of vegetable materials which usually result in breaking of the glycosidic ring. In the IR spectrum of these composites, a decrease in the intensity of the bands related with the aromatic –OH at 3300 cm^{-1} and an increase in the intensity of the –CH peaks at 2850-2920 cm^{-1} were observed. Moreover, the absorption bands of carbonyl groups and aromatic C=C groups at 1716 cm^{-1} and 1512 was disappeared from the spectrum due to the hydrolysis. Also with silane treatment, new peaks were observed. The overall FTIR results showed that the vegetable additives were successfully incorporated into TPU matrix.
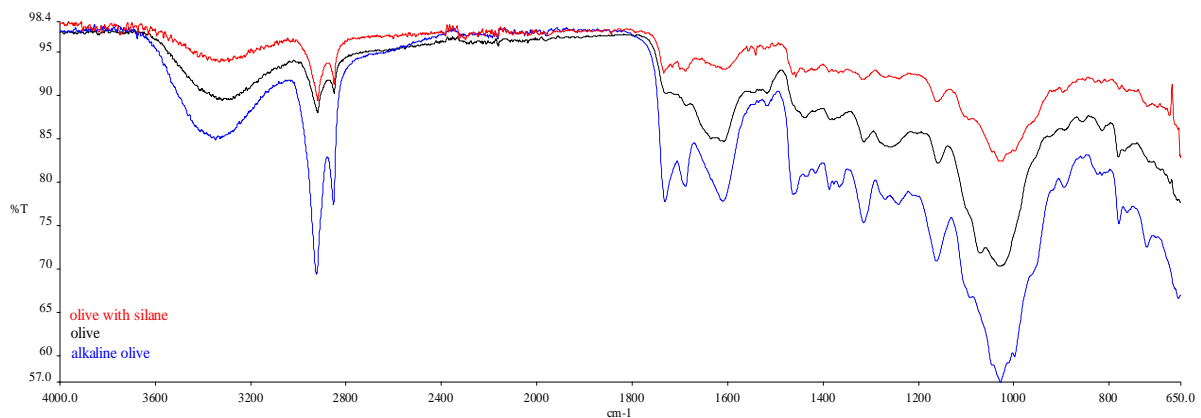

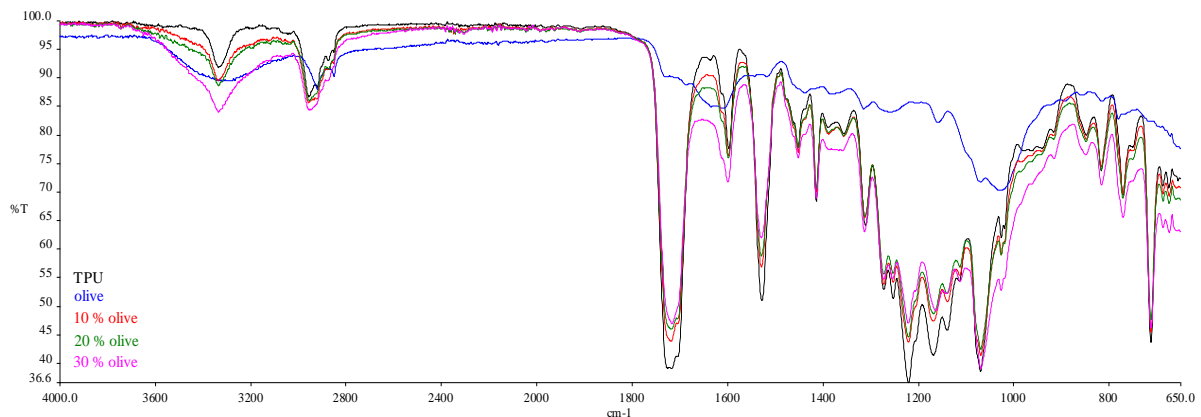**Figure 1.** IR spectra of surface treated and un-treated bio-fillers

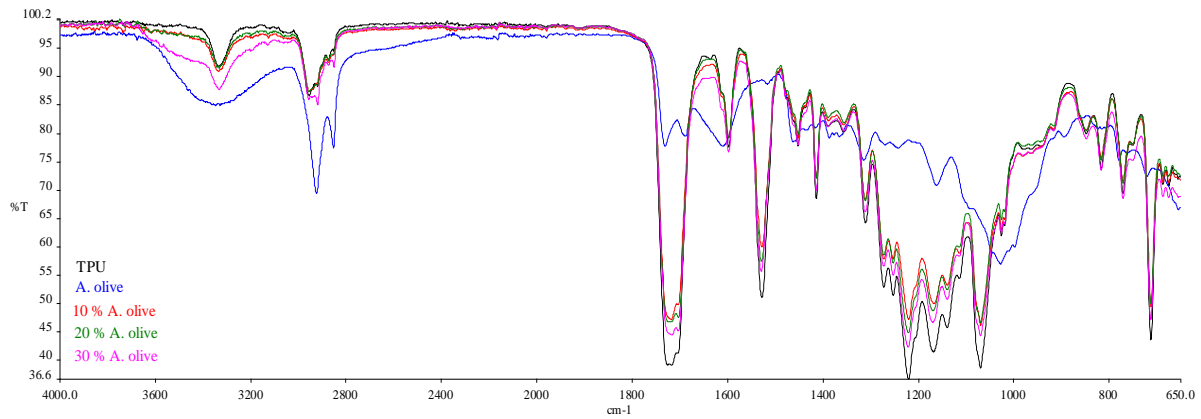**Figure 2.** IR spectra of composites containing olive leaves

Figure 3. IR spectra of composites containing alkaline treated olive leaves
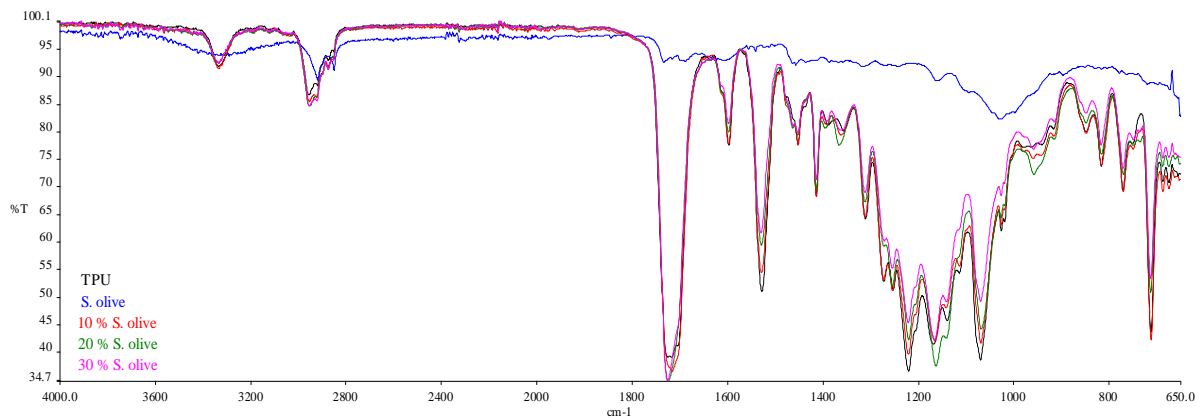

Figure 4. IR spectra of composites containing silane treated olive leaves

TGA results

The thermogravimetric curves of TPU matrix, vegetable additives and their composites are given in Figure 5-7 and some data of their thermal behavior summarized in Table 1. From the results it can be seen that the vegetable materials start to decompose at lower temperatures than TPU. For instance the temperature of 10% mass loss has been found to be at 281°C for TPU whereas for all the vegetable materials were found to be between 217-254°C. On the other hand the decomposing temperatures of 50% and higher mass loss were found to be much higher for vegetable materials than TPU matrix. The composites showed to be affected from both component since their thermal stability at initial and medium temperatures was slightly lower than pure TPU and higher at increased temperatures (>390°C).
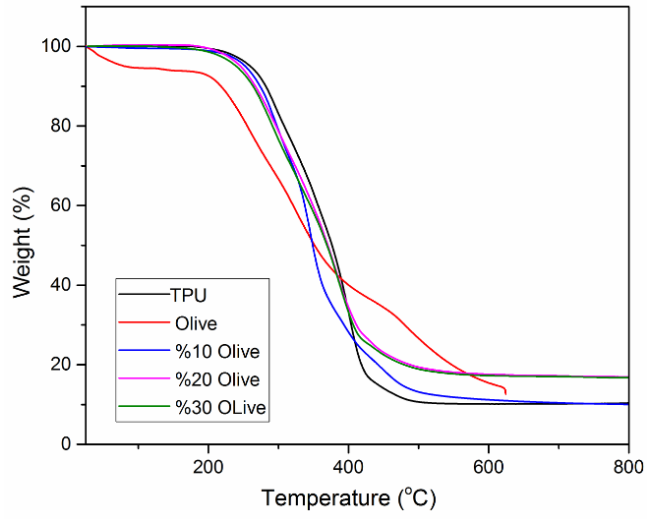
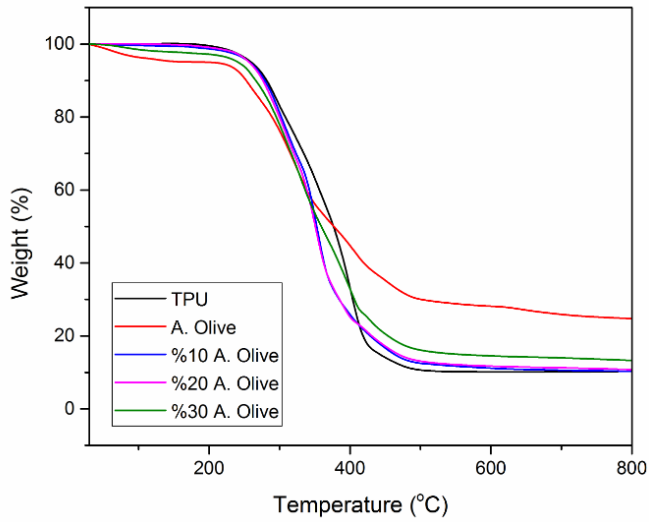**Figure 5.** TGA curves of TPU, olive leaves and its composites

Figure 6. TGA curves of TPU, alkaline treated olive leaves and its composites
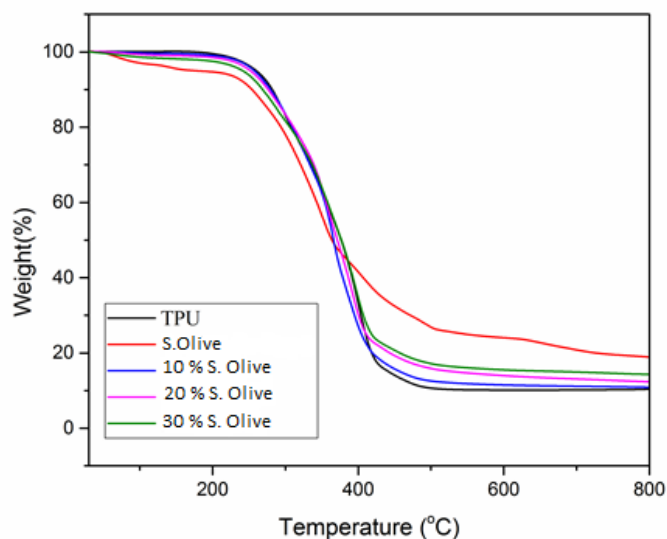
Figure 7. TGA curves of TPU, silane treated olive leaves and its composites

Table 1. Some values about thermal behavior of TPU, and acorn based composites

MATERIAL	$T_{\%10}$ (°C)	$T_{\%50}$ (°C)	Tp(°C)	Weight Loss (%)
TPU	281	376	400	10.50
Olive Leaves	217	351	326	12.53
Alkaline Treated Olive Leaves	252	377	324	24.26
Silane Treated Olive Leaves	254	364	350	17.55
10% Olive Composites	273	348	345	9.78
20% Olive Composites	267	370	382	16.66
30% Olive Composites	263	369	385	16.50
10% A. Olive Composites	279	352	353	10.00
20% A. Olive Composites	276	350	350	10.40
30% A. Olive Composites	266	359	328	10.42
10% S. Olive Composites	279	366	365	10.60
20% S. Olive Composites	276	371	391	11.71
30% S. Olive Composites	268	377	397	13,87

DSC results

The data obtained from differential scanning calorimetry analysis of TPU, additives and composites are summarized in Table 2. From the DSC analysis, the glass transition temperature of pure polyurethane matrix was found to be at -44°C (T_g) and melting temperature at 157 °C(T_m). In the thermograms of composites both thermal characteristics of the components were observed. The glass transitions of TPU segments were observed between -40 and -47 °C, endothermic water removal peak of additives was observed at around 100 °C and melting peak of TPU segments was observed

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between 150-160°C. The surface treatments did not show significant effect on DSC behavior of the composites.

Table 2. The results of DSC analysis of TPU, additives and composites

Material	Tg (^{o}C)	Pic 1 (^{o}C)	Pic 2 (^{o}C)
TPU	-44	-	157.45
Olive Leaves	-	74.43	148.42
A. Olive	-	79.29	152.15
S. Olive	-	71.12	150.22
%10 Olive Composites	-43.34	-	167.13
%20 Olive Composites	-40.14	58.59	155.19
%30 Olive Composites	-42.59	79.34	155.22
%10 A. Olive Composites	-43.55	-	157.87
%20 A. Olive Composites	-42.50	-	156.82
%30 A. Olive Composites	-	-	37.10
%10 S. Olive Composites	-45.59	100.95	157.81
%20 S. Olive Composites	-46.94	30.71	147.97
%30 S. Olive Composites	-46.46	104.84	219.60

Antimicrobial results of composites

The antimicrobial results of the biocomposites are shown in Table 3. The bio-composites had no antimicrobial effect on *S. aureus and B. cereus* organisms. But positive results were obtained from *E. coli* and *B. cereus* microorganisms and the highest inhibition zone was obtained from 30% alkali treatment of olive bio-composite for both strains. Also, the other bio-composites gave similar results for these two organisms.

Table 3. Inhibition zones of the bio-composites

	S. aureus	*K. pneumoniae*	*E. coli*	*B. cereus*
10% S. Olive	-	0.4	0.6	-
20% S. Olive	-	0.5	0.4	-
30% S. Olive	-	0.6	0.4	-
10% Olive	-	0.25	0.4	-
20% Olive	-	0.55	0.4	-
30% Olive	-	0.45	0.55	-
10% A. Olive	-	0.4	-	-
20% A. Olive	-	0.45	0.5	-
30% A. Olive	-	0.85	1.9	-

4.   Conclusion

In this study, the use of vegetable fillers as a potential reinforcement material in polymer composites for the production of footwear sole material was investigated and following conclusions have been drawn;

1.   The vegetable fillers were incorporated into the polyurethane matrix successfully and partial structural modifications were occurred as a result of the surface treatments.

2.   Although the thermal resistance of composite materials at low temperatures was found slightly lower than the TPU, higher thermal resistance values were obtained at higher temperatures.

3.   Homogenous dispersion of vegetable fillers within the polymer matrix was achieved successfully and the obtained bio-composite materials were found to be a good candidate to use as bio based footwear sole material.

4.   Antimicrobial effect of biocomposites on *K. pneumonia* and *E. coli* was found and the highest inhibition zone was obtained from the alkali treatment of 30% olive leaf containing bio-composite.

5.   Acknowledgement

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SELF ASSEMBLED COLLAGEN-PVDF-GRAPHENE OXIDE THIN FILM AS PIEZO - ELECTRIC   SENSOR

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Collagen -PVDF- Graphene Oxide thin films were made by self-assembling aqueous solution of Polyvynyldifluoride (PVDF) and collagen with graphene oxide as substrates in Poly vinyl Chloride (PVC) trays at room temperature. SEM micrographs showed that the surface of the films were uniform and distributed with the nano particles. X-ray diffraction (XRD) studies showed that the PVDF films settled in two dimensional phase. Raman results together with XRD studies confirmed that the synthesized films are single phase PVDF graphene with good stoichiometry. FTIR studies of the films showed a strong piezo spectra obtained at low temperature (25°C) exhibit better resolved Piezo electric peaks of the CV caused by the combination. The dominant peaks observed in the FTIR spectra, Characteristic spectra confirmed high quality of the Collagen-PVDF- Graphene thin film piezo electric sensor.   The report has been summarized by highlighting recent nano generator developments and future prospects including high power nano generators, energy storage/regulation systems and fundamentals on piezoelectricity. The property enhancements were directly related to the strong and specific interfacial interaction that results in the adsorption of macromolecular chains of PVDF onto the GOn surface. This article highlights Nano fiber based Nano electric generators, experimental characterizations using collagen from waste leather, PVDF (polyvinylidene fluoride) and Graphene-Oxide. Collagen exhibits the polar uni axial orientation of molecular dipoles in its structure and can be considered as bio electrets, a sort of dielectric material exhibiting quasi permanent space charge and dipolar charge. (Fukada et al). Piezoelectric Nano generators made of PVDF (polyvinylidene fluoride) or PZT (lead zirconate titan ate) and fabricated by means of electro spinning processes such as conventional, modified or near-field electro spinning (NFES) are the key focuses of this paper. This low cost manufacturing route is to induce piezoelectric behavior in PVDF polymer and to improve its properties for new technological applications.

Key words

Collagen, PVDF, Graphene oxide, Piezo electric sensor, Characterization.

Introduction

Nanotechnology is a group of emerging technologies in which the structure of matter is controlled at the nanometer scale to produce novel materials and devices that had useful and unique properties (Jeremy Ramselen, 2009). Nanostructures had very different characteristic and novel properties with respect to the bulk material and also they have physical and chemical properties, in particular large surface area to volume ratio and quantum size effects. They also permit applications such as nanoscale devices, sensors and scanning probes which are not possible with standard structures (Giovanni Attolini *et al*. 2010).Semiconductor Nanowires (SWs) and Carbon Nanotubes (CNT) had shown particular promise. In contrast to Nanotubes, controlled Semiconductor Nanowires can be synthesized in the single crystal form with all key parameters including Chemical composition and properties (Mark S. Gudiksen *et al*. 2001). Thus, Semiconducting Nanowires represented the best defined class of nanoscale building blocks. Nano fiber based piezoelectric energy generators had scalable power sources, applicable in various electrical devices and systems by scavenging mechanical energy from the environment.Piezoelectric Nano generators made of PVDF (poly vinylidene fluoride) or PZT (lead zirconate titan ate) and fabricated by means of electro spinning processes such as conventional, modified or near-field electro spinning (NFES) are the key focuses of this paper. Material and structural analyses on fabricated nano fibers using tools such as XRD (X-ray diffraction), FTIR (Fourier transform infrared), and SHG (second harmonic generation), PFM (piezo response force microscopy) and Raman spectroscopy towards the fundamental characterizations of piezoelectric nano fibers were also described.

Materials and method

Materials

Graphene had been synthesized in various ways and on different substrates. In the following, we summarize the synthesis and methods. Graphene had been synthesized in Hummer's method.

Collagen is a family of highly developed fibrous protein found in all multicellular animals. It is the most abundant protein found in mammals constituting 25percent of the total protein mass. Collagen is the main fibrous component of skin, bone, tendon, cartilage and periodontium.PVDF and graphene oxide nano sheets can be mixed in conditions enabling the formation of a homogeneous solution in DMF, resulting in a stable solution at room temperature. By casting solutions of PVDF and PVDF/GOn mixture on glass plates, the high quality and flexible graphene oxide Nano sheets (GOn)-filled PVDF nano composite films had been produced. Because of the high compatibility between GOn and PVDF, GOn are well dispersed and distributed within the PVDF matrix.

Methods

It can be made from large scale and pilot scale in future for more energy conservation. This is an alternative source of energy from the waste material is recycled. Input given to the voltage booster is very small in terms of milli volts and microampere that can be boosted to 3V to 6V DC and current 500mA. Electrodes such as Tin-Anode and Gold plated-Cathode were used. The thin film is made as a piezo electric cell. The collagen is already proved that it is a piezo electric material. Further we intended

to develop these materials as a source of electrical energy or we wanted to convert pressure to voltage. It can be connected in parallel and series connection to generate the voltage and current. The aim is to develop a prototype piezoelectric cell for conservation of electrical energy. Later on this energy generated in terms of milli Volts and microamperes that can be boosted using a voltage booster (Self acting voltage booster).As a basic building block of other carbon allotropes, graphene can be wrapped to generate 0D fullerenes rolled up to form 1D carbon nanotubes and stacked to produce 3D graphite. It has been making a profound impact in many areas of science and technology due its remarkable physicochemical properties. These include a high specific surface area, extraordinary electronic properties, and excellent thermal and electrical conductivities. Collagen is a semiconducting material. It synthesized from waste leather materials. It is a natural semiconducting material having resistance of below 20Kohm. We are mining suitable binding materials such as hydroxyl ethyl cellulose, carbon nanoparticles, graphene, contacting ink synthesizing using these materials. Piezo materials are included such as PMMA, this material is made into a block of 10/10cm size. We are incorporating or fusing electrodes in these materials.

Synthesis of graphene

Preparation of graphene oxide

1g of graphite is added to 23ml mixure of zero degree conc.H_2SO_4 and 3g $KMNO_4$.Keep it in an ice   bath (stirring) then 1 hour maintained the temp at below 20degreeCelsius. It is stirred at 35 degree for ½ hour 46ml was slowly added, the temp was raised to 98degree Celsius and heated for 15 minutes then added 140ml of distilled water and 10ml of 30% H_2O_2 added then kept the above content in centrifuge and residue washed with 5% HCL (5times) later on, washed with acetone and dried at 65degree Celsius for 12 hours.

Reduction of grapheme oxide

GO 100mg in 100ml distilled water then sonicated for ½ hour, added NH_3 to increase the PH => 12, Then added 250ml RB flax (100degree Celsius)   reflex after 24 hours black precipitate was formed later Washed it with water and acetone then dried at 60degree Celsius.

Synthesis of collagen

 500g of chrome shaving added to 500ml of NaOH solution for 2 hours. Then washed it cleanly and added 50 ml Conc.H_2SO4.It was mixed and kept it for 30 minutes. Washed it cleanly until it becomes white color then grinded it and kept it in cold room.

Results and discussion

Characterizations of synthesized graphene oxide
FT-IR Analysis

FT-IR stands for Fourier Transform Infrared, the preferred method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through the sample. Some of the infrared radiation is absorbed by

the sample and some of it is passed through (transmitted). The resulting spectrum represented the molecular absorption and transmission, creating a molecular fingerprint of the sample.Assignation (cm-1): for graphene oxide 1719 C=O (carbonyl/carboxyl); 1621 C=C (aromatics); 1337 C-O (carboxyl); 1228 C-O (epoxy); 1039 C-O (alkoxy). In comparison peaks, oxygen functional groups were almost entirely removed from reduced graphene oxide. Y-axis is transmittance and X-axis is Wave number.

Raman spectroscopy

The G, graphene peak is the result of plane optical vibrations and had a literature value of 1598 cm-[1]. The D peak is located at 1358 cm-1 and it was due to the first order resonance. This peak was absent in defect free graphene nanosheets. X-axis is Raman Shift and Y-axis is intensity.

The main features in the Raman spectra of carbons were the so-called G and D peaks, which lie at around 1560 and 1420 cm−1 respectively for visible excitation. In amorphous carbons a peak at around 1060 cm−1 (T peak) was seen in UV excitation [14]. Except for UV excitation, the Raman spectra of carbon films were dominated by the sp2 sites, because visible excitation always resonated with the π states. Thus even for highly sp3 amorphous carbon samples, the visible Raman spectra were due to sp2 vibrations. Only for diamond or samples containing a significant fraction of diamond phase, the diamond sp3 peak 26.5° for pristine graphite, 9.1° for graphene oxide, 13.5° for graphene HG-0.5, 13.7° for graphene HG-2, and 13.3° for X-ray diffraction patterns for graphene hydrogel are shown in figure 9. The diffraction peaks were graphene HGS-2, corresponding to a layer-to-layer distance of 3.36Å, 9.68Å, 6.55Å, 6.45Å, and 6.64Å, respectively. The interlayer distance for graphene oxide is significantly larger than for pristine graphite due to the intercalating oxide functional groups. The following popper user interface control may not be accessible. Tab to the next button to revert the control to an accessible version. The interlayer distance for graphene hydrogel was lower compared with graphene oxide, indicating the removal of oxide functional groups from the graphene sheets after the hydrothermal process. Meanwhile, the appearance of a characteristic peak of graphite for non sonicated graphene hydrogel, which shifted slightly to the left, suggested the existence of π–π stacking between graphene sheets due to the recovery of a π-conjugated system from the graphene oxide nano sheets upon hydrothermal reduction. Their interlayer spacing was slightly higher than for graphite, which was 3.63 Å and 3.39Å, respectively, for graphene HG-0.5 and graphene HG-2. The diffraction peak and interlayer distance of graphene HG-2 is the closest to that of graphite because of its relatively high concentration.

XRD analysis

Left, XRD pattern of graphite, graphene oxide and reduced graphene oxide. Right, fitting curve for reduced graphene oxide (002) peak. In the XRD pattern of GO, the strong and sharp peak at 2θ = 11.7ºcorresponds to an interlayer distance of 7.6 Å (d002). Reduced graphene oxide showed a broad peak that can be fitted by using a Lorentz an function into three peaks centered at 2θ = 20.17º , 23,78º and 42.88º, corresponding to interlayer distances of 4.47, 3.82 and 3.53 Å, respectively. These XRD

results were related to the exfoliation and reduction processes of GO and the processes of removing intercalated water molecules and the oxide groups.

SEM (Scanning Electron Microscopy) Analysis

To study the morphology of obtained graphene oxide, the green product was first get dispersed by the Di methyl formamide solvent and after it got coated on the surface of Aluminium foil. The coated Aluminium foil was dried for solvent evaporation and then SEM image was taken with 10.0KV applied Voltage. Through self-assembly at the liquid/air interface, monolayer graphene oxide sheets aggregated into a semi-transparent GO membrane. It presented a scanning electron microscopy (SEM) image of a typical GO membrane (heating period of the hydrosol: 40 min), which showed that this membrane is of uniform thickness (10mm) and relatively smooth surface. The thickness could be precisely controlled in a range of 0.5–20mm by adjusting the evaporating time of the hydrosol. It showed the top surface SEM image, from which the individual graphene oxide sheets could be clearly identified as 'building blocks' for the membrane. The cross-sectional image, as shown in Figure, exhibits a compact layer-by-layer stacking of graphene oxide sheets.

SEM Images of Collagen Graphene film with different magnification

SEM Analysis

SEM is more convenient for imaging large areas of conducting samples. The electron beam directed at the sample typically had an energy ranging from 0.5 keV to 40 keV, and a spot size of about 0.4 nm to 5 nm in diameter. The image, which was formed by the detection of backscattered electrons or radiation, could achieve a resolution of ~ 10nm in the best machines. Due to the very narrow beam, SEM micrographs have a large depth of field yielding a characteristic three dimensional appearance. Examples of SEM images of suspended graphene devices are shown in Figure10. A very useful feature available with SEM had the possibility to write sub-micron size patterns by exposing an e-beam resist on the surface of a sample. The disadvantage of using the SEM for imaging is electron beam induced contamination due to the deposition of carbonaceous material on the sample surface. This contamination was almost always presented after viewing by SEM, its extent depending on the accelerating voltage and exposure. Contaminant deposition rates could be as high as a few tens of nanometers per second

Characterization of piezoelectric sensor

CV Method:

The characterization has been performed using CV mammogram

CV of Collagen Graphene PVDF thin film

Fig 11 shows the collagen based gold nanoparticles impregnated thin film for which the CV mammogram is shown for different concentration.

Conclusion

It is an innovative product using electrodes such as Tin-Anode and Gold-Cathode; this is made as a piezoelectric cell. Thus collagen is already proved that it is a piezoelectric material. Further we want to develop these materials as a source of electrical energy or we want to convert pressure to voltage. The as-prepared nano composites exhibited a purely piezoelectric -polymorph for GOn level as low as 0.1 wt. %, while a mixture of polymorph is found below that concentration. The tensile properties of nano composites were remarkably improved by the incorporation of GOn into PVDF matrix. Adding 2 wt. % of GOn results in an increase of 13.7%for the Young's modulus and of 92% for the tensile strength. The thermal stability of PVDF was increased by 65ºC at 2 wt. % GOn content.

As the surface area increases, the electro and mechanical properties are increased. It can be connected in parallel and series connection to generate the voltage and current. This is to develop a prototype piezoelectric cell for conservation of electrical energy. Later on this energy generated in terms of milli Volts and micro amperes that can be boosted using a voltage booster (Self acting voltage booster).It can be made large scale and pilot scale in future for more energy conversions. Since we are making this materials cost is very much reduced. Raw materials are cheap and we can benefit in enormous. This is an alternative source of energy from the waste material, is recycled. It can be made as a cottage industry and small scale industry investing around 10 lakhs Indian rupees.

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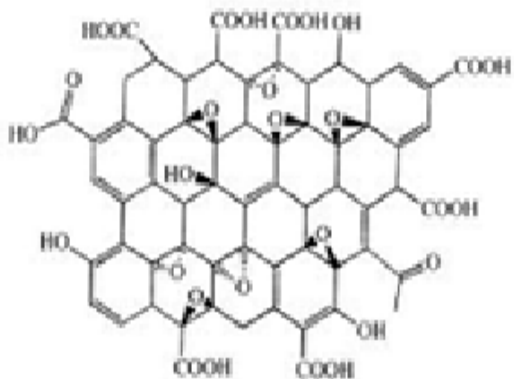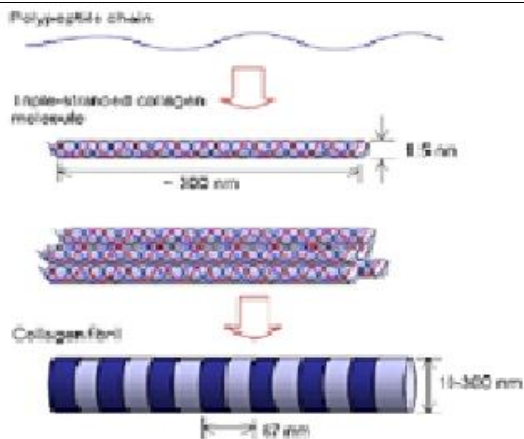
Figure.1. Structure of graphene oxide

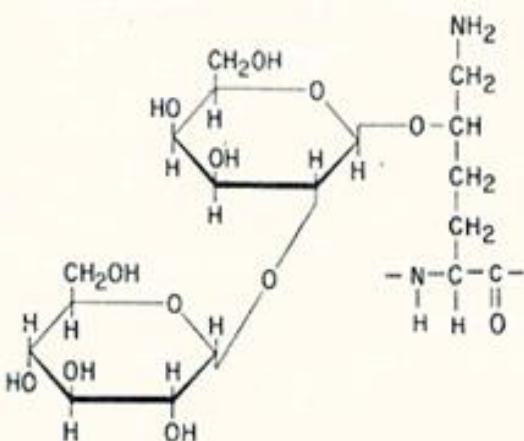**Figure 2**. Flow scheme for preparation of collagen fibril
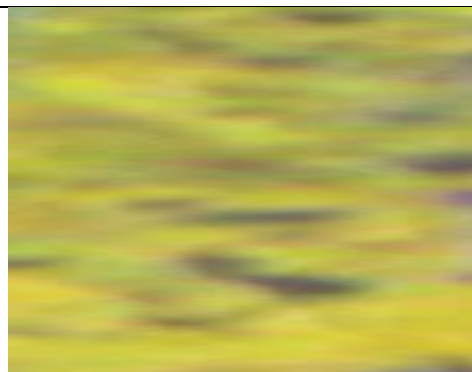

Figure 3. General structure of the collagen

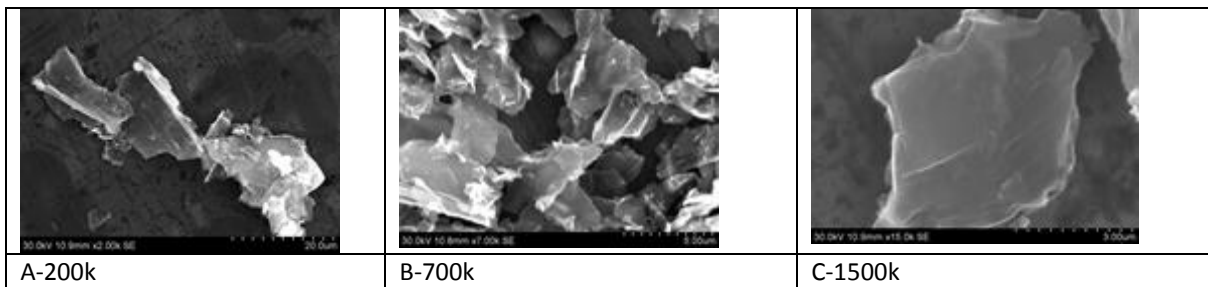**Figure. 4**: Collagen PVDF gold piezo electric cell

Figure 5. FTIR spectra of synthesized grapheme oxide

Figure 6. Raman Spectroscopy of graphene collagen thin film. The Y axis represents the Raman shift and the X-axis represents the Intensity.(Control)

Figure 7.Experiment Raman Spectroscopy of Collagen Graphene PVDF thin film

Figure.8 Results of XRD Analysis on the sample
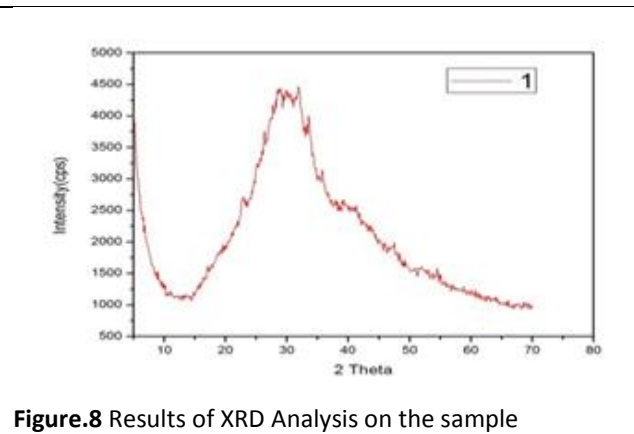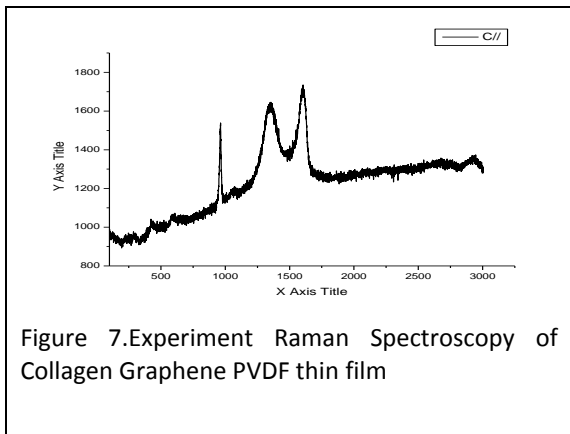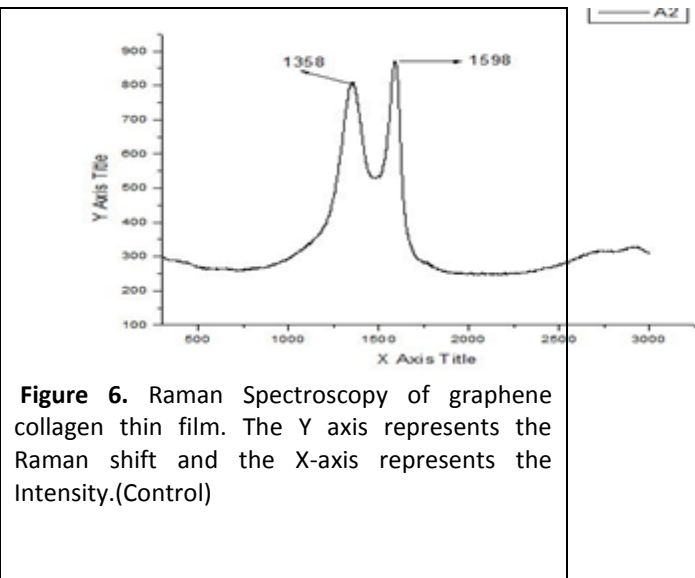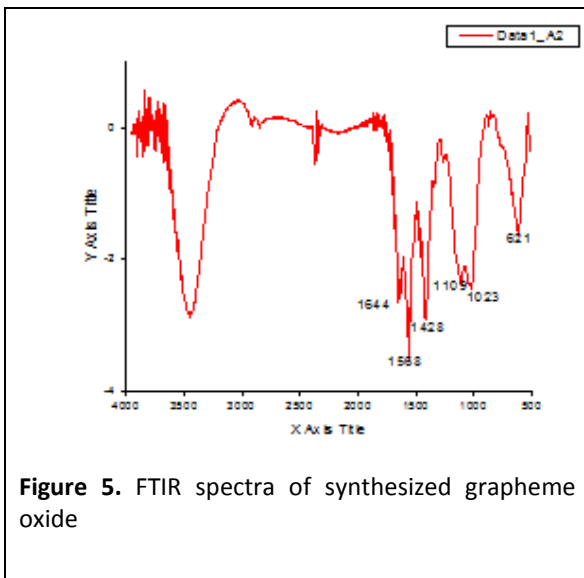

| A-200k | B-700k | C-1500k |

Figure 9. SEM (Scanning Electron Microscopy) Analysis of graphene nano particles embedded in collagen thin film
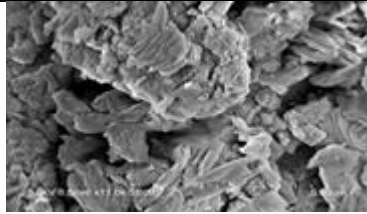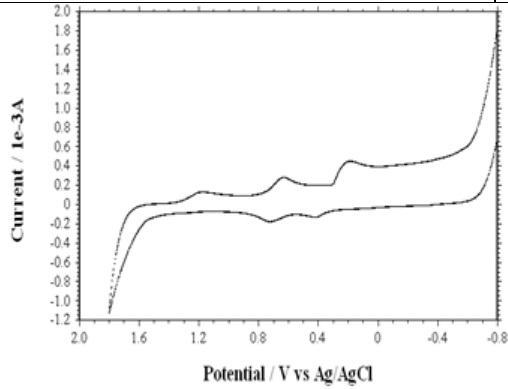
Figure 10. SEM of grapheme particles

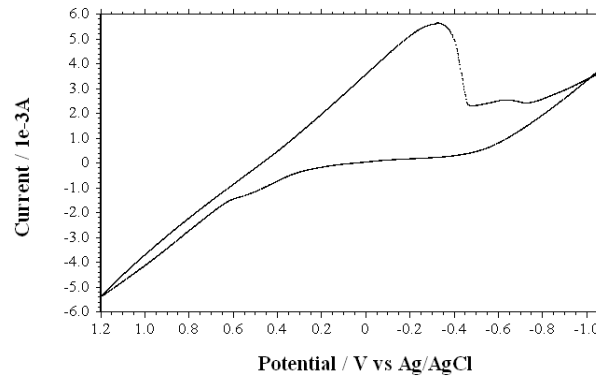**Figure 11a**. Collagen to GN dissolved in different concentration (Control)

Figure 11b. Collagen to GN dissolved in different concentration (Experimental)

NATURAL PHENOLICS PREVENTING AGING IN LEATHER

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Gallic acid esters are a class of polyphenolics that have been found in our previous work to be very suitable antioxidants for leather securely preventing the formation of Cr(VI). In fact, alkyl gallates with long carbon chain have an efficiency that is far superior to tara or gallic acid. In this work we compare gallic acid derivatives with DL-tocopherol (Vitamin E) and tocopherol derivatives, discussing the results in antioxidant efficiency in leather in the framework of the polar paradox and the efficiency of the very phenolic groups. The final aim of this work is the improvement of sustainability of leather by increasing quality and durability of leather articles and a better protection against the formation of Cr(VI) by natural ingredients.

Keywords: antioxidants, Cr(VI), tocopherol, gallic acid esters

1. Introduction

Leather is a high quality material, which, for considerations of sustainability should preserve its natural beauty for many years. Very good mechanical and chemical fastness and its durability make leather a material which is used for much longer time than synthetic alternatives. However, there are aging processes occurring in the leather deteriorating its quality. Normally, the extent of leather aging depends on environmental factors, such as heat or light. Its mechanism has been studied in detail and it is a generally accepted fact that aging of leather starts with a reaction at the fatliquor. The initial step of this process is that a hydrogen radical is abstracted at the ⍺-position to a conjugated double bond of the fatliquor (Candar et al. 2001; Segura et al. 2000) forming a carbon cantered radical. Followed by this initiation step, more radicals including oxygen cantered peroxy radicals are formed in a cascade type reaction. As a result, many unwanted deterioration processes are occurring in the leather matrix, at the collagen and at many other components present in the leather. The entire aging process is thus a chain-reaction, called autoxidation, which can be divided in initiation, propagation and termination.

The result of the autoxidation is distinguished by different phenomena by which the extent of aging can be quantified: they can be divided into organoleptic, mechanical or purely chemical degradation signs.

There are basically two possibilities to make leather more durable. One can

(a) Prevent the radical formation (initiation). Although difficult, by optimizing the storage conditions of leather or by using suitable UV blocking finishing or special UV absorbers, aging processes can be significantly slowed down.
(b) Introduce substances, which terminate the chain reaction without creating new radicals. These substances, called primary anti-oxidants, normally react with the oxygen cantered peroxy-radicals formed during autoxidation and which, due to their high reactivity, are of foremost importance for the degradation of leather

The antioxidants use for the protection of leather are basically of three different groups

- Phenolic compounds
- Aromatic amines
- Hindered amines

There are different approaches for using antioxidants. Often, antioxidants are part of the fatliquor composition, what is very reasonable, since their deposition in close vicinity to the fatliquor molecules guarantees a high probability that they show an optimum effect. On the other hand, many vegetable and some synthetic type tanning agents are themselves acting as phenolic type antioxidants. Thus, a proper retannage can give a substantial protection against at least some signs of aging.

As far as natural antioxidants in leather are concerned, studies with hydrolysable tannins, especially tara, for preventing Cr(VI) formation have been done (Font et al. 1999). Hydrolysable tannins contain a high number of H-donating phenol groups, which are known to be very efficient primary antioxidants. Also other natural compounds containing polyphenolics have been found successful for the suppression of Cr(VI) formation, such as plant extracts of walnut leaves (Bayramoglu et al. 2012), tannic acid (Colak et al. 2014), gallic acid(Devikavathi et al. 2014), tocopherol(Liu and Latona 2003) and bayberry extract(Ma et al. 2012). Our recent work was focused on the antioxidant activity of gallic acid esters and tara hydrolysates (Kilikli et al. in print). As far as synthetic antioxidants are concerned, there is a general recommendation made by Font et al. (1999) to make use of 1:1 mixtures of phenolic and aminic antioxidants. In fact, fatliquor producers are testing the best synergetic mixtures of antioxidants for the very formulation and optimize their incorporation into the final product thus improving the quality and durability of the leather articles their products are used for.

In the current paper we would like to compare different natural antioxidants regarding their efficiency in various aging protocols.

The following antioxidants are compared with each other

- Gallic acid
- Hexadecyl gallate (C16 Gallate)
- fully synthetic phenolic antioxidant (SAOx). A non-yellowing, commercially successful antioxidant was chosen

- DL-tocopherol (Vitamin E)
- DL-tocopheryl acetate (Vitamin E acetate)
- Trolox

Notably, the aim is to investigate the structure-efficiency relation of the different compounds. For this reason, the comparison was done based on the same concentration of phenolic OH-groups rather than the same amount.

2. Materials and methods

Anhydrous gallic acid (for synthesis) was purchased from Merck KGaA (Germany). Hexadecyl gallate was bought from TCI Europe N.V. (Belgium). **Tara Powder FP** of Exandal S.A. (Peru) with a tannin content of 52% and a particle size of <100 µm was used. DL-Tocopherol (Vitamin E) was purchased from Klüver & Schulz (Germany), Trolox 96% from Acros Organics (Belgium), DL-Tocopheryl Acetate 97% (Vitamine E Acetate Care) was supplied by BASF (Germany). The Synthetic Antioxidant (SAOx), Benzenepropanoic acid, 3,5-bis (1,1-dimethyl-ethyl)-4-hydroxy-C7-C9 branched alkyl esters (Irganox 1135), was supplied by BTC Europe GmbH (Germany). All solvents used were of either analytical or spectroscopic grade (Sigma Aldrich).

Total phenolics of tara was determined following the established method(Chambia et al 2013) using gallic acid as a standard. Absorbance was measured at 755 nm and the result was expressed as gallic acid equivalents (GAE). For all other antioxidants the total phenolic content was calculated based on chemical structure with the following formula:

(1) $GAE = 170.12 \text{ g mol}^{-1} * n_{(OH)} / M_w * 3$

With n(OH) being the number of phenolic OH groups (gallic acid: $n_{(OH)} = 3$) and M_w the molecular weight of the antioxidant (gallic acid: $M_w = 170.12 \text{ g mol}^{-1}$).

Leather trial. For the leather trial, a commercially successful sulfited fish oil based fatliquor of 80% concentration having a fish oil content of 50% was used. The different antioxidants were mixed into the fatliquor after being pre-dissolved in a small amount of isopropanol (3% based on amount of fatliquor). The reference consisted of the same fatliquor without any antioxidant, but having the same portion of isopropanol incorporated.

Spanish split wet blue bovine leather was washed (60 min, 0.2% formic acid, 1% ethoxylated fatty alcohol), re-chromed (4% Cr-Sulfate 33% Basicity, 1% Alumn silicate), neutralized (2% Na-formate, 2% Na-bicarbonate, 110 min) to pH 6.5 and then fatliquored with 2x7% of aforementioned fatliquor at 50ºC for 2x45 min. After that, the fatliquor was fixed with 3 additions of 1% of formic acid to a bath pH of 3.7. The leathers were horsed-up air dried horizontally. All % are based on shaved weight.

Cr(VI) measurement in leather were conducted following DIN ISO 17075:2008-02, after a pre-ageing of 24h at 80ºC with air flow and a repose time of 1h. The leathers were cut to pieces of approximately 4x4mm before aging. Inhibition was calculated as

(2) % Inhibition = (C − S)/C* 100,

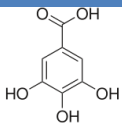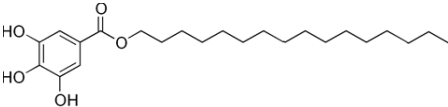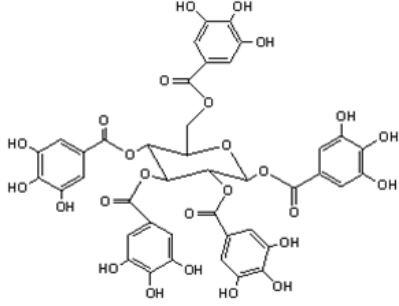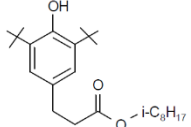where C and S are the test results of the sample without and with antioxidant, respectively.

Shrinkage temperature T_s was measured in a mixture of glycerin:water 1:1 following DIN EN ISO 3380. For the assessment of aging, the decrease in shrinkage temperature by a thermal treatment for 24h at 80ºC was determined. Leathers used for the assessment of shrinkage temperature contained 0.2% GAE of antioxidants.

Odor was measured after 2h at 80ºC using leather samples containing 0.2% GAE of antioxidant. The evaluation of rancid type odor was done by 4 experienced lab technicians and expressed as scores from 1 (very noticeable) to 5 (not noticeable).

Heat yellowing was done for 288h at 100ºC with air flow, followed by the determination of yellowness index according to ASTM E313. The leather used for heat yellowing contained 0.1 % GAE of antioxidant. Results are expressed as difference in Yellowness Index, ꓒYI

3.   Results and discussion

As already explained, the trials with antioxidants were done based on the same **total phenolic content** (expressed as gallic acid equivalents, GAE) and not based on the same amount. Having in mind that it is the phenolic moieties that are scavenging free radicals, this molar approach gives a better understanding for the efficiency of the very molecule. The GAE values and the chemical structure of the antioxidants used are depicted below:

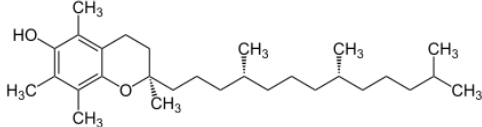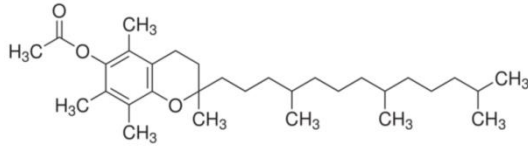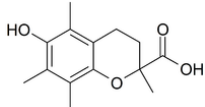| Component	GAE	Chemical Structure
Gallic acid	1.00	
Hexadecyl Gallate	0.40	
Tara	0.49	(fragment)
SAOx	0.15	

DL-Tocopherol	0.13	
DL-Tocopheryl acetate	(0.12)	
Trolox	0.23	

Table 1. Values of gallic acid equivalents (GAE) and chemical strtucture of the samples tested

Results

Assessment of reduction of Cr(VI) formation in leather. In many of our previous trials with antioxidants we had found that the measurement of proneness to Cr(VI) formation, a chemical sign of aging, is in fact a very precise means to determine the capability of an antioxidant to prevent aging since very exact and reproducible values are obtained. The results given are based on average values of samples taken from different areas of the respective leathers.
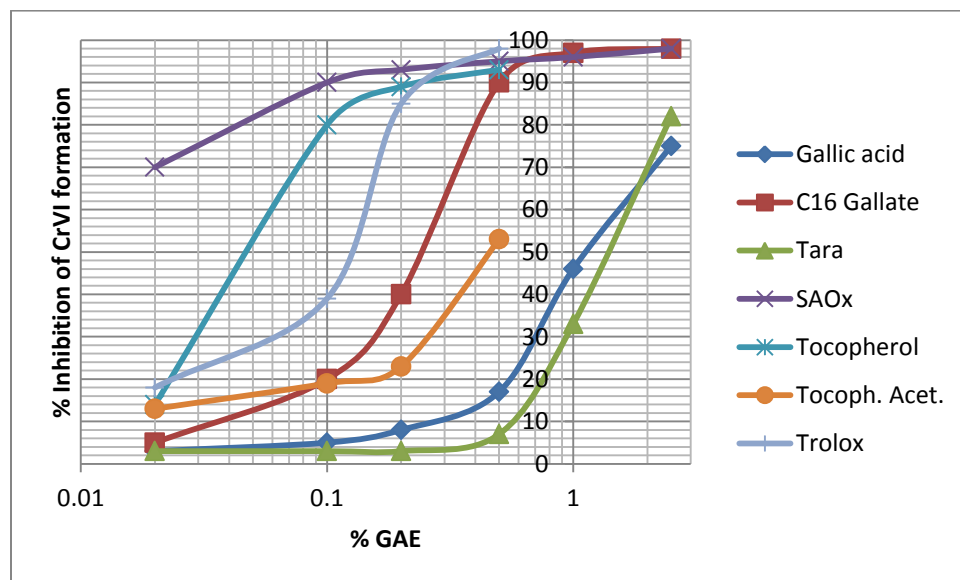
Fig. 1. Results of inhibition of Cr(VI) formation using 0.5% GAE antioxidant based on fatliquor weight

Measurement of decrease shrinkage temperature. The decrease of shrinkage temperature, a physical sign of leather aging, by thermal treatment can be attributed to a partial rupture of chemical bonds in the collagen or between collagen and the tanning agent, possibly by direct attack of oxygen centered peroxide radicals. Thus, the evaluation of the effect an antioxidant has on the decrease of shrinkage temperature is another means for the determination of the efficiency of an antioxidant. Results obtained with 0.2% GAE of antioxidants are depicted below
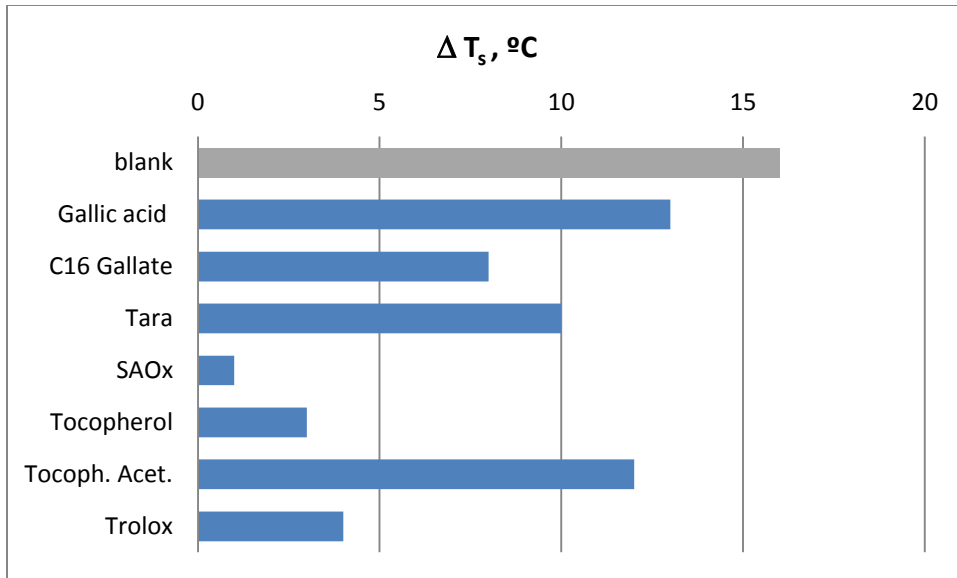
Fig. 2. Decrease in shrinkage temperature in relation to the different antioxidants used

A possible evolution of **rancid odors** by oxidation processes is of importance for the quality and usage time of a leather article. These odors originate from the formation of aldehydes and ketones, often stemming from the reduction of peroxides of the fatliquor, natural grease or other components of the leather matrix.

Results for the assessment of odor with the use of 0.2% GAE of the different antioxidants are displayed below.

Fig. 3. Odor formation with the use of different antioxidants

Heat Yellowing is the probably most prominent method for assessing aging in leather. Generally, yellowing is due to the formation of chromophoric groups, especially conjugated double bonds, in the

course of autoxidation. When investigating different antioxidants, the interpretation on the basis of heat yellowing values is at times misleading, since some very powerful antioxidants yellow themselves, give an intrinsic coloration to the leather or interact with other components in the leather matrix. The trials presented here were done with 0.1% GAE of antioxidant: a relatively small quantity has been chosen in order to keep the yellowing of the antioxidant itself low.

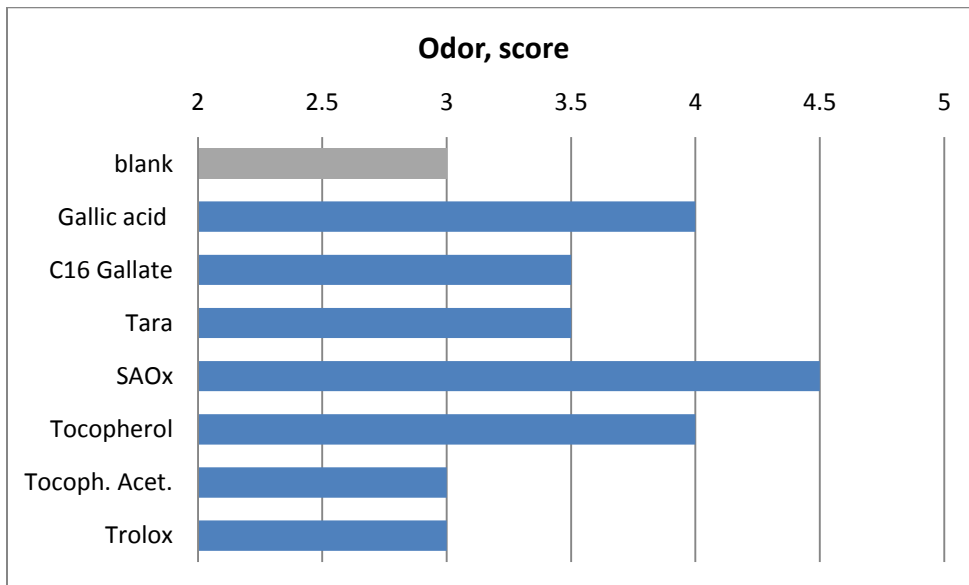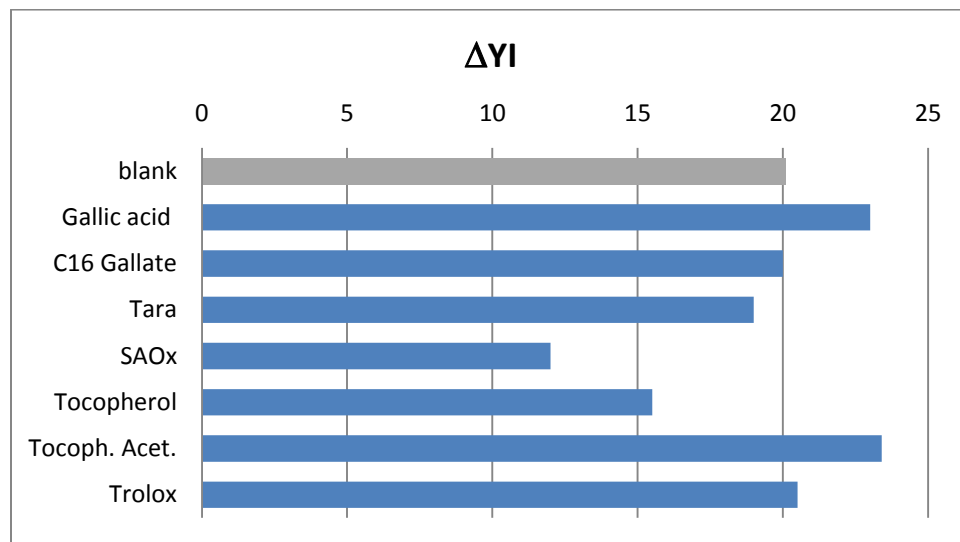*Fig. 4. Heat yellowing by the use of different antioxidants*

Discussion

When used based on the same phenolic content, the efficiency of tara and gallic acid for the suppression of Cr(VI) formation are similar. As had been demonstrated in our previous work, long chain alkyl gallates are very efficient antioxidants for leather fatliquors. In fact, with the increasing lipophilic character of the alkyl gallates they have a higher tendency to be dissolved in the oil droplets of the emulsion. When the leather dries, the alkyl gallates are deposited together with the oil. Notably, due to the fact that mobility of the components in dry leather is very limited, it is of foremost importance that the antioxidant is evenly distributed on the very oil and does not form clusters or is associated in other parts of the leather. The general empirical principle, which is behind this explanation is called the polar paradox, which predicts that the efficiency of a non-polar antioxidant is better in a polar medium and vice versa (see Laguerre et al. 2015). A polar antioxidant would have a high affinity to the aqueous phase, which of course does not oxidize at all. Notably, a polar antioxidant works very well for a bulk oil, since it is situated at the interface of the oil and tiny air bubbles inside of the oil, where the very oxidation is taking place.

Thus, as a general principle, the polar paradox seems to fully apply for prevention of Cr(VI) in leather, taking into account that the fatliquoring is done in a polar environment, and therefore non-polar antioxidants have a higher efficiency.

In the other aging phenomena investigated in this paper, the behavior is more complex. Gallic acid gives rise to a relatively strong yellowing, tara and hexadecyl gallate yellow less. On the other hand, for the

prevention of rancid odors, gallic acid was found to perform slightly better than tara or hexadecyl gallate. As far as the decrease in shrinkage temperature is concerned, the long chain alkyl gallate performs better than tara of gallic acid. There might be, however, an interaction of the polyphenolics with the collagen, competing with the influence of the substance as antioxidant.

Regarding the Vitamin E derivatives, their efficiency in suppressing Cr(VI) formation when calculated on the same phenol content is in general significantly superior to gallic acid derivatives. This can be explained by the methyl substituents adjacent to the phenolic OH-group which decrease the electron density in the phenolic ring thus making the abstraction of the phenolic H-radical energetically favorable. In fact, Vitamin E is known to be one of the most powerful natural antioxidants. Trolox had been developed as a water soluble analog of Vitamin E. Again, as the polar paradox would predict, the higher polarity is a disadvantage when used in emulsions. Having a carboxylic group it is also possible that trolox binds to the cationic centers in the collagen and thus actually competes with the fatliquor. With gallic acid esters it had been found, though, that this can also be of some advantage. Notably, the Cr(VI) suppressing efficiency of gallic acid had been found to be better than of ethyl gallate, although from point of view of the polar paradox it would be vice versa. A possible explanation is that the amount of antioxidant actually present in the leather is increased by ion-ion interactions at the anionic moiety. The finding with Vitamin E acetate, on the other hand, is a little confusing. This antioxidant is normally used for applications in living organisms, where the ester bond is enzymatically hydrolyzed leaving the phenolic group open. The Vitamin E acetate used contains only up to 1% of free tocopherol, so this would not explain its considerable efficiency in Cr(VI) suppression. One possible explanation might be that in the conditions of leather treatment, supposedly by acid catalysis during fixation, some of the ester bonds may break up giving free Vitamin E. It would also be possible that it is the reductive action rather than the radical scavenging activity, which gives lower Cr(VI) values with Vitamin E acetate.

In the other tests for aging, it turned out that Vitamin E does always perform better than trolox, what would be in line with the findings and explanation given above. For Vitamin E aceteate, on the other hand, very little until no protection against other signs of aging has been found. This altogether would give rise to the conclusion that Vitamin E acetate does not form free Vitamin E during leather processing and that the effect which had been found in Cr(VI) suppression could be attributed to electrochemical reduction rather than radical scavenging.

The synthetic antioxidant is a tailor-made molecule with an enhanced solubility in oil, what, according to the polar paradox makes it very suitable for use in emulsions. When the emulsion dries out, the antioxidant which is dissolved inside of the oil droplets will be deposited in vicinity to the tiny oil drops. Regarding general reactivity it has to be mentioned that the bulky alkyl substituents in o- and p-position to the phenol group increases drastically its reactivity reducing the O-H bond dissociation enthalpy.

4. Conclusion

In Cr(VI) suppression, Vitamin E and trolox perform generally better than gallic acid derivatives, surely due to the effect of the methyl substitution reducing the electron density at the phenolic ring. For both gallic acid and tocopherol derivatives the finding would be that protection increases with the decrease

in polarity of the molecule, something that can be explained in terms of the polar paradox, the antioxidants being used in emulsions. Notably, the polar paradox paradigm has a special importance in leather, due to the practical immobility of the components under the conditions of aging of dry leather. In the investigation of the other aging phenomena the processes occurring are more complex and only partially understood. Vitamin E and trolox give a lower decrease in shrinkage temperature. In yellowing, the very strong yellowing of gallic acid is seen as a disadvantage. The highly oil-soluble, non-polar synthetic antioxidant used for comparison has an excellent efficiency in all aging protocols investigated.

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Segura R, Palma J-J, Izquierdo F, Prevencion del envejecimiento en el cuero. *Boletin AQEIC* 2000, **51**, 141-149

ISOLATION OF Endo-β-1,4-Xylanase: A TOOL FOR NEW HOLISTIC PARADIGM TOWARDS WHITE LEATHER PROCESSING

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The linear polysaccharide β-1, 4-xylan (hemicellulose), one of the major components of plant cell walls, is considered as by-product in paper making process. The enzymatic hydrolysis of β-1, 4-xylan by xylanases into xylose can be useful for preparation of different value added product with wide industrial applications. However, the principal producer of xylanase is filamentous fungi. Hence, xylanase can be explored in various industrial applications including greener processing of hides and skins. It can be used for the soaking, greener cleaning of hides and skin, bleaching of pigments from skin in leather industries. In this work, a Three Phase Partitioning (TPP) method has been used for isolation of this enzyme from *Agaricusbisporous*which isanedible basidiomycetemushroom. The isolated enzyme extract was assayed to find out the xylanase activity. The enzyme production was optimized under variable experimental conditions such as ammonium sulphate concentration, ratio of culture filtrate to tertiary butanol (v/v) and pH. It was found that all the three experimental variables influence the degree of enzyme production and its activity. Maximum enzyme was obtained in the interfacial phase at 50 % ammonium sulphate saturation (w/v) when the other conditions were maintained constant, whereas at 1:2 ratio of culture filtrate to *t*-butanol (v/v) and at pH 6 keeping the remaining experimental variables constant individually the same result has been achieved. This work might pave the way towards a greener eco-friendly enzyme based leather processing.

Keywords- Hemicellulose, xylanase, basidiomycete, leather processing

1.   Introduction

Bio refinery of lignocellulosic biomass is gaining considerable economic importance as it can be platform intermediate chemicals with respect to current ones. Xylose, the 2nd most abundant sugar present in lignocellulosic biomass after glucose, is the hydrolysed product of xylan (Basinskiene et al. 2006). Xylanase catalyzes the hydrolysis of glycosidic linkage of linear polysaccharide β-1,4-xylan, leading to the formation of xylose, xylobiose and others sugars (Aspinall et al. 1959). Indeed, xylanase is ubiquitous among microorganisms (fungi, bacteria and actinomycetes) belonging to several ecological niches (Adhi et al. 1989).It plays a major role in micro-organisms thriving on plant sources for the degradation of plant matter into usable nutrients, increasing the sustainability of lignocellulosic biomass. Several efforts

are underway to achieve an efficient commercially viable process development for xylose production and purification.

Three-phase partitioning (TPP) is one of the novel bio separation approaches for industrially important enzymes with the enhancement of catalytic power of the enzyme (Agnihotri et al. 2010). The method involves the addition of a salt followed by the addition of a water miscible organic solvent to the crude protein extract (Amnison 1992). In TPP, proteins are excluded from two immiscible liquid phases into a middle zone that becomes concentrated by low speed centrifugation into a thin disk (Roy et al. 2000). Kosmotropy, electrostatic forces, conformation tightening and protein hydration shifts have been suggested as the physico-chemical basis for underlying TPP of protein (Kalyanpur 2000).

As a ''proof of the concept'', TPP approach was adopted for the commercial downstream processing of xylanase using agro-residues in a cost effective manner (Wati 2009). *Agaricus bisporus* is an edible basidiomycete mushroom native to grassland in Europe and North America. It represents a very attractive lignocellulosic feedstock in bio refinery schemes. It is shown that xylanase preparation, subjected to TPP, resulted in recovery of xylanase activity as well as significant purification of the enzyme. Various parameters affect the efficient partitioning of xylanase. Therefore, concentration of ammonium sulphate added, pH of the culture medium and ratio of volume of *t*-butanol to the culture filtrate were varied to optimize in order to get highest purity fold and yield in partitioning (Calci 2009).

2. Materials and methods

2.1 Microorganism and Inoculum Preparation.

The fungus *Agaricus bisporous* was used for the submerged fermentation of xylanase production system.The *A.bisporous* was maintained on potato dextrose agar slants at 4 °C. The active *A. bisporous* was inoculated in a complex medium containing 10% potato extract, 1% glucose, 0.15 % KH_2PO_4 and 2%malt extract. The mycelial culture of *A. bisporous* was grown at 25 °C for 7 days.

2.2. Medium Preparation for enzyme production

Xylanase production medium containing 2 % tamarind kernel powder, 0.2% yeast extract, 0.15 % KH_2PO_4, 0.037% $CaCl_2,H_2O$, 0.05 % $ZnSO_4, 7H_2O$, 0.05 % $MgSO_4, 7H_2O$, 0.05% H_3BO_3, 0.05% $MnCl_2, 5H_2O$, 0.0036%$NaMoO_4$, H_2O, 0.0036 $FeSO_4, 7H_2O$ and 10 % (v/v) spore suspension. TKP (Tamarind Kernel Powder) was used asa carbon source fermentation was carried out at about 30°C for a week in shake flask culture and culture filtrate was used for the enzyme protein partitioning system.

2.3. Three-phase partitioning of xylanase

Three phase partitioning uses *t*-butanol and ammonium sulphate to precipitate xylanase from aqueous solution (Roy et al. 2002). The crude culture filtrate was mixed with ammonium sulphate at 25 °C and then vortexed gently to dissolve the salt. The mixture was vortexed gently for 1 min and then allowed to stand for 1 h at room temperature. Afterwards, the mixture was centrifuged at 5000 rpm for 10 min again at room temperature and the three phases were observed. Tertiary butanol is normally completely miscible with water, but upon the addition of enoughsalt, such as ammonium sulphate, the solution separates into two phases, a lower aqueous phase and an upper organic *t*-butanol phase. Presence of protein in the original aqueous phase leads to the formation of an intermediate phase containing xylanase–*t*-butanol co-precipitates between the lower aqueous and upper *t*-butanol phase. They float above denser aqueous salts because bound *t*-butanol increases the buoyancy. Concentration

of ammonium sulphate added, pH of the culture medium and ratio of volume of *t*-butanol to the culture filtrate were optimised as the floating is the function of these process parameters (Sharma 2001).

2.4. Xylanase activity assay

For xylanase activity determination, xylan was used as a substrate. 0.2 gm of Xylan was dissolved in 10 ml of distilled water. Four test tubes were marked as blank, control, $test_1$ and $test_2$. In the blank test tube, 2ml of distilled water and 3ml of DNSA was added and mixed together and kept aside. The remaining three marked test tubes containthe assay samples. The assay samples contain 3ml of DNSA, 200 µl of acetate buffer (pH 5.0), 1.4 ml of the substrate and 200 µl of an appropriate enzyme solution volume. The reaction mixtures were incubated at 40 °C for 10 min, and reducing sugars liberated were determinedby measuring O.D.

3. Result and discussion

In order to optimize best three-phase partitioning system for purification of endo-xylanase from culture filtrate, effect of various process parameters such as percent saturation of ammonium sulphate, crude extract to *t*-butanol ratio and pH of the culture medium were analyzed.

3.1. Effect of ammonium sulphate concentration

In order to determine the effect of ammonium sulphate concentration on partitioning, the assay was carried out over the concentration range of 30-70 %.The relative activities (%) were expressed as the ratio of the xylanase activity obtained at a certain temperature to the maximum activity obtained at the given temperature range. Fig 1 shows the partitioning of endo-xylanase into the interfacial phase with different percent saturation of ammonium sulphate with *t-butanol* equal in volume to the starting crude enzyme solution. The results demonstrate that optimized ammonium sulphate concentration for the enzyme extraction in TPP was found to be 50%.
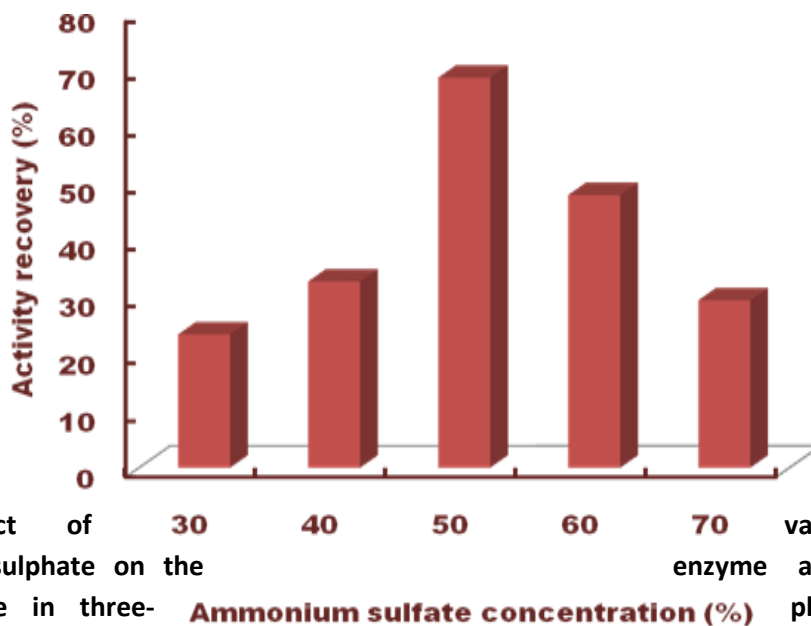

Fig. 1.Effect of varying conc. of ammonium sulphate on the enzyme activity recovery of endo-xylanase in three-phase partitioning system.

3.2. Effect of culture filtrate to *t*-butanolconcentration

Various amounts of t-butanol was added to crude extract and saturated with 50% ammonium sulphate in the volumetric ratio viz. 1:0.5, 1:1, 1:1.5 and 1:2 (shown inFig. 2) shows the optimization of crude extract to t-butanol ratio for the recovery of enzymes. The best result was obtained at 1:2 ratio of ammonium sulphate and *t*-butanol interfacial phase.
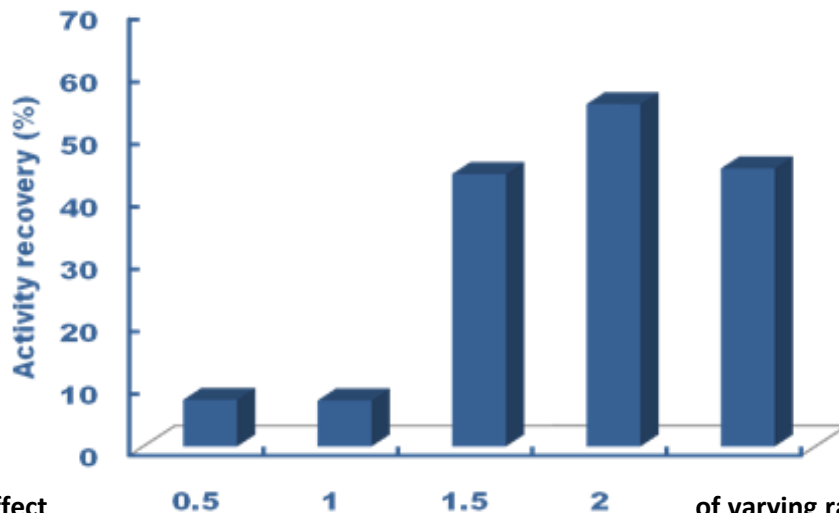

Fig. 2: Effect of varying ratio of culture filtrate to *tert-butanol* on the enzyme activity recovery of endo-xylanase in three-phase partitioning system.

3.3. Effect of pH on the activity and stability of partitioned xylanase

The effect of pH on the activity of xylanase was investigated by incubating samples with acetate buffer of different pH, ranging from 2.6 to 7.0 at 37 °C. The crude culture filtrate of xylanase was brought to 50% ammonium sulphate saturation. The pH was adjusted to different pH value in the desired range, followed by addition of *t*-butanol in the ratio of 1:1 (v/v). After incubation, the residual activity (%) with respect to control was assayed under standard activity assay conditions. Fig. 3 shows the effect of pH on the enzyme recovery. It has been found that maximum enzyme was recovered at pH6.
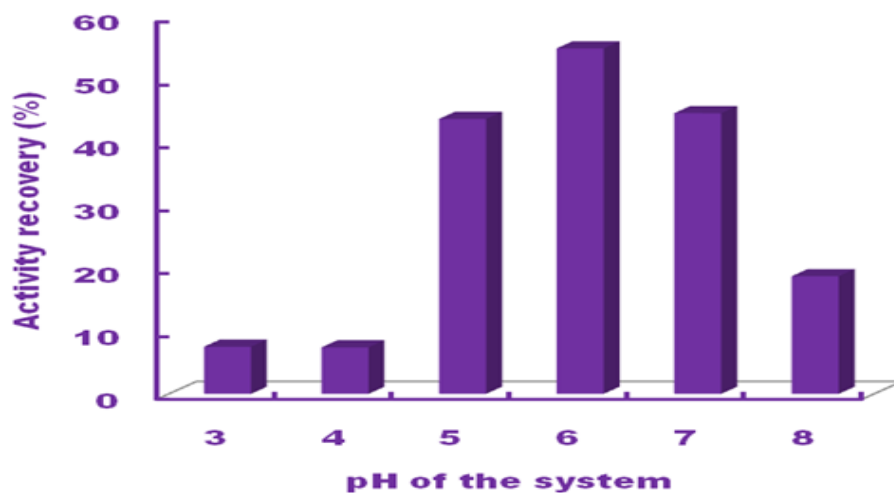

Fig. 3.Effect of pH of the system on the enzyme activity recovery of endo-xylanase in three-phase partitioning system.

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4.   Conclusion

Three – phase partitioning has so far been shown to be useful for downstream operation in protein recovery. It is a simple, quick and economical technique and scaling up is convenient. I can conclude from the present set of researches that with necessary optimization, the combination of 50 %(w/v) ammonium sulphate saturation with 1:2 ratio of xylanase to *t*-butanol (v/v) at ph 6.0 was optimal for attaining the best recovery of xylanase of *A.bisporous*. As TPP is a scalable process, the process described here has the potential to be carried out at the production level. The data given in the experiments also shows the efficiency of TPP as an initial step for bio separation of xylanase.

5.   Acknowledgement

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OPTIMIZING OPERATING PARAMETERS OF AN ELECTROCOAGULATION PROCESS FOR THE TREATMENT OF TANNERY EFFLUENT

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Electrochemical treatments are becoming a reliable option for treating the wastewater effluent of the tanning industry. It has been reported that the electrocoagulation process is being efficiently used to remove organic and inorganic contaminants rapidly and at low cost, significantly reducing levels of chemical oxygen demand (COD), turbidity and chromium in these effluents.

In this work, the team carried out the treatment of a real tannery effluent obtained from pilot plant tannery laboratory at CIATEC. The studies were performed by electrocoagulation using iron and aluminum electrodes to determine the optimum operating parameters for a pilot-scale plant treatment process. The one hour experimental studies were conducted at different current densities (28, 111 and 444 mA/cm^2), on 1 L of effluent held at a 7.0 pH. Following treatment COD, total organic carbon (TOC), turbidity, total chromium, solids, pH and conductivity were evaluated. With a current density of 28 mA /cm^2, the removal of COD and TOC was increased using iron electrodes, reaching 69% removal of COD and 60% TOC. Turbidity showed a 99% decrease with aluminum electrodes at the same current density.

The processes costs were also evaluated, the most economic treatment used iron electrodes and a current density of 28 mA/cm^2. Considering total treatment effectiveness, then, the optimum operating parameters were found using iron electrodes at a current density of 28 mA /cm^2 for 1 hour.

Keywords: electrocoagulation process, wastewater treatment, iron and aluminum electrodes.

1. Introduction

The tanning industry is one of the most important productive activities in the city of Leon, producing approximately two-thirds of the total number of skins tanned in Mexico. In the tanning process, an average of 35 liters of wastewater is generated per kg of treated skin when considering the overall process. Usually, this tannery effluent does not meet the environmental regulations to be discharged directly into the receiving water (Aravindhan, 2004).

Due to the presence of compounds and complex chemicals inhibiting the activity of microorganisms, efficient biological treatments are difficult to apply. In chemical treatments such as coagulation, in which aluminum sulfate and ferric chloride reagents are added, sometimes toxic sludge is formed, creating an additional problem. Membrane separation methods involve a high cost, so they are often reject by the companies as unsustainable (Fahim, 2006).

Among the different wastewater treatment technologies available, the electrochemical processes constitute the most important emergent approach to resolve the water contamination problem. There are several reasons to explain this fact, and among these, the reagents can be generated in situ through the surface of the electrodes applying a constant current (Gao, 2005).

Electrocoagulation (EC) is an electrochemical treatment technology in which the cation coagulant of iron or aluminum, is generated by applying a direct current to the electrodes. During the process, the produced metal hydroxides are capable to adsorbe pollutans. The EC efficiency depends of the interaction of different variables as: (i) physicochemical characteristics of water (pH, conductivity, total solid, etc.); (ii) applied current density; (iii) material, distance and configuration of the electrodes and, (iv) hydrodynamic parameters. Understanding these interactions could aid to design optimal units in a range of predetermined operating conditions (Isarain, 2014).

The available literature reveals that the treatment mechanism by electrocoagulation depends on the waste nature and the design of EC device. It also appears that in order to evaluate the treatment efficiency, various criteria have to be considered: TOC, COD, turbidity and concentration of toxic species e.g chrome. The aim of the study was to investigate the efficiency of the technique to treat tannery wastewaters, analyzing the effects of cathode materials and current density, to determine the optimal conditions for pollutants removal, giving special attention to the compared variations of COD and TOC levels and turbidity.

2. Materials and methods

Wastewater utilized in this study was supplied by leather pilot plant located in CIATEC, Leon, Guanajuato, Mexico. Collected in a closed container and preserved in accordance with Mexican standard NMX-AA-003-1980. The main characteristic of these wastewater samples before treatment is presented in Table 1.

The experimental setup used for the electrocoagulation studies was a typical electrochemical reactor of two electrodes arranged parallel to each other with a gap between the anode and cathode plates, using a non-conducting horizontal support to avoid any short-circuits. The active electrode surface area was 72 cm^2 (6cm×12 cm) of iron or aluminium. In all cases, the two electrodes were made of the same material and the distance between the anode and cathode plates were set up at 3.0 cm. The electrodes were operated in mono-polar mode and connected to a DC power source to supply constant cell current of 2 A (B&K Precision 1621A). The volume of wastewater was one liter. To achieve good mass transfer, a magnetic agitator (Corning PC-420D) with a 600 rpm stirring rate was used.

Table 1. Initial characteristics of wastewater

Sample characteristics	
Temperature (°C)	19.2
pH	3.73
Conductivity (ms/cm)	41.3
COD (mg/l)	1033.33
TOC (mg/l)	393.80
Total solids (mg/l)	6000
Total suspended solids (mg/l)	701.67
Settleable solids (ml/l)	240
Turbidity (NTU)	525
Total Cr (mg/l)	313.2
Total Fe (mg/l)	337.27

All physicochemical parameter measurements were made for each non- and treated sample. Sodium hydroxide was supplied by Karal in order to adjust the initial solution's pH. The pH was measured by using a digital pH meter (Extech instruments, 407227). The COD is an indicator of the effluent pollution degree and is used by regulatory agencies to gage overall treatment plant [19]. The COD was determined by the opened reflux method established in Mexican standard NMX-AA-030/1-SCFI-2012. Wastewater mineralization was monitored using TOC decay which in turn was measured using a Shimadzu TOC-L analyzer. Before analysis, samples were withdrawn from the solutions treated by EC and filtered with 0.45 μm PTFE filters purchased from Whatman. A digital conductivity meter (Hach, P1.4) was used for measurements of the samples ionic conductivity. Turbidity (Nephelometric Turbidity Unit, NTU) was determined with a turbidity-meter Hach, 2100P.

Concentrations of Cr, Al, and Fe were determined by atomic absorption (ThermoScientific, AAS iCE 3000). Dilution and acidification of the solution samples was performed with nitric and hydrochloric acid in a volume proportion 3:2 respectively for total dissolution of the metal species. In most cases, the liquid fractions had to be filtered using conventional 0.45 μm filters to remove the suspended solids, prior to injection into the atomic absorption apparatus.

3. Results and Discussion

The supply of current to the electrocoagulation process determines the amount of Fe^{2+} or Al^{3+} ions released from the electrodes. The value of the current density establishes the coagulant production rate, adjusts the rate and size of the bubble production, therefore this affect's the growth of flocs. This parameter was varied in the range 28-444 mA/cm^2.

Figure 1 shows the effect of current density on COD and TOC removal using both types of electrodes: iron (Fig. 1a) and aluminum (Fig. 1b) respectively. A decrease in current density from 444 to 28 mA/cm^2 yields to improved efficiency of COD and TOC removal from 57% to 69% and 48% to 60% for the iron electrode after 60 min of treatment (Fig. 1a). Likewise it is observed in Fig. 1b for the aluminum electrode with a COD and TOC removal of 59% to 72% and 38% to 57%, respectively in the current

density decrease from 111 to 28 mA/cm^2. Nevertheless, in this case, an increase in current density from 111 to 444 mA/cm^2 yields to improved efficiency of COD (90%) but not for TOC removal (42%).

It has been reported that a too large current density would result in a significant decrease in current and treatment efficiency and in order for the electrocoagulation system to operate for a long period of time without maintenance, its current density is suggested to be 20-25 mA/cm^2 unless there are measures taken for a periodical cleaning of the surface of electrodes.
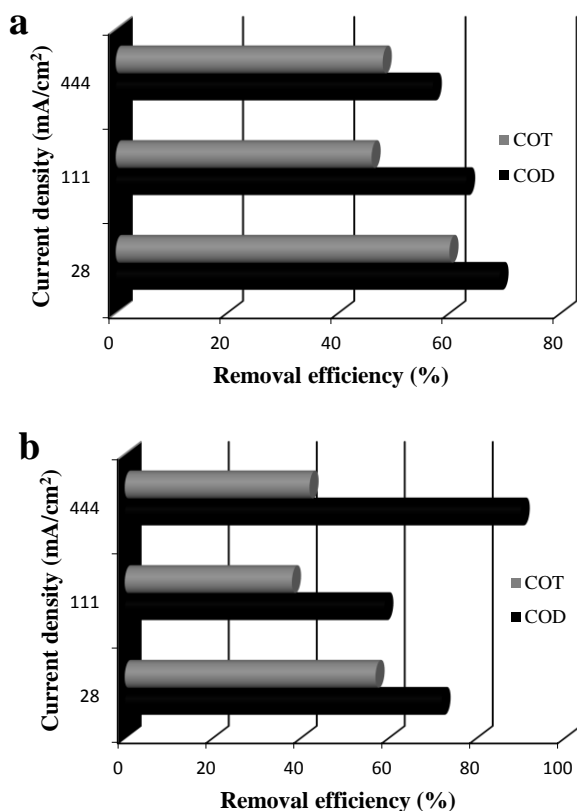

Figure 1. Removal efficiency of COT and COD after 60 minutes of electrocoagulation process using (a) iron and aluminum (b) electrodes at different current density (444, 111 and 28 mA/cm^2) for 1.0 L of tannery wastewater.

Table 2 shows the final parameters obtained after the electrocoagulation treatment. With all treatments a reduction of turbidity greater than 95 % was achieved. The most economic treatment used iron electrodes and a current density of 28 mA/cm^2. Considering total treatment effectiveness, then, the optimum operating parameters were found using iron electrodes at a current density of 28 mA /cm^2 for 1 hour.

Table 2. Final parameters of tanning wastewater treated by electrocoagulation

Material electrode	Iron			Aluminium		
Current density (mA/cm^2)	28	111	444	28	111	444
Removal percentage (COD)	69	63	57	72	59	90
Removal percentage (COT)	60	46	48	57	38	42
Turbidity (NTU)	15.2	206	71.9	0.08	0.15	2.21
Total Cr (mg/l)	0	0	0	0	62.81	0
Processes costs (USD)	0.347	0.454	0.829	0.552	0.753	1.256

4. Conclusion

The treatment by electrocoagulation of tannery wastewater was investigated using two different material electrodes (iron and aluminium) at different current densities (28, 111 and 444 mA/cm^2). Using a current density of 28 mA /cm^2, the removal of COD and TOC increased using iron electrodes, reaching 69% removal of COD and 60% TOC. Turbidity showed a 99% decrease with aluminum electrodes at the same current density. The processes costs were also evaluated, the most economic treatment used iron electrodes and a current density of 28 mA/cm^2. Considering total treatment effectiveness, then, the optimum operating parameters were found using iron electrodes at a current density of 28 mA /cm^2 for 1 hour.

5. Acknowledgements

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CHARACTERIZATION OF LEATHER BUFFING DUST MODIFIED BITUMINOUS BINDER FOR FLEXIBLE PAVEMENT

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Bitumen comprises saturates, aromatics, resins and asphaltenes (SARA), has been widely used as road binder. Basically, bituminous binder undergoes two types of aging viz short term aging (ASTM D 2872) at high temperature during mixing, transportation and laying, and long term aging (ASTM D 6521) at ambient temperature during in-service. India is one of the major producers of leather in the world with around 2000 tanneries across the country, processing around 7 lakh ton of raw hides/ skins annually. One ton of wet hide yields only 150 to 200 kg of finished leather with 800 to 850 kg of solid waste in different form. Buffing dust (BD) is a micro fine solid particulate comprising of collagen, oil, tanning agent, dye chemicals, synthetic fat etc. The objective of this study was to develop a new bituminous binder using the chrome containing BD into a useful product, thereby reducing environmental pollution. A mixture of VG-30 bituminous binder, SBS powder, warm mix additive (Sasobit) and BD, was thoroughly mixed in the laboratory to develop a new binder. Super pave binder tests, Rotational viscometer and Dynamic Shear Rheometer (DSR) was used to test the performance grading (PG) testing protocol as per ASTM D 6373, and the new binder was designated as PG-88. The physico-chemical properties such as FTIR, DSC/TGA, SEM/EDAX, rheological property and aging were assessed. From the DSR value, it was found that PG 88 gave better fatigue resistance (G*.sinδ) and rutting resistance (G*/sinδ) resulting, it can be used as an alternative binder for flexible pavements.

Keywords: Bitumen, buffing dust, rheology, fatigue and rutting resistance, flexible pavement

1. Introduction

Bitumen is a complex mixture comprises saturates, aromatics, resins and asphaltenes (SARA), has been widely used as road binder because of its good viscoelastic properties (fig.1). The deterioration of bituminous pavement is mainly due to the aging of bitumen, which contribute reduction of durability and service life of flexible pavement (M.E.Abdullah et.al 2014). Basically, bituminous binder undergoes two types of aging process throughout its service life. The mechanisms of aging include oxidation, evaporation and physical hardening. Physical hardening is a reversible process, which changes the

rheological properties of bitumen without altering its chemical composition. At ambient temperatures, physical hardening is very slow, but it can speed up at low temperatures (Xianhu Lu et.al).

Bitumen constitutes 4 to 6% by weight of mixture but active part in any type of bituminous mixes. It also influences pavement performance at low and high pavement temperatures. Due to continuously increasing traffic volumes, axle loads and temperature cycling, flexible pavements are subjected to fatigue cracking and rutting. Therefore, the properties of bitumen may be modified by certain additives such as polymer, crumb rubber, sulpher etc. Binder modification enhance the properties viz. fatigue resistance, stiffness modulus, rutting resistance, stripping potential, temperature susceptibility and oxidation potential (Praveen kumar et.al 2012, M.E.Abdullah et.al 2012).

Presently, addition of fiber to bitumen plays a prominent role in modification. The addition of fiber increases the toughness and fracture resistance of hot mix and to act as a stabilizing additive to prevent drain down of the bitumen (Stuart, K. D 1992, Kamaraj et.al 2006). Krummenaver et.al (2009) investigated the usability of leather saw dust in asphalt micro surfacing layer. Leather saw dust was used as such without pre- treatment (Kamaraj et.al 2016).

India is one of the major producers of leather in the world with around 2000 tanneries across the country, processing around 7 lakh ton of raw hides/ skins annually (Kameswari et.al 2012). One ton of wet hide yields only 150 to 200 kg of finished leather with 800 to 850 kg of solid waste in the form of fleshing, chrome shavings, cuttings, trimmings and buffing dust (Goel et.al 2010). Buffing dust (BD) is a micro fine solid particulate comprising of collagen, oil, tanning agent, dye chemicals, synthetic fat etc. (Swarnalatha et.al 2006, 2008). BD contains chromium, it is carcinogenic in nature and it causes clinical problems like respiratory treat ailments, ulcers, perforated nasal septum, kidney malfunction and lung cancer in humans exposed to the environment containing BD particulates (Leonard et.al 1980). With addition of warm mix additive, the mixing and compacting temperature of the bituminous mixes can be reduced by 30% which in turns saves energy (Ambika Behl et.al 2011).

The objective of this study was to develop a new bituminous binder using BD. The study aims to convert toxic chrome containing BD into a useful product, thereby reducing environmental pollution. The product was characterized for its physic-chemical properties using FTIR, DSC/TGA, SEM/EDAX, rheological property and aging were assessed.

2.   Material and methods
Warm Mix additive - Sasobit Sasobit is a fine crystalline long chain aliphatic polymethylene hydrocarbon produced from natural gas. The sasobit used in this study is in pastille form of size 4mm diameter.
Buffing dust - Buffing dust is in the form of fine particles generated as leather industry waste. It is a proteinous solid with chromium, synthetic fat, oil, tanning agents, and dye chemicals.
Styrene Butadiene Styrene (SBS) Poly (Styrene-Butadiene-Styrene), or SBS, is a hard rubber that is used in the production of soles of shoes, tyre treads, and in other places where durability is important. It is a type of copolymer called a block copolymer.

Bitumen - The bitumen used in this study is VG 30 grade bitumen which is the most commonly used for road construction in India. It was tested for its property after adding SBS, Sasobit and buffing dust to it at varying proportions.

Aggregate - The aggregates used in this study were collected from various quarries near Chennai.

Blending of Bitumen - VG 30 grade of bitumen and SBS at dosage of 5% was first blended then added warm mix additive (2%) to reduce the mixing temperature up to 110° C and 100° C respectively for a period of 1 hour. With the above blend, buffing dust finally blended at a dosage of 1% by weight of bitumen at the temperature of 100° C for a period of 1 hour.

2.1   Instrumental Analysis

2.1.1   Scanning Electron Microscopy coupled Energy Dispersive X-ray (SEM/EDAX)

The surface morphology of the carbon residue obtained after pyrolysis was studied using SEM. The instrument used for the analysis was of model Hitachi S-3400 N. The coating given to the sample was of gold. The time required for the setting of coating in the sample was 60s.

2.1.2   Rotational viscosity test

This method is used to determine the viscosity of asphalt at application temperatures as per ASTM D 4402. The measured viscosity at elevated temperatures can be used to determine whether the binder can be handled and pumped at the refinery, terminal, or hot mix asphalt plant facility. The rotational viscometer (RV) test was used to evaluate the difference in viscous behavior between the control and modified asphalt binder. The temperatures over which the RV test was conducted were 120, 135, 150, and 165 and 180 °C using a Brookfield viscometer (Model DV-II+).

2.1.3   Dynamic Shear Rheometer (DSR) Test

A stress-controlled HAAKE dynamic shear rheometer (DSR) was used with 25 mm parallel plates to characterize the mechanical properties of the control and modified asphalt binders prepared under temperatures ranging from 46 to 70 0C and loading frequency at 10 rad/s (1.59 Hz). During testing, samples were conducted in a control and homogeny environment. The Super pave performance grading (PG) testing protocol was used to evaluate the control and modified asphalt binder samples. High service temperature (HT) for a binder is determined as the temperature at which the G*/Sin δ is greater than 1 kPa for unaged binder and greater than 2.2 kPa for the RTFO. In addition to the performance requirements at high and low service temperature; there is a limiting maximum stiffness at the intermediate service temperature (IT) to alleviate fatigue cracking, at which the binder's G*Sin δ after PAV aged binder condition does not exceed 5000 kPa.

2.1.4   Fourier transform infrared (FTIR) spectroscopy

Chemical characterizations of modified binders before and after ageing were performed using FTIR spectroscopy, Infinity 60AR (Mattson, resolution 0.125 cm-1). The FTIR can detect molecular vibrations that may be in the form of two atoms in a diatomic molecule experiencing a simple coupled motion to each individual atom in a large polyfunctional molecule undergoing motion. It also can detect the

variation of the main functional groups in the asphalt based on the infrared spectra corresponding to molecular structures of different molecular group. In this study, five percent by weight solutions of asphalts were prepared in carbon disulfide. Scans were performed using circular sealed cells (ZnSe windows and 1 mm thickness). All spectra were obtained by 32 scans with 5% iris and 4 cm-1 resolution in wave numbers ranging from 1900 to 500 cm-^{1}. In this study, sulfoxide band and carbonyl band in the infrared spectra related were investigated to determine aging effect of modified asphalt binders.

2.1.5   Differential scanning calorimetry (DSC)

The pure asphalt binders were analyzed by DSC on a Waters TA 2010 equipment to determine its glass transition temperature (Tg), in which the samples were rapidly cooled down to −100∘C and then evaluated upon heating at 5∘C/min. Differential scanning calorimetry or DSC is a thermo analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. Both the sample and reference are maintained at nearly the same temperature throughout the experiment.

2.1.6   Thermo Gravimetric Analysis

TGA was performed using Universal TGA Q50 V20.6 build 31. The 5-10 mg of the sample was taken in the sample holder and the TGA controller is programmed and the sample holder is kept inside the analyser. The sample temperature was maintained at 50°C for a minute and then increased from 50°C to 800°C at 40 °C/min. The inert atmosphere was maintained with nitrogen flow of 20 mL/min. The TGA for the carbon residue was also carried out in presence of air.

2.1.7   Standard ageing procedure

Short-term ageing was addressed by the thin film oven test (TFOT). Binders were aged during 5 h at 163 C in air as described in the standard ASTM D1754. This technique has been validated for some time for unmodified asphalts for which it is considered to be more severe than actual jobsite conditions; in the case of modified asphalts, its validity is still open to doubt [16]. However, in the absence of anything better, it has been used with considerable success to compare different PMAs during laboratory studies.

Long-term ageing was addressed by the pressure ageing vessel (PAV). Binders were aged during 20 h at 100 C under 2.1 MPa of air as described in the standard ASTM D6521. In the case of unmodified asphalts, the simulated ageing provided is recognized to be equivalent to several years of service in a road, but how long this equivalence is depends on the asphalt recently, a study of several experimental pavements found this simulated ageing to be equivalent to 4 years.

2.1.8   Inductively Coupled Plasma (ICP) - Optical Emission Spectrometry (OES)

ICP-OES In optical emission spectrometry (OES) the sample is exposed to a source of high energy, such as an inductively coupled plasma (ICP). At temperatures of T = 5000 to 10000 K, elements emit light of a spectrum being characteristic for each element. This emitted light is collected by photomultipliers or LEDs. Since the intensity of the emitted light is directly proportional to the concentration of the element in the sample, quantification by calibration is possible. Modern ICP-OES instruments may analyze up to

70 elements in one step. Since Inductively coupled plasma – optical emission spectrometry (ICP-OES) is a multi-element method, it is ideal for scanning liquid samples in order to obtain overall information on the inorganic composition of the sample under study.
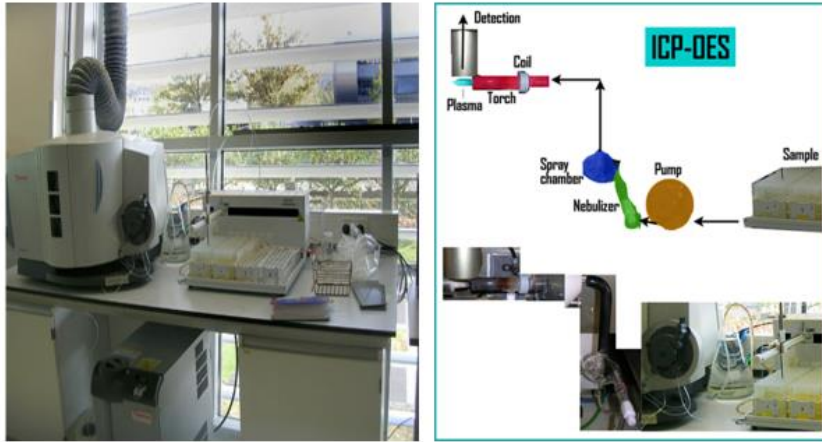
2.1.9 Chemical structure of constituent materials (SARA, SBS powder and leather powder)
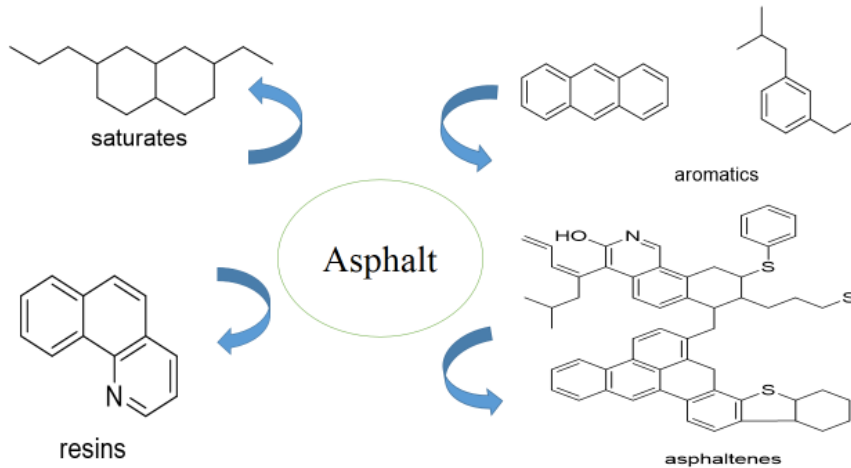

Fig.1.a Representative Structures of the four bitumen fractions: saturates, aromatics, resins and asphaltenes (SARA).
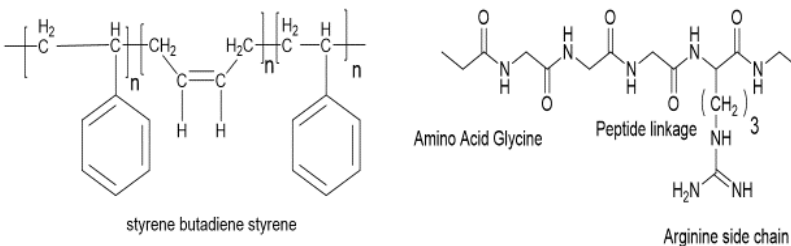
Fig.1.b   Representative structures of the SBS powder and leather

3.   Results and discussion

3.1   Scanning Electron Microscopy (SEM) and Energy dispersive X-ray (EDX) analyses.

The broken fibrous structures of pyrolysis sample are shown in Fig 2.   The plate like formation of pyrolysis sample distributed over bitumen can be viewed from Fig shows that pyrolysis residual ash sample blends well with bitumen and small aggregates particles are dispersed over it. EDX profiles corresponding to the SEM images confirmed the presence of trivalent chromium at 5.5eV in the pyrolysis sample.
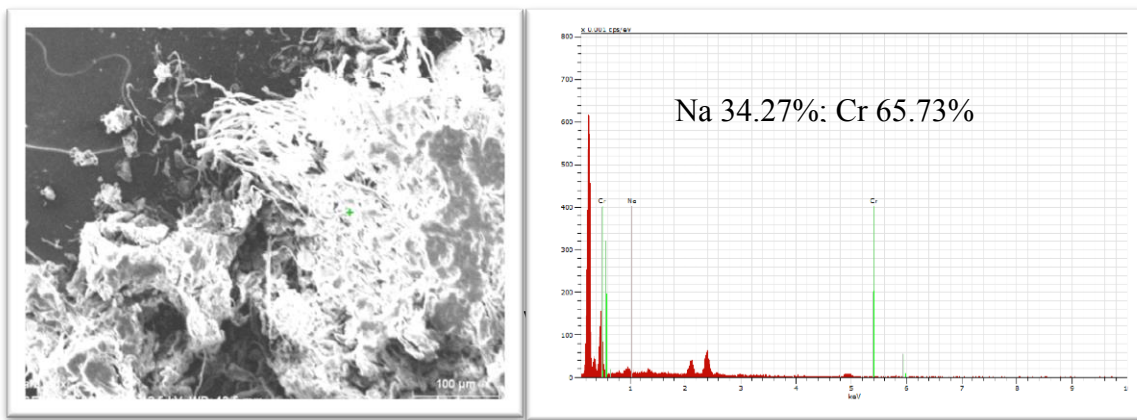
4.

Na 34.27%: Cr 65.73%

Fig 2.   SEM/EDAX analysis of BD.

3.2   Brookfield viscosity results

Figures 3.1 and 3.2 indicated that the modified bitumen (PG-88) viscosity have increased compared to PG-RTFOT and PG-PAV binder. The increment was obvious at lower temperature, 90°C compared to higher temperature. Strategic Highway Research Program (SHRP) has fixed the maximum steady state viscosity of 3 Pas at 135°C to provide potential satisfactory result during industrial processing which involved handling, laydown and compaction. Results obtained showed that the viscosity of modified bitumen fulfilled the SHRP requirement. The viscosity of asphalt binder at high manufacturing and construction temperatures generally above 135°C is important because it can control the following: • Pump ability. The ability of the asphalt binder to be pumped between storage facilities and into the HMA manufacturing plant; • Mix ability. The ability of the asphalt binder to be properly mixed with and to coat aggregate and other HMA constituents in the HMA manufacturing plant and • Workability. The ability of the resultant HMA to be placed and compacted with reasonable effort.

3.3   Dynamic Shear Rheometer (DSR) results

From the test results the newly modified binder designated as PG 88 as per ASTM D 6373-13, the test results given in Table 1.
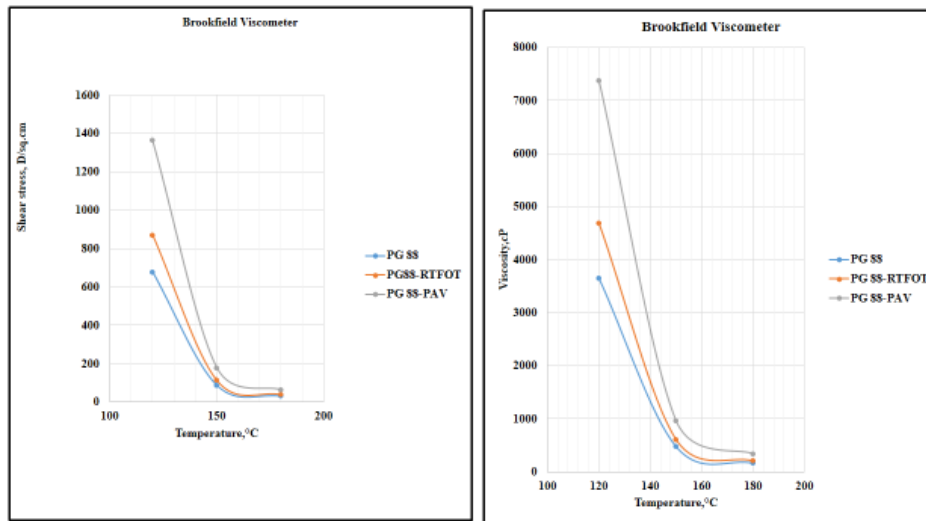
Figure 3.1 and 3.2.   Viscosity of modified binders

Table 1.   Performance graded asphalt binder specification as per ASTM-D-6373-13

Performance Grade	PG-82	PG-88
Average 7 day max.pavement design temp, °C	<82	<88
Min. Pavement design temperature, °C	> - 34	> -34
Original binder		
Viscosity, D-4402, max. Pa.s, test temp. °C	135	165
Dynamic shear, ASTM D-7175, g*/sin δ, min 1.00kpa, 25 mm plate, 1mm gap, test temp. At 10 rad/s, °C	82	88
Rolling thin film oven (test method ASTM-D 2872)		
Mass change, max %	1.00	1.00
Dynamic shear, ASTM D-7175, g*/sin δ, min 1.00kpa, 25 mm plate, 1mm gap, test temp. At 10 rad/s, °C	82	88
Pressure aging vessel (PAV) residue (test method ASTM-D 6521)		

PAV aging temperature, °C	100	100
Dynamic shear, ASTM D 7175, g*.sin δ, max 5000kpa, 8 mm plate, 2mm gap, test temp. At 10 rad/s, °C	28	31

3.3.1 Complex modulus (G*) and Phase angle (δ):

Figures 4. 1 and 2 illustrate the complex modulus and Phase angle, respectively, versus different temperature. All specimens showed a decrease in complex modulus values, respectively an increase in phase angle, as the temperature increases. In General, the bitumen exhibited slightly higher complex modulus throughout the temperature range compared to original binder. The higher complex modulus indicated that binder strength have increased which can be correlated to higher rutting resistance.
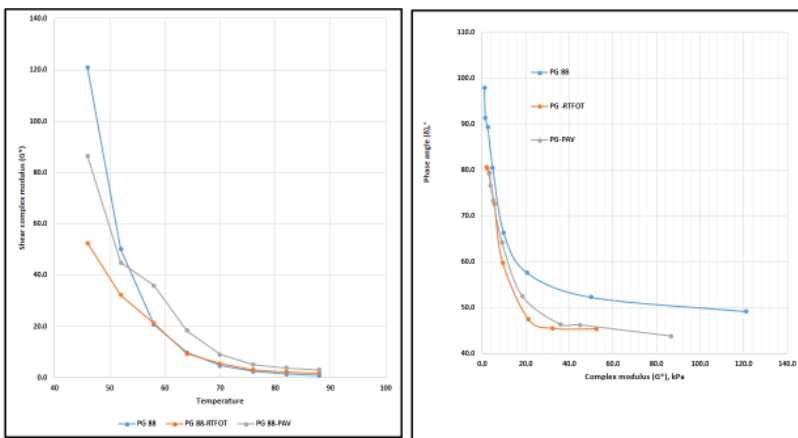

Fig 4.1 and 4.2   Rheology of modified binder

Figure 4.2 showed that phase angle for modified bitumen was lower compared to original bitumen. As temperature increase around 70°C, original binder totally loss it elasticity (phase angle equals to 90°C). However, improvement was noticed in modified binder where the binder can retain its elastic respond or delay loss of its elasticity respond at high temperature.

3.3.1.1 Rutting Resistance

The effect of the BD modified asphalt binder on the rutting resistance (G*/ sin δ) was evaluated using DSR. In Super pave specifications, G*/sin δ from the DSR test is used as a key factor to define the rutting resistance of an asphalt binder at a high-performance temperature. In Fig 7, all G*/sin δ values are higher than 1.0 kPa (unaged condition) and 2.2 kPa (RTFO aging condition) at 64°C. The G*/sin δ for PG 88, PG 88- RTFOT and PG 88- PAV shows better resistance of rutting.
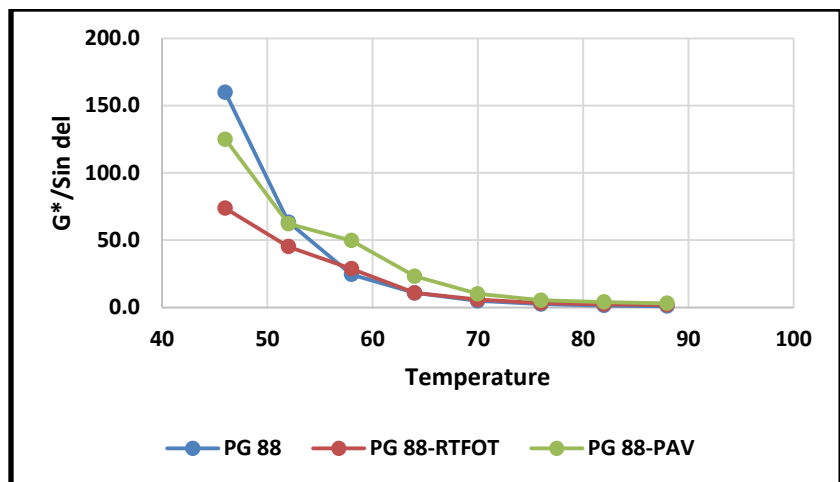
Fig. 7   G*/sin δ values of modified binders

3.3.1.2 Fatigue Resistance

Fatigue resistance (G*.sin δ) of modified asphalt binder after conditioned (RTFO + PAV residual) were determined using the DSR at 25°C and the results are shown in Fig. 8. The G*.sin δ values meet the standard specification of Super pave™ requirements (maximum 5000 kPa). The lower G*.sin δ values are generally considered to be desirable characteristic from the stand point of fatigue cracking resistance.

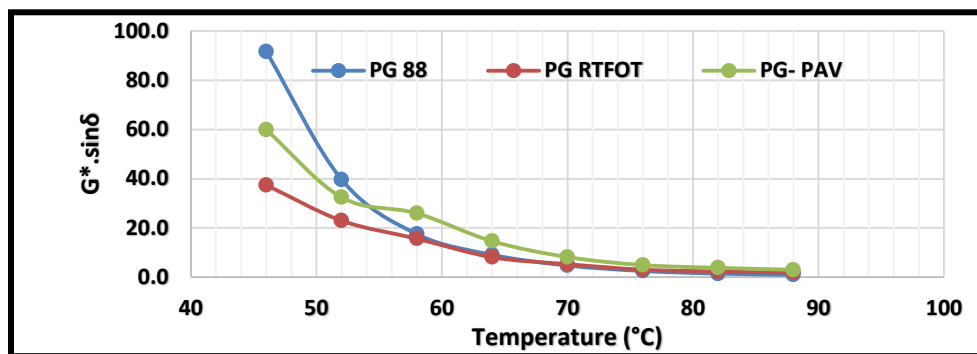Fig. 8   G*.sin δ values of modified binders

3.4   FTIR results

Fig. 5 shows the IR spectra of PG 88, PG 88-RTFOT and PG 88-PAV, shows the presence of OH in asphaltene at frequency of $3444 cm^{-1}$, the frequency at $2919 cm^{-1}$ to $2845 cm^{-1}$ shows the presence of C-H alkenes in saturates, the frequency at $1724 cm^{-1}$ shows the C=O as carbonyl group in peptide. The frequency at $1623 cm^{-1}$ shows the N-H bending amino acid in the leather powder and also C=C alkene in aromatic compound in the bitumen. The frequency at $1450 cm^{-1}$ to $1370 cm^{-1}$ shows the C-H bending alkene in saturates. The frequency at $1032 cm^{-1}$ - $962 cm^{-1}$ shows the C-S and S=O (sulphonyl group) in asphaltene. The frequency at $862 cm^{-1}$ – $698 cm^{-1}$ shows the =C-H- bending aromatic, resin and asphaltene in SARA. The intense peak at $875 cm^{-1}$ – $712 cm^{-1}$ are attributed to the Cr (III) species. It is

concluded that the addition of BD along with the mixture of VG 30, SBS powder and warm mix additive not changed any functional group of SARA.

3.5 Thermal Analysis

3.5.1 DSC/TGA

The DSC curve of PG 88s was examined to evaluate its physical characteristics, which depend on aspects of temperature and heat flow (mW). Figure 6.1 shows the phase transitions observed. PG 88 shows both exo (-) and endo (+) thermic peak at 103.53°C (-0.2069 mW) and at 190°C (0.1724 mW). PG 88-RTFOT shows both exo (-) thermic peak at 102.67°C (-0.9395 mW) and at 178.3°C (-0.7454 mW). PG 88 –PAV shows both exo (-) and endo (+) thermic peak at 106.37°C (-0.2882 mW) and at 265.85°C (0.2389 mW). Thermo gram of modified bitumen (Fig 6.2) shows four areas of weight loss. The TGA curve of PG-88 binder, first weight loss of nearly 2.68% at 269.99°C was due to elimination of moisture content, second loss of nearly 47.41% at 500.48°C, third loss of nearly 22.26% at 655.90°C and finally a complete loss beyond 700°C was attributed to decomposition of organic compounds of residual ash and volatilization of low boiling point components of bitumen. The PG-88 RTFOT binder, first weight loss of nearly 5.93% at 307.05°C was due to elimination of moisture content, second loss of nearly 16.42% at 427.13°C, third loss of nearly 30.82% at 511.38°C and finally 23% loss beyond 700°C was attributed to decomposition of organic compounds of residual ash and volatilization of low boiling point components of bitumen. The PG-88 PAV binder, first weight loss of nearly 3.19% at 277.87°C was due to elimination of moisture content, second loss of nearly 17.66% at 439.73°C, third loss of nearly 23.07% at 521.33°C and finally 20.55% at beyond 700°C was attributed to decomposition of organic compounds of residual ash and volatilization of low boiling point components of bitumen.
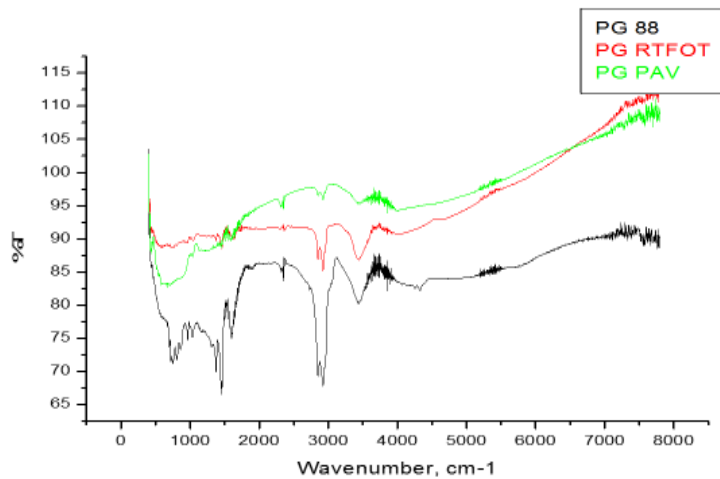

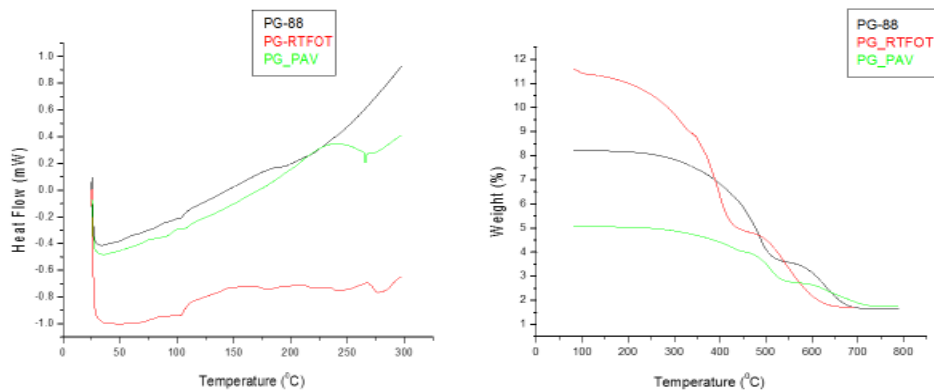Fig.5   IR spectra of modified binder

Fig.6.1 and 6.2   DSC/TGA of modified binder

3.6   Inductively Coupled Plasma (ICP) - Optical Emission Spectrometry (OES)

From table 1, the ICP-OES spectrometry shows the amount of water soluble chromium in the modified binder. The chromium is in the level in the BD is about 198 mg/kg, whereas the BD mixed along with warm mix additive the ppm level drastically reduced to 1 ppm to 3ppm after long term aging.

Table 1. Water soluble chromium in modified binder

Sl.No	Sample ID	Cr (mg/kg)
1.	BD	198.2
2.	PG 88	1.3
3.	PG 88-RTFOT	2.0
4.	PG 88-PAV	3.4

CONCLUSION

The newly modified binder was not affected any functional group of SARA. The thermo gram showed good stability beyond 500°C, which the binder never experience in service period. From the complex modulus (G*) and phase angle (δ), showed better rutting (G*/sin δ) and fatigue (G*.sin δ) characteristics of binder. The newly prepared modified binder confirms PG-88 as per ASTM D 6373-13. The water soluble chrome was 3.4mg/l, so any alternate method may be formulated to use the buffing dust in bituminous mixture in future in a secured manner.

Acknowledgement

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ENVIRONMENTAL SCIENCE AND TECHNOLOGY INNOVATIONS IN LEATHER RESEARCH TOWARDS SUSTAINABLE DEVELOPMENT IN LEATHER INDUSTRY: A SCIENTOMETRIC STUDY

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The '**Scientometric study'** is an analysis of literature with mathematical and statistical methods in information use and seeking pattern approaches in a particular field of Information products and services for Knowledge Indicators towards Organisational Development. The **Impact** of Scientific Research on Scientific Community could be testified by citation analysis. The citation analysis is an analytical methodology under Scientometrics used to evaluate research programmes. In view of this, Leather Science Abstracts*(LESA)* and Indexing International Periodical Publication Knowledge Resources/Information Products and Services serve as a tool for *Knowledge Indicators* in Environmental Science and Technology innovations in Leather Research towards sustainable development in Leather Industry. In view of this, the **LESA** Digital Documentation Knowledge Resources available in the public domain on www.clrilesa.in according to the **Digital India Concept,Digital India-An Initiative by the Honourable Prime Minister of India and be the Part of the Initiative for institutional and national development**.

The present case study is dealt with LESAdatabase on Scientometric analysis of literature related to Environmental Science and Technology research trends in leather research**.** Analyses **4500** citations appended to **331** research contributions published in Environmental Science and Technology during the period from**(2001-2005)** in LESA database. The design of the study is a *'Citation Analysis Approach* **(CAA).** The purpose of the study is to investigate through citation analysis on **Dissemination of Knowledge** in Environmental Science and Technology innovations in leather research for sustainability of leather. Descriptive Statistics was used in analysing the data. About **24** Sub-fields were identified under environmental science and technology for sustainability of leather. Out of 331 citations, **48** citations(14.5%) are from *'Leather production and Pollution Control aspects in leather industry'*. Based on the survey, the highest number citations/research contributions totalling **67** (20.24%) has emanated from 'Leather Institutions'.

Based on the study, the environmental science and technology research contributions are more **128**(38.67%) from **'India'** and stand at number one ranking under Country wise distributions of research contributions. It is evident from the study that the majority of the research papers/contributions **214**(64.65%) are from co-authored pattern/collaborative efforts in leather research. The analysis indicated that the Scientists preferred to publish research papers in Joint authorship(64.65%) having

0.65 degree of collaboration. The Scientometric Software, 'Scientosoft', technology application tool used for this purpose. The Single authored research Contributions **117**(35.34%) is also well recognized feature in environmental science and technology for sustainability of leather.

The study encourages the researchers to initiate collaborative efforts for inter-disciplinary research activities particularly in Environmental Systems Design Modelling and Optimization, Environmental Monitoring, Wastewater Treatment Technology, **Environmental Impact Assessment(EIA)** and Environment and Health aspects in Leather Industry for sustainable development.

Keywords: Scientometric study, Impact on Environmental Science and Technology innovations, Leather Science Abstracts *(LESA)*database, Knowledge Indicators/ Knowledge Dissemination, Leather Research, Sustainable Development.

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1. INTRODUCTION

Information and *Documentation* Knowledge Management play a vital role in　　　　Content Analysis and Content Management for subject inclusion in leather and allied fields including the present paper theme concept study on Environmental Science and Technology *ENVIST* in Leather Science Abstract*(LESA)* and Indexing the International periodical publication services. The LESA Digital Documentation Knowledge Resources available in www.clrilesa.in according to the **Digital India Concept, Digital India- An Initiative by the Honourable Prime Minister of India and be the Part of the Initiative for institutional and national development**. The LESA periodical Publication Knowledge Resource serves as a tool for Knowledge Indicators both for Current as well as retrospective **R & D** supporting services towards Knowledge Access and Sharing the Information for Research Approach Objectives**(KASIRAO)** in leather research for Sustainable Development in Leather Industry.

2. SCOPE AND OBJECTIVES

The scope and objectives have been identified through *'Scientometric Studies'* impact on Environmental Science and Technology (*ENVIST*) innovations for knowledge dissemination and knowledge access in leather research towards sustainable development in Leather Industry in the following respects.

❋　　　To examine the growth of *'Leather Research'* published in Leather Science Abstract (LESA) and Indexing periodical publication database knowledge resource for the period from **2001-2005.**

❋　　　To identify the *sub-fields-wise* distribution of **research Contributions/Citations** in **ENVIST innovations** in LESA database.

❋　　　To identify the *rank-wise* distribution of subject contributions in relation to **ENVIST innovations** in LESA database.

❋　　　To analyse the *author-wise* distribution of research contributions in LESA database.

❋　　　To evaluate the *institution-wise* distribution of research contributions in LESA database.

❋　　　To identify the *geographical-wise* distribution research contributions in LESA database.

3. HYPOTHESES

Based on the objectives, stated in the **'Scientometric Studies'** in relation to *ENVIST* innovations in Leather Research using *LESA* database, the following Hypotheses were framed:

✵ There exists a considerable level of variation in different sub-fields in **ENVIST**innovations in Leather Research aspects.

✵ There exists a significant level of difference between Leather Research performance of Indian and other countries research contributions in **ENVIST**towards sustainable development in Leather Industry.

4. LIMITATIONS OF THE STUDY

The study is confined to the '**Scientometric Analysis'** of literature in **ENVIST** innovations in Leather Research covered in LESA database for the period from **2001-2005.**

5. SIGNIFICANCE OF THE STUDY

✵ The study of the nature brings the state-of-the-Art of the Leather Research activities in ENVIST and its**(Scientometric study)** application in **ENVIST** sub-fields for **knowledge indicators** towards sustainable development in Leather Industry.

✵ The study would pave the way to other academic and **R&D** institutions to provide similar services in their respective discipline towards organizational development.

6. METHODOLOGY

The study is an exploratory research and relies upon review of Literature/Data available with the Knowledge Resources in **LESA** data base. The **LESA** database source served as a tool for data collection, analysis and interpretations towards this research study.

7. LESA : A DESCRIPTIVE ANALYSIS

Leather Science Abstract (LESA) is a monthly international abstracting and Indexing Current Awareness Service **(CAS)** periodical publication, Published by the Documentation Department since 1968 in CSIR-CLRI, containing the subjects such as Footwear Technology, Environmental Science and Technology **(ENVIST),** Wool Technology, Leather Chemicals and auxiliaries, Leather finishing materials, Leather properties and quality control are under **'leather science and technology'** heading and the subjects like footwear and leather goods are under **'leather products'** heading for knowledge dissemination in these fields for **K**nowledge **A**ccess and **S**haring **I**nformation for **R**esearch **A**pproach **O**bjectives**(KASIRAO)** towards sustainable development in leather industry.

8. ANALYSIS AND DISCUSSION

The Scientometric study serves as a tool for scattering of research articles over journals, growth of literature, productivity and impact of research. Based on the impact of research, the application of *'Scientometric study'* serves as a tool for knowledge indicators in "Leather research with access to LESA database" on Subjects, Authors, Documents, Institutions and Geographical-wise distribution research contributions pertaining to volume 34-38, issues(1-12) and the years from 2001-2005 under survey. The research study results as discussed in the ensuing sections from 8.1-8.4.

8.1   **Volume–wise and total break up of Authorship Pattern research contributions in Environmental Science and Technology (ENVIST)**

Table-1 Provides the authorship-pattern research contribution in relation to **ENVIST**.

Table -1

Volume–wise and total break up of Authorship Pattern research contributions in Environmental Science and Technology(ENVIST)

S. No.	Vol. No.	Year	Single Authors	Two Authors	Three Authors	More than Three	Citation from Periodical Sources	Total of Multiple Authors	Degree of collaboration	Total No. of research contributions
1	34	2001	21	19	12	10	1	42	0.67	63
2	35	2002	19	14	8	16	5	43	0.69	62
3	36	2003	15	10	7	9	5	31	0.67	46
4	37	2004	31	20	16	19	3	58	0.65	89
5	38	2005	31	11	11	13	5	40	0.56	71
Total			**117**	**74**	**54**	**67**	**19**	**214**	**0.65**	**331**

The **author- wise** analysis of citations indicated that the majority research papers/contributions **214**(64.65%) are from co-authored pattern/collaborative efforts in leather research and the analysis also indicated that the scientist preferred to publish research in joint authorship(65.65%) having 0.65 degree collaboration in the authorship pattern.

The **'*Scientosoft'*** Technology tool used for this purpose. The single authored research contribution **117**(35.34%) is also well recognized features in the **ENVIST** innovations.

The singled authored and co-authorship patterns presented in table-1 establish that the number of multi-authored research contributions(**'N' marc**) in ENVIST in leather research is more. The number single authored research contributions(**'N' sarc**) in Environmental related fields in leather research are low under study. The degree collaboration calculated using   V. KASIRAO Formula invented by the author. V. Kasirao, the Formula maybe stated as follows:

DC   = $\dfrac{'N'marc}{'N'marc + 'N'sarc}$

Where DC   =   Degree of Collaboration in a particular discipline

'N' marc = No. of multi-authored research contributions in a particular discipline for certain period of study.

'N' sarc = No. of single-authored research contributions for the same period

How the Degree of collaborations**(DC)** can be calculated in a particular field of database source of study is indicted below:

S. No.	Subject Areas in Environmental Science and Technology (ENVIST)	Coverage for Total No. records n=331 (Year – 2001 -2005)						RANK	Percentage
		2001	2002	2003	2004	2005	Total		

Database Name(for example): *Leather Science Abstract **(LESA)** and Indexing Monthly International* periodical publication services in leather and allied fields towards leather industrial development in CLRI.

Calculations:

$$DC = \frac{'N'marc}{'N'marc + 'N'sarc}$$

$$= \frac{214}{214+117} = 0.65$$

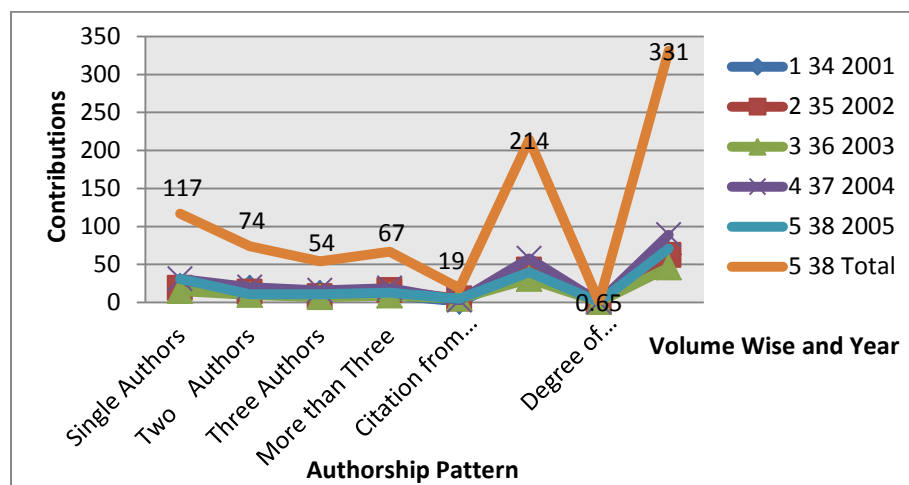**Authorship-pattern research contribution in relation to ENVIST**

Fig: 1 Authorship - Pattern

8.2 Subject wise distribution of research contributions in ENVIST

Table -2 Provides the subject wise distribution of research contributions in ENVIST Under study.

Table – 2

Subject wise distribution of research contributions in ENVIST

1.	Ammonium and chromium removal of heavy metal in waste water treatment plant	13	2	3	7	5	30	II	9.06
2.	Tannery effluent and biomass in leather industry	1	1	1	-	1	4	XVII	1.21
3.	Recovery and economic utilization in leather industry	3	-	-	-	2	5	XVI	1.51
4.	Chrome tanning and leather industry	4	1	1	3	4	13	IX	3.93
5.	Solid and liquid waste and tannery management	4	6	2	2	4	18	V	5.44
6.	Recycling system and tannery	4	2	-	1	2	9	XII	2.72
7.	Environmental management and Eco system in leather industry	3	-	2	4	2	11	XI	3.32
8.	Tannery effluent and tannins on soil fungi	-	3	-	2	1	6	XV	1.81
9.	Design and common effluent treatment plant in leather industry	3	2	4	6	1	16	VII	4.83
10.	Waste water control process, pollution control and finishing method in leather industry	5	5	5	6	5	26	III	7.85
11.	Ultra filtration processing in leather industry	1	-	-	1	1	3	XVIII	0.91
12.	Environmental protection in leather industry	2	3	1	3	3	12	X	3.62
13.	Leather production and pollution control in leather industry	7	11	10	14	6	48	I	14.50
14.	Cleaning technology, environment management and	4	3	-	2	2	11	XI	3.32

S. No.	Subject Areas in Environmental Science and Technology (ENVIST)	2001	2002	2003	2004	2005	Total	Rank	Percentage
	pollution control in leather industry								
15.	Environmental legislation and finishing in leather industry	2	-	1	1	1	5	XVI	1.51
16.	Biological degradation in leather industry	-	3	4	6	3	16	VII	4.83

It is observed from Table **2** in ENVIST with regard to sub-field area wise citation analysis of records in LESA database in ENVIST indicate that out of **331** citations in Environmental Science and Technology(ENVIST) innovations in leather research towards sustainable development in leather industry, **48** records(14.50%) are from 'Leather production and pollution control in leather industry, **30**

S. No.	Subject Areas in Environmental g Science and Technology (ENVIST)	Coverage for Total No. records n=331 (Year – 2001 -2005)						Rank	Percentage
		2001	2002	2003	2004	2005	Total		
17.	Antibacterial activity in leather industry	1	4		3	-	8	XIII	2.42
18.	Sewage treatment in leather industry	-	1	1	4	1	7	XIV	2.11
19.	Physical and chemical analysis in leather industry	1	6	2	4	4	17	VI	5.14
20.	Different dyes and toxicity in leather industry	2	4	3	4	1	14	VIII	4.22
21.	Safety and Health Environment **(SHE)** in leather industry	2	5	-	7	8	22	IV	6.64
22.	Ecology aspects and effluent in leather industry	-	-	2	4	8	14	VIII	4.22
23.	Biotechnology and methanol cultured seed sludge in leather industry	-	-	4	4	3	11	XI	3.32
24.	Environmental impact and development in leather industry	1	-	-	1	3	5	XVI	1.51
		63	62	46	89	71	331		

records/citations (12.08%) are from 'Ammonium and chromium removal of heavy metal in waste water treatment plant, **26** records(7.85%) are from Waste water control process, pollution control and finishing method in leather industry and followed by other sub-field knowledge indicators in **ENVIST** as stated in the above table. The research contributions are more from *'Leather production and pollution control'* and stand at number one in ranking under survey.

8.3 Institution-wise Distribution Research Contributions/ Knowledge indicators in Environmental Science and Technology *(ENVIST)* **Access with** *LESA* **Database**

Table- 3 provides the Institution-wise Distribution Research Contributions/ Knowledge indicators in Environmental Science and Technology (ENVIST) Access with LESA Database under study. Table.3 Institution-wise Distribution Research Contributions in ENVIST

Table 3 - Institution-wise Distribution Research Contributions in ENVIST

It is observed from Table 3 indicated that Eight groups of institutions were identified under study. The number of research Contributions/Citations **67**(20.24%) are more from *LeatherInstitutions* and the contributions **57**(17.22%) from *CLRI* and followed by other institutions as stated in the above table.

S. No.	Type of Institutions	Coverage for Total No. records n= 331 (Year – 2001-2005)						Percentage	Rank
		2001	2002	2003	2004	2005	Total		
1.	CLRI	11	12	8	16	10	57	17.22	III
2.	CSIR	3	4	2	1	2	12	3.63	VIII
3.	Universities	4	7	1	9	9	30	9.06	V
4.	Academic Institutions	13	8	1	19	15	56	16.92	IV
5.	Research Institutions	5	4	6	7	3	25	7.55	VI
6.	Leather Institutions	11	13	18	14	11	67	20.24	I
7.	National Research Institutions	5	3	4	10	2	24	7.25	VII
8.	Others	11	11	6	13	19	60	18.13	II
	Total	63	62	46	89	71	**331**	**100.00**	
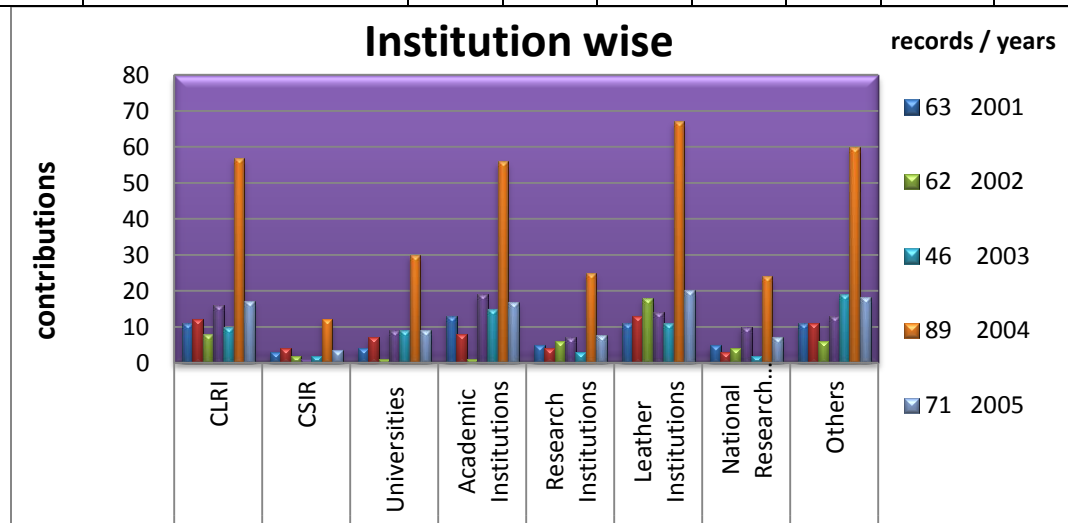
Fig: 2 Institution Wise distributions of citations

8.4   Geographical-wise Distribution of Research Contribution in LESA Database in ENVIST

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Table - 4 provides the geographical-wise distribution of research contribution in LESA database in ENVIST under study.

Table-4

Geographical-wise Distribution of Research Contribution in LESA Database in ENVIST

S. No.	Name of the Countries	Coverage for Total No. records n= 331 (Year – 2001 - 2005)					Total	Percen tage	Rank
		2001	2002	2003	2004	2005			
1	UK	10	13	8	8	5	44	13.29	III
2	India	28	18	21	43	18	128	38.67	I
3	U.S.A	7	14	13	20	24	78	23.56	II
4	Germany	1	2	0	6	6	15	4.53	V
5	China	6	1	0	4	6	17	5.14	IV
6	Spain	3	3	1	1	3	11	3.32	VII
7	Australia	0	1	0	0	0	1	0.30	X
8	Canada	0	1	0	0	0	1	0.30	X
9	Italy	1	1	1	2	7	12	3.63	VI
10	Netherland	5	4	0	4	2	15	4.53	V
11	Russian	0	1	0	0	0	1	0.30	X
12	Polish	1	0	1	0	0	2	0.60	XI
13	Japan	0	1	0	0	0	1	0.30	X
14	Taiwan	0	1	1	1	0	3	0.91	VIII
15	Finland	0	1	0	0	0	1	0.30	X
16	Poland	1	0	0	0	0	1	0.30	X
		63	62	46	89	71	331	100.00	

The Geographical-wise distribution of research Contribution in *ENVIST* access with LESA Database Knowledge Resources indicate that out of **331** resource/citations in **LESA** database, **128** records(38.67%) are from **India,78** records(23.56%) are from **USA**,   44 records(13.29%) are from **UK** and followed other countries as indicated in Table-6.   The Contributions are more from '**India**' and stand at number one in ranking.
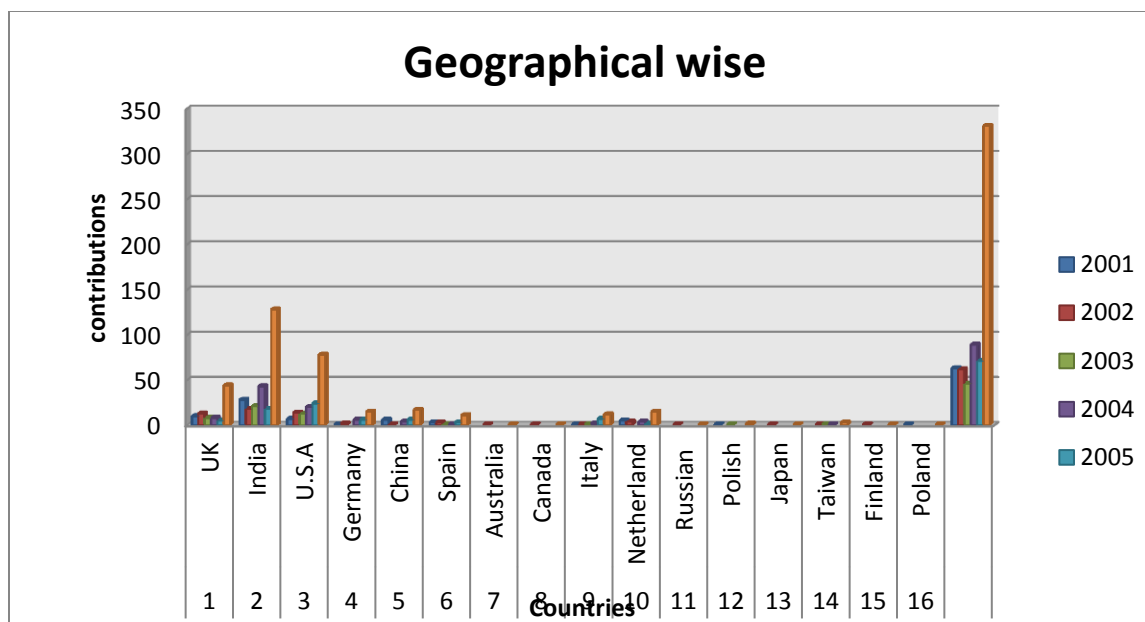
Fig: 3 Geographical Wise distributions of citations

9. FINDINGS OF THE STUDY

❋   The study shows that the publication of articles/research contributions in Environmental related fields as cited in various periodicals knowledge resources have witnessed an escalating trend from study for the period from **2001- 2005.**

❋   The study shows that the majority of the researchers preferred to publish research papers in joint authorship(64.65%) having 0.65 degree of collaboration.

❋   About 24 sub-fields were identified in relation to ENVIST out of 331 Citations/ research contributions, 48 citations(14.5%) are from **"Leather Production and Pollution Control"** aspects in leather industry under study.

❋   The highest number of citations totalling 67(20.24%) has emanated from **"Leather Institutions"**.

❋   The research contributions are more 128(38.67%) from 'India' and stand at number one in ranking under geographical-wise distribution of research contributions under study.

10.   CONCLUSION:

The study reported above led to the following concluding remarks:

❋   The Leather Science Abstract **(LESA)** and Indexing periodical publication services play a vital in CLRI to know-how in leather and allied fields and show-how for Knowledge Dissemination and Knowledge Access and Sharing the Information for research Approach Objectives **(KASIRAO) in leather research** towards sustainable development in leather industry.

❋   The citation study approach(CSA) is an imperative measure to identity the emerging trends in Leather Science and Technology **(LEAST)** innovations in leather and allied fields with access to LESA towards organizational development.

❋ The **'Scientometric Study'** would create an awareness concerned scholars regarding core authors, core journals that publish the literature, areas where more research is being conducted and areas where more research needs to the done towards organizational development.

❋ The abstracting and indexing services of the type covered in this study will enable other academic and **R&D** institutions to provide similar services in their respective fields towards organizational development.

❋ The **'Scientometric study'** as a tool for knowledge indicators on **"Impact Factor" (IF)** analysis in a particular field of research interest towards organisational development. The Impact Factor is a quality indicator used to evaluate the researchers, scientific journals and Institutions. It is also useful in comparing various journals that are in the same field. In view of this, the present study relation to **ENVIST** innovations in leather research as a supporting source for sustainable development for leather industry.

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AN INVESTIGATION ON FUNGICIDAL MECHANISM OF BIOCIDES ON NEW *ASPERGILLUS VERSICOLOR* STRAIN-TANCK1 ISOLATED FROM LEATHER WATCH STRAP

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A new fungal species growing on leather watch strap has been isolated and characterized by 18s rRNA sequencing and found to be *Aspergillus versicolor* (GenBank accession number KX814964). Three fungicidal formulations used in leather industry were evaluated for their efficacy in controlling the growth of this fungal isolate. Among the three biocide formulations, 2-(Thiocyanomethylthio) benzothiazole was found to effectively inhibit the fungal growth with minimum dosage (MIC of 31.2µg/ml) when compared to potassium dimethyldithiocarbamate (MIC- 1250µg/ml) and 2,2,dibromo-3-nitrilo propionamide (MIC- 625µg/ml). The mode of action of the fungicides at 2-fold MIC dosage on 3-5days old fungal mycelium was also studied. There is an increase in the membrane permeability in the biocide treated samples, which is evident from cellular release and decrease in cellular ergosterol content. The effect of biocide on cell wall seems to be very negligible as evidenced from little change in the chitin content. Scanning electron microscopy study reveals that there is a considerable change in the mycelium with the collapse of hyphae structure and the effect was observed to be very pronounced in the case of dimethyldithiocarbamate and 2,2,dibromo-3-nitrilo propionamide, where the hyphae seem to have totally collapsed with considerable reduction in thickness. The spores also seem to have shrunk in size with biocide treatment. It appears from the results that all three biocides bring about fungal inhibition through membrane damage with almost negligible effect on the cell wall.

Key Words: *Aspergillus versicolor*, Chitin, Ergosterol, Fungicides, Leather Watch Strap, 18S rRNA And SEM,

Introduction

In many manufacturing industries dealing with materials prone to microbial attack, biocides are used for imparting protection during processing as well as increasing the shelf life of the products. Leather industry is one such industry as it deals with skin matrix which is prone to attack by different types of microorganisms during processing as well as its lifetime (Orlita 1968b; Zyska 2000).

The microorganisms encountered in leather industry have been extensively reviewed (Cordon et al. 1965; Orlita 2004; Chandra Babu et al. 2011). After tanning, the hides and skins become permanently resistant to bacterial degradation but are prone to fungal attack and hence should be treated with suitable biocides with fungicidal efficacy. Pickled pelt and wet blue leather are internationally traded commodities which require protection against fungi for enabling longer storage and transportation. Even the finished leather is susceptible to fungal attack and hence treatment with an effective fungicide during wet finishing becomes necessary to impart permanent preservation for continued protection during usage.

Some of the biocides used earlier in leather industry including organo mercuric compounds like Phenylmercuric acetate (PMA) and chlorinated phenols like Pentachlorophenol (PCP) have been phased out due to toxicological consideration (Hauber and Germann 1997; Didato and Yanek 1999) and newer biocides with least toxicity have been introduced.

Fungal species encountered in leather industry have been profiled and the common species identified mostly belong to families of *Aspergillus* and *Penicillium* and some species of *Trichoderma, Paecilomyces,* and *Cladosporium* are also implicated in certain conditions (Orlita 2004). Other species of fungi reported include *Verticillium glaucum*, *Verticillium tenerum, Basipetospora,Fusarium chlamydosporium*, *Trichosporon* and *Scopulariopsis brevicaulis(*Birbir 1994; Orlita 2004*)*.

In the present investigation, a new fungal species hitherto not commonly encountered in leather industry has been isolated from leather watch strap. A detailed study has been performed to identify the species and to screen the efficacy of fungicides in inhibiting this new strain. An attempt has also been made to understand the mechanism of the fungicidal action on the isolated strain.

Materials and methods

The watch strap infested with the fungal growth as received from a reputed Indian Watch strap brand has been used in the study. Media and other chemicals were purchased from Hi-Media (India). Three biocides formulations based on 2-(Thiocyanomethylthio) benzothiazole (TCMTB), potassium dimethyldithiocarbamate, 2,2,dibromo-3-nitrilo propionamide (DBNP), respectively were sourced from reputed Leather Chemical Supply Houses.

Phenotypic and genotypic characterization

To study the phenotypic characterization, the fungal isolate was grown on SDA and the radial growth was observed by inoculating spore suspension of 10µl (1-5x10^6 CFU ml^{-1}) on the centre of an agar plate, incubated at l26±2ºC. Genomic DNA was isolated from fungal mycelial mats grown on sabouraud dextrose broth (SDB) for 5-7 days as per the procedure of Liu et al. (1997). PCR reactions were carried out according to the standard protocol (White et al. 1990).

Study on the efficacy of leather fungicides in controlling the growth of the fungal species

Three commercial fungicide formulations used in leather industry were screened for their efficacy in the inhibition of the new species. The stock solutions of all the formulations were prepared at a concentration of 1000 µg mL^{-1} using sterile distilled water as per the laboratory standard protocol (Andrews 2001).

Spore suspension for antifungal susceptibility test was prepared as per the standard method (Petrikkou 2001). The minimum inhibitory concentration (MIC) for all fungicides against the isolated species was determined by Macrobroth dilution method (M38-A) with slight modification as reported earlier (Fothergill 2002).

Determination of Soluble Protein

Soluble protein in the biocide treated and untreated fungal cells, was determined by Lowry's method.

Ergosterol assay

The effect of fungicides on the fungal cell membrane permeability was determined by ergosterol assay method (Arthington-Skaggs et al. 1999). The ergosterol content and sterol intermediate 24(28) dehydroergosterol (DHE) in all the samples was calculated as % of dry weight of the cell as described by Khan et al. (2013).

Cellular release content

The cellular release was measured according to the method previously reported (Paul et al. 2011). The mycelial suspensions were treated with 2 fold MIC of each fungicide and incubated in a shaker incubator at 28± 2ºC. About 2ml of samples were drawn at different time intervals and centrifuged at 12000 rpm for 2 min, the absorbance for supernatant was measured at 260nm.

Fungal Cell wall preparation and determination of chitin content

Fungal cell walls were prepared from the mycelium pellets according to the method of Pessonie et al. (2005). The chitin content in the cell walls was determined by the method of Nilsson and Bjurman (1998). Chitin content was calculated as glucosamine hydrochloride ($\mu g\ mg^{-1}$ of dry weight of fungal cell walls) from a standard curve obtained with varying concentrations of glucosamine hydrochloride (5-30 $\mu g\ mL^{-1}$).

Scanning Electron Microscope (SEM) Analysis

SEM analysis was performed to study the effect of fungicides on the fungal morphology. The samples were prepared as per standard protocol using an Edwards E-306 sputter coater and examined in Bruker S-3400N at a magnification of 10,000x and a voltage of 10Kv.

Results and Discussion

Phenotypic and genotypic characterization

Colonies growing on SDA (Fig. 1) were identified based on morphology, reverse and surface coloration of colonies (Pitt and Hocking 1994; Cheesbrough 2000; Abbey 2007).

a) Colony morphology   (b) Reverse side
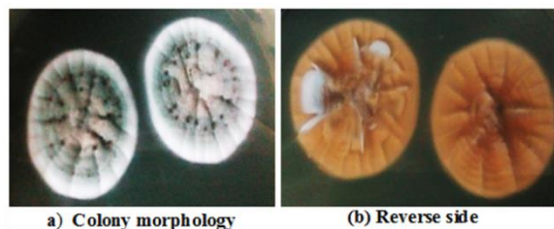
Fig. 1 (a) Seven days old *Aspergillus versicolor* colony on SDA showing green velvety appearance with white edges and deep red exudates (b) The reverse of the same colony on SDA

Microscopic examination of the isolate appeared to have reduced conidiogenous structures like *Penicillium* sp. having brush-like conidial head (Fig. 2), which indicated that this species might be a *Penicillium* species.

(a)   (b)
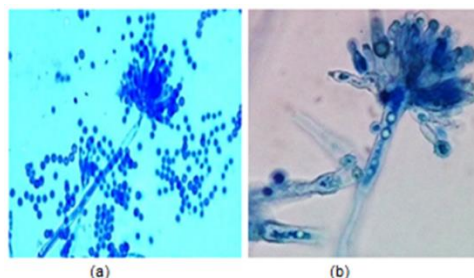
Fig. 2 Microscopic Morphology of *A. versicolor* at (a) 40X and (b) 100X magnification

A consensus sequence of 757 bp was obtained by 18s rRNA sequencing and submitted to GenBank and the accession number of KX814964 was obtained and strain designated as *Aspergillus versicolor* TANCK1. Evolutionary history was inferred using neighbour-joining method (Kumar et al. 2016) and the fungi was rooted to *Aspergillus versicolor* strain PSFNRO-2with 99% similarity. From leather industry point of view, *A. versicolor* strain is not commonly encountered and only one review paper has reported the occurrence of *A. versicolor* F-581 strain on leather (Orlita 2004).

Susceptibility of *Aspergillus versicolor* strain to biocides

TCMTB formulation showed good inhibition at lesser concentration (31.2 µg ml^{-1}) when compared with other two formulations based on potassium dimethyldithiocarbamate (1250 µg ml^{-1}) and 2,2, dibromo-3-nitrilopropionamide (DBNP-625 µg ml^{-1}).

Understanding the mode of action of biocides on fungal cells

Variation in the level of chitin content in all the three fungicides treated samples compared to untreated samples was found to be negligible with p-value > 0.05 (Table 1). The results indicate that the major target of the fungicides may not be the cell wall and the target could be cell membrane. A decrease in the total cell protein and ergosterol content was observed when treated with three fungicides when compared with that of untreated cells (Table 2 & 3). Reduction in ergosterol content was observed to be maximum in the case of dithiocarbamate with 56.67% closely followed by DBNP with 51.38%. The reduction was found to be the lowest with TCMTB (11.05%), which is surprising considering the fact that the lowest MIC is the lowest for this fungicide against the strain.

Table 1 Chitin content in the fungal cell walls in the presence and absence of various fungicides

Fungicides	Chitin content (µg/mg dry wt of cells)	Chitin content (µg/ml)
Control (Untreated)	0.73±0.04	10.55±0.05
TCMTB	0.69±0.05	10.32±0.22
Dithiocarbamate	0.77±0.02	8.50±0.40
DBNP	0.66±0.04	9.03±0.04

Table 2 Total protein content in the presence and absence of various fungicides

Fungicides	Total protein Content (µg/mg dry wt of cells)	Total protein content (µg/ml)
Control (Untreated)	2.64±0.59	37±0.026
TCMTB	1.14±0.36	17±0.008
Dithiocarbamate	1.42±0.30	15.5±0.010
DBNP	1.117±0.038	15.2±0.029

Table 3 Ergosterol content in the fungal cell membrane in the presence and absence of fungicides with % reduction in each case compared to control

Fungicides	Ergosterol content (%)	Reduction in %
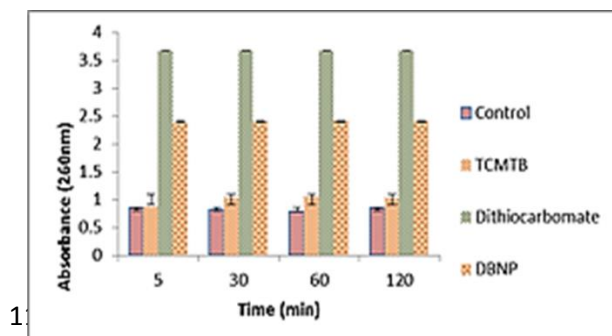
Fig. 3 Release of cytoplasmic cellular constituents in control and fungicide treated cells

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Control	0.1942±0.006	-
TCMTB	0.1240±0.011	11.05±1.01
Dithiocarbamate	0.0709±0.010	56.67±0.99
DBNP	0.0402±0.011	51.38±1.11

In cellular release study, the release of cytoplasmic constituents with respect to time of incubation was followed (Fig. 3). The dithiocarbamate treated cells showed maximum release with OD_{A260} of 3.67 in 5 min release of intracellular constituents when compared with that of the control samples with OD_{A260} of0.85 for the same incubation time. Therefore, it indicated that the fungicidal target is primarily the cell membrane.

SEM Analysis

The effect of fungicides on the fungal morphology was also studied (Fig. 4). The control (untreated) samples grown in SDB had normal mycelial growth with biseriated conidial structures and tubular homogenous hyphae (Fig. 4a). There is a considerable change in the mycelium with the collapse of hyphae structure with fungicidal treatment and the effect was observed in the case of dithiocarbamate and DBNP (Fig. 4c & d) compared with TCMTB (Fig. 4b). Similar effect was also observed earlier in the case of *P. italicum,* where changes in hyphae and increase in cell permeabilization were also reported (Bajapai et al. 2013). The spores also seem to have shrunk in size with biocide treatment. This supports the inference made from the cell wall studies that the biocidal action is brought about by

the collapse of the cell membrane.
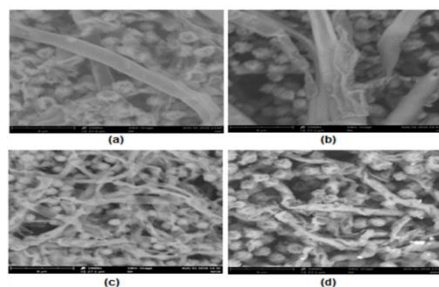
Fig. 4 Scanning electron microscopic images of *A. versicolor* TANCK1 (a) control (b) treated with TCMTB, (c) treated with DBNP, (d) treated with dithiocarbamate

Conclusion

It is inferred from the results that all the three fungicides bring about inhibition through disruption of cell membrane without much effect on cell wall. It is proposed to carry out gene expression studies to understand the genotypic changes brought about in *Aspergillus versicolor* by the fungicidal treatment*,* which may lead to development of biocidal resistance.

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A STUDY ON DENSE GAS DISPERSION MODEL

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The Dense Gas Dispersion Model (DEGADIS) is a mathematical dispersion model that can be used to model the release of toxic chemicals into the atmosphere. It's range of applicability includes continuous, instantaneous, finite duration, and time-variant releases; negatively-buoyant and neutrally-buoyant releases; ground-level, low-momentum area releases; ground-level or elevated upwardly-directed stack releases of gases or aerosols. The simulations are carried out over flat, level, unobstructed terrain for which the characteristic surface roughness is not a significant fraction of the depth of the dispersion layer. DEGADIS can be used as a refined modeling approach to estimate short-term ambient and the expected area of exposure to concentrations above specified threshold values for toxic chemical releases. It is especially useful in situations where density effects are suspected to be important and where screening estimates of ambient concentrations are above levels of concern. Exposure to an ammonia-charge atmosphere may induce various types of physical effects such as Ocular effects:, Skin effects, Respiratory effects. APO is an accident which occurs following the inhalation of vesicant gases (Cl_2, NH_3, SO_2) by breaking down the walls of the pulmonary alveoli which are then inundated with blood plasma. Studying the atmospheric dispersion of ammonia is of major interest for two reasons. Firstly, ammonia is a very toxic, corrosive, flammable and explosive substance under certain conditions. Secondly, ammonia is a very widely used substance with numerous applications, due to its chemical or physical properties. With respect to its chemical properties, the main application of ammonia is the manufacture of fertilizers. Ammonia represents the main concentrated source of nitrogen for agriculture, which consume approximately 85 to 90% of production. Industry uses ammonia as a raw material for the manufacture of explosives, fibers and plastics. It is also used in the manufacture of paper, rubber, in refineries, the leather industry and the pharmaceutical industry.

Keywords: Dense Gas Dispersion, Emission control, Simulation and Modeling

Introduction

A large number of industrial activities involve the use of toxic materials that if released, could cause fatalities and distress in the local population. It is necessary to calculate the consequence of any releases of material which involve estimation of release rates, dispersion for a range of credible

scenarios, followed by an assessment of the toxicological effects of the resulting concentrations of the material.

Accidental releases of any hazardous material are not generally predictable with any degree of accuracy. Some methods of hazard identification and scenario development are necessary preliminaries to any consequence analysis. An important part of any consequence analysis involving the release of toxic material is the calculation of the dispersion of any vapor released to the atmosphere.

The main agent in dispersing airborne releases is the turbulence within the atmosphere. Many gases with positive or neutral buoyancy will disperse rapidly and therefore will not affect personnel at ground level. Standard methods are available for dispersion in such situations. Dense vapor such as chlorine, phosgene and MIC will remain close to the ground and cause a problem over a greater distance.

Many gases with positive or neutral buoyancy will disperse rapidly and therefore will not affect personnel at ground level. Standard methods are available for dispersion in such situations. Wind speed generally, a higher will result in improved dilution and hence shorter hazard ranges. However, in certain circumstances a higher wind speed may result in a gas cloud being carried further downwind, with resultant increased hazard potential.

The action of atmospheric turbulence is most efficient in unstable conditions. When the atmosphere is strongly stable, the turbulent mixing is much reduced and hazardous cloud sizes are increased.

Materials

Deliming process and the release of Ammonia during Deliming[1]

Deliming is one of the most important unit operations in leather processing. Conventional deliming process employs ammonium salts which generates considerable amount of ammonia during the process, making tannery environment unhealthy. Therefore ammonia free deliming is necessary in view of environmental concern. Deliming is carried out inorder to remove the lime from the pelt. It is a process of neutralizing lime. The objectives of deliming are

- Remove the bound lime
- Lowering the pH, in preparation for bating
- De_swelling the pelt, partially reversing the swelling

If the pelts are delimed and limed pelts are directly introduced to vegetable tanning liquor, lime present in the pelt will react with vegetable tanning and produced calcium tannate. This is the percipitate which will deposit at the surface of the pelt to produce grain cracking. Conventional deliming is based on ammonium salts in which significant amount of ammonia is liberated and also contribute significantly to total Kjeldahi nitrogen (TKN) as reported by UNIDO. Excessive liberation of ammonia is of great environmental as well as health concern. There is a significant reduction in both ammonia concentration and TKN in deliming sectional stream of wastewater due to ammonia free systems,

suitable for biological wastewater treatment methods. Ammonia liberated as (Kg/t raw hide) for Ammonia free systems(AFS) is 0.070 as compared to 2.69 for the conventional ammonium salts(AS) based systems. Similarly ammonia in waste water is 58 ppm for AFS as compared to 2243 for AS. Whereas, TKN (Kg/t raw hide) another important parameter is 0.6-1.5 for AFS as against 2.6-3.9 for AS process.

Liberation of ammonia in Ammonium salts based deliming

Use of ammnium chloride as deliming agent

$$Ca(OH)_2 + NH_4Cl \text{-----------------------} > CaCl_2 + NH_3 + H_2O \qquad (1)$$
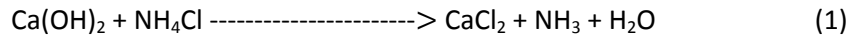
In the above reaction(1) calcium chloride formed is completely soluble in water. When Ammonium Sulphate is used for deliming purposes it reacts with calcium hydroxide and produces calcium sulphate(2). The reaction involved is given below:

$$(NH_4)_2SO_4 + Ca(OH)_2 \text{-----------------------} > CaSO_4 + 2 NH_3 + 2 H_2O \qquad (2)$$
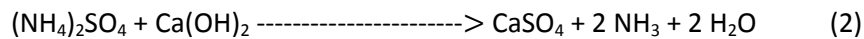
This ammonia liberated during the process lead to workers exposure to high concentration of ammonia gas while opening the drum during or after deliming process . This would also lead to prevalence of ammonia in and around the tannery premises.

There are environmental health hazards associated with ammonia [2] . Based on the reports of National Institute for Occupational Safety and Health (NIOSH) with regard to ammonia in work places, OSHA's former exposure limit for ammonia was 50 ppm as an 8-hour Time weighted average(TWA). OSHA proposed to revise this limit to 25 ppm TWA and to add 15-ppm 15-minute Short-term exposure limit based on the limits established by the American Conference of Governmental Industrial Hygienists(ACGIH). Exposure limit values and Health factor associated with Ammonia gas are given by OSHA, US Department of Labour.

Methods

Turbulent structure of the atmosphere

The wind and in particular its turbulent nature, is the chief agent in dispersing any gas releases. It is therefore useful to understand the structure of the atmosphere and the characteristics of turbulence. Winds are generated by large scale pressure differences, which, in turn are caused by differential solar heating of land and sea masses.

The structure of the wind at any location is then determined by the underlying terrain, a rougher terrain causing more turbulence and resulting in lower mean wind speeds, but with potentially higher gusts. An excellent review of the atmospheric processes involved in the production of strong winds is given by Cook(1985). Pasqual(1962) has defined a range of stability categories from A to F, the most significant of which are:

A Unstable – highly turbulent but relatively low wind speed

D Neutral – moderate turbulence, generally moderate to strong winds

F Stable – very little turbulence, with low wind speed

Essentials of Dispersion

The dense gas is affected by release conditions, topography and type of surroundings, and also the atmospheric ambient conditions. The essentials of dense gas dispersion include:

1. Source data
2. Meteorological data
3. Receptor data
4. Site information

The transport and dispersion of gas released in atmosphere is dependent on chemical, the release scenario and the desired averaging time. The gas dispersion modeling considers these stages:

1. Initial acceleration and dilution
2. Dominance of internal buoyancy
3. Transition from dominance of internal buoyancy to dominance of ambient turbulence
4. Dominance of ambient turbulence

Dispersion of gas released in atmosphere occur as a result of turbulence in the atmosphere, therefore it is significant to influence by the meteorological condition. Based on the chemical nature of gas, the dispersion of vapour clouds is classified into two groups.

Dense gas dispersion modeling is categorized into two classes:

1. Instantaneous release modeling
2. Continuous release modeling

Instantaneous release modeling

The instantaneous release of dense gas, can occur in the event of the catastrophic failure of vessel containing, gas or liquid capable of flashing instantly. In the case of instantaneous release of dense gas, cloud shape most frequently used is cylindrical type.

The initial volume of cloud, usually represented as a vertically oriented cylinder of height, h and radius, r placed in the flow field. The cloud is moved downwind with a velocity determined from the wind vertical profile. The following equations were used:

Mass conservation equation:

$$dm/dt = dmg/dt + dma/dt \qquad\qquad (3)$$

where dma/dt(3) is the rate of mixing of air with vapour cloud and dmg/dt = 0 for instantaneous case.

Gaussian air pollutant dispersion equation[2]

Sir Graham Sutton derived an air pollutant plume dispersion equation in 1947 which did include the assumption of Gaussian distribution for the vertical and crosswind dispersion of the plume and also included the effect of ground reflection of the plume. The Complete Equation(4) for Gaussian Dispersion Modeling of Continuous, Buoyant Air Pollution Plumes shown below:

$$C = \frac{Q}{u} \cdot \frac{f}{\sigma_y\sqrt{2\pi}} \cdot \frac{g_1 + g_2 + g_3}{\sigma_z\sqrt{2\pi}} \qquad (4)$$

Where f =crosswind dispersion parameter = $\exp\left[-(z+H)^2/(2\,\sigma_z^2)\right]$
G = vertical dispersion parameter = g1+g2+g3
C = Concentration of emissions, in g/m^3
Q = Source pollutant emission rate, in g/s
H = Height of emission plume centerline above ground level in m
σ = Standard deviation of the emission distribution in m
L = height from ground level to bottom of the inversion aloft in m

Briggs plume rise equations

The Gaussian air pollutant dispersion equation requires the input of H which is the pollutant plume's centerline height above ground level—and H is the sum of Hs (the actual physical height of the pollutant plume's emission source point) plus ΔH (the plume rise due the plume's buoyancy).
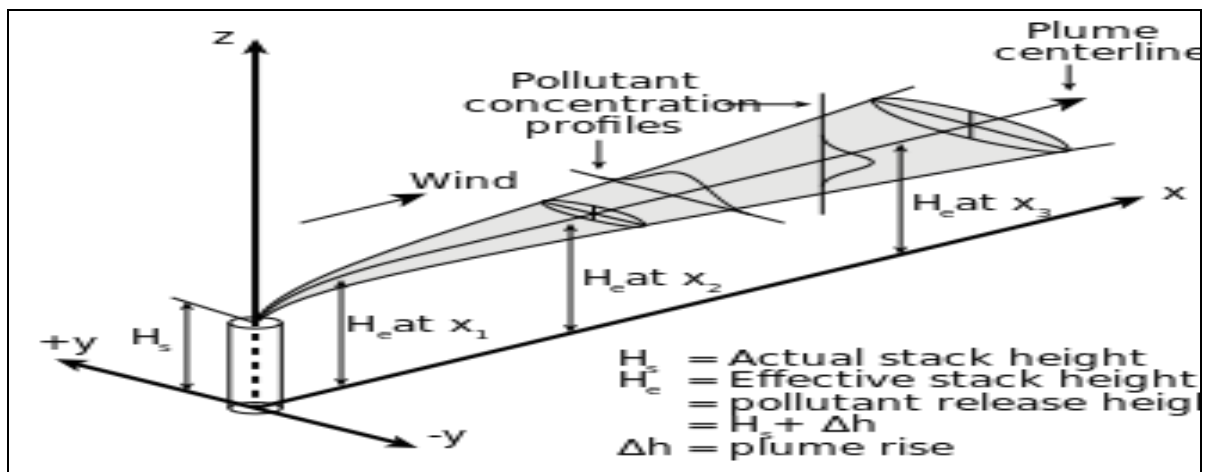
Fig 1.0 Visualization of a buoyant Gaussian air pollutant dispersion plume

Briggs divided air pollution plumes into these four general categories:

- Cold jet plumes in calm ambient air conditions
- Cold jet plumes in windy ambient air conditions
- Hot, buoyant plumes in calm ambient air conditions
- Hot, buoyant plumes in windy ambient air conditions

where:

Δh = plume rise, in m

F = buoyancy factor, in $m^4 s^{-3}$

x = downwind distance from plume source, in m

x_f = downwind distance from plume source to point of maximum plume rise, in m

u = wind speed at actual stack height, in m/s

s = stability parameter, in s^{-2}

Effect of ammonia on human beings[3]

- 50 – 10 ppm (V) (weighted average for eight hours) can be detected by the odour
- 750 ppm (V) (weighted average for eight hours) can be detected by persons who are accustomed to work in ammonia atmosphere
- 150 – 200 ppm gives general discomfort and eye pain
- 400 – 70 ppm results in eye irritation, ears and throat irritation more severe
- 2000 ppm causes burns, blisters, strangulation, asphyxia and ultimately death

Case study 1:
CALCULATION MODEL : DISPERSION ---- 1-BUTENE

SOURCE TYPE   : CONTINUOUS
AMOUNT OF GAS BETWEEN TWO LIMITS
STABILITY CLASS ; VERY STABLE
DESCRIPTION OF ROUGHNESS – LENGTH:
RESIDENTIAL AREA (AREA WITH DENSE BUT LOW BUILDING,WOODED AREA, INDUSTRIAL SITE WITH NOT TO GREAT OBSTACLES)

SOURCE HEIGHT	=	0.0	M
WIND VELOCITY	=	2.0	M/S
SOURCE STRENGTH	=	25.0	Kg/sec
TEMPERATURE	=	288	K
AVERAGE TIME	=	400	S

AMOUNT OF GAS = 4.11E+03 KG

BETWEEN 0 AND 546 M

UPPER LIMIT = 0.09 M^3/M^3

LOWER LIMIT = 0.02 M^3/M^3

Case study 2:

MAXIMUM CONCENTRATION IN THE DIRECTION OF THE WIND

STABILITY CLASS : VERY STABLE

DESCRIPTION OF ROUGHNESS – LENGTH:

FARM LAND

SOURCE HEIGHT   =   1.0   M

WIND VELOCITY=   2.0   M/S

SOURCE STRENGTH   =   25.0   Kg/sec

TEMPERATURE =   288   K

AVERAGE TIME =   600   S
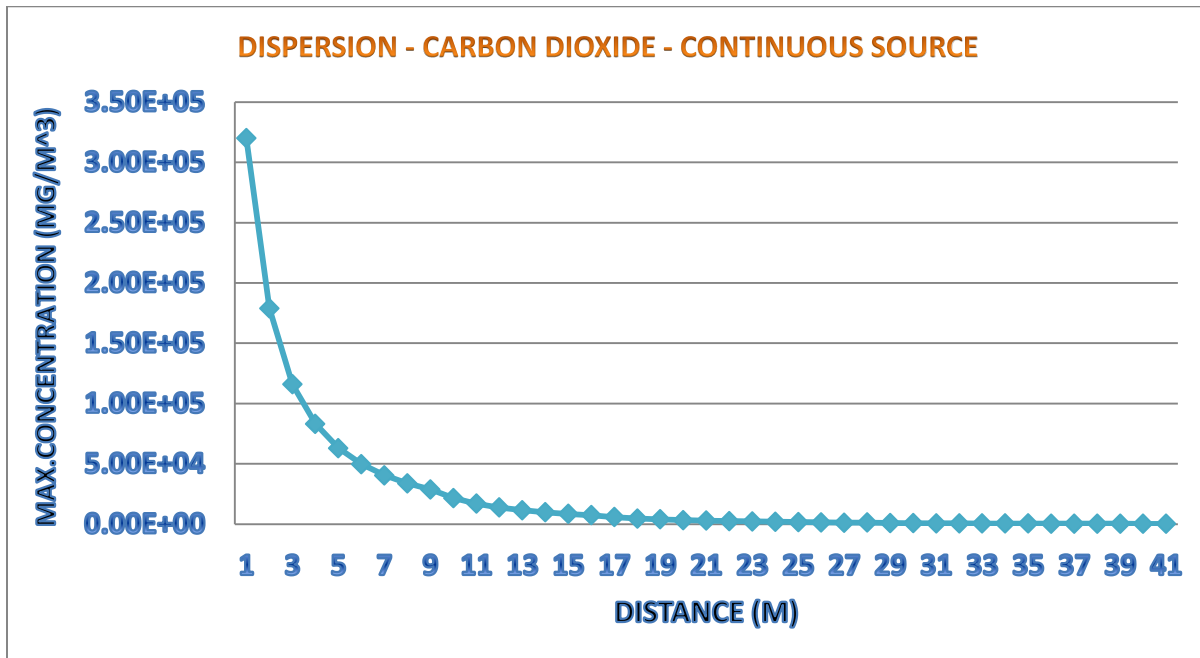
Fig 2.0 Dispersion Calculation model: Distance vs. Max Concentration

Case Study 3:

INSTANTANEOUS SOURCE MODEL : VINYL CHLORIDE

MAXIMUM CONCENTRATION AT A GIVEN POINT
STABILITY CLASS : NEUTRAL
DESCRIPTION OF ROUGHNESS LENGTH : CULTIVATED LAND

SOURCE HEIGHT   =   0.0   M
SOURCE CONCENTRATION = 1.0 M^3/M^3
WIND VELOCITY=   5.0   M/S
SOURCE STRENGTH   =   3000.0 Kg
TEMPERATURE =   293   K

X(M)	Y(M)	Z(M)	T(S)	CONCENTRATION (MG/M^3)
150	50	0	30	4.74E-03
200	50	0	40	5.20E-01
250	40	0	50	1.75E+02
300	25	0	60	5.97E+03
500	0	0	100	9.85E+03

Results

Following graphs[4] illustrate the Concentration of Chlorine, Ammonia at various locations when there was a release of these gases occurred and these results are collected from Use of Dispersion Modeling Software in Ammonia Refrigeration Facility Design, Martin L. Timm, PE , World Wide Web.
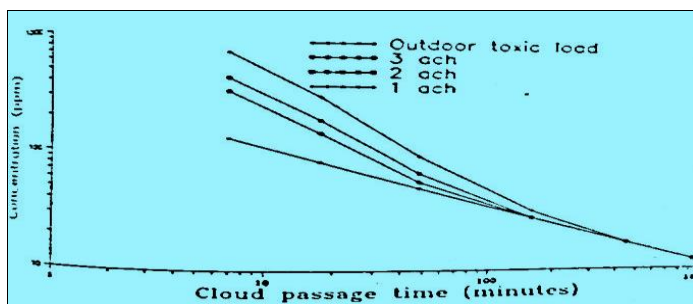
Fig 3.0 Indoor Chlorine concentration criteria for dangerous toxic load
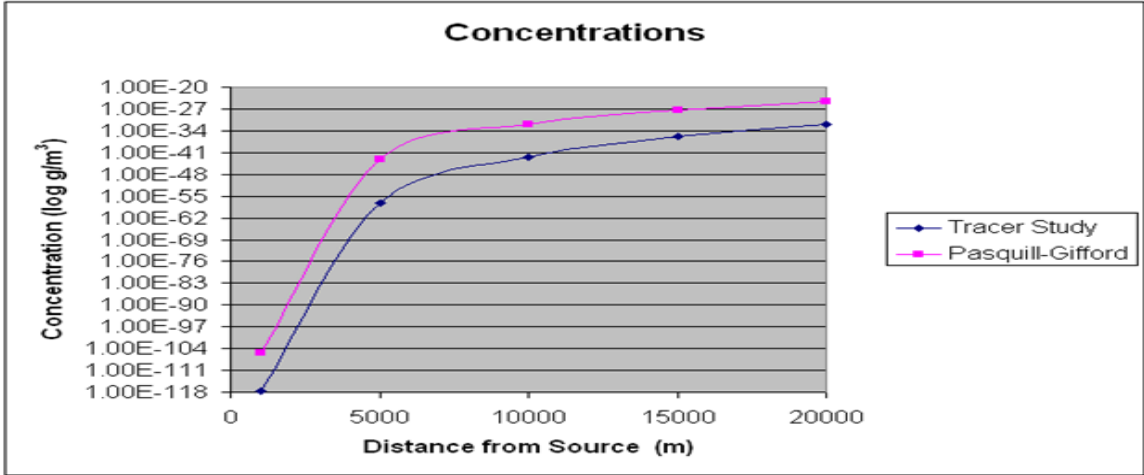
Fig 4.0 Source strength based on Pasquill and Tracer study
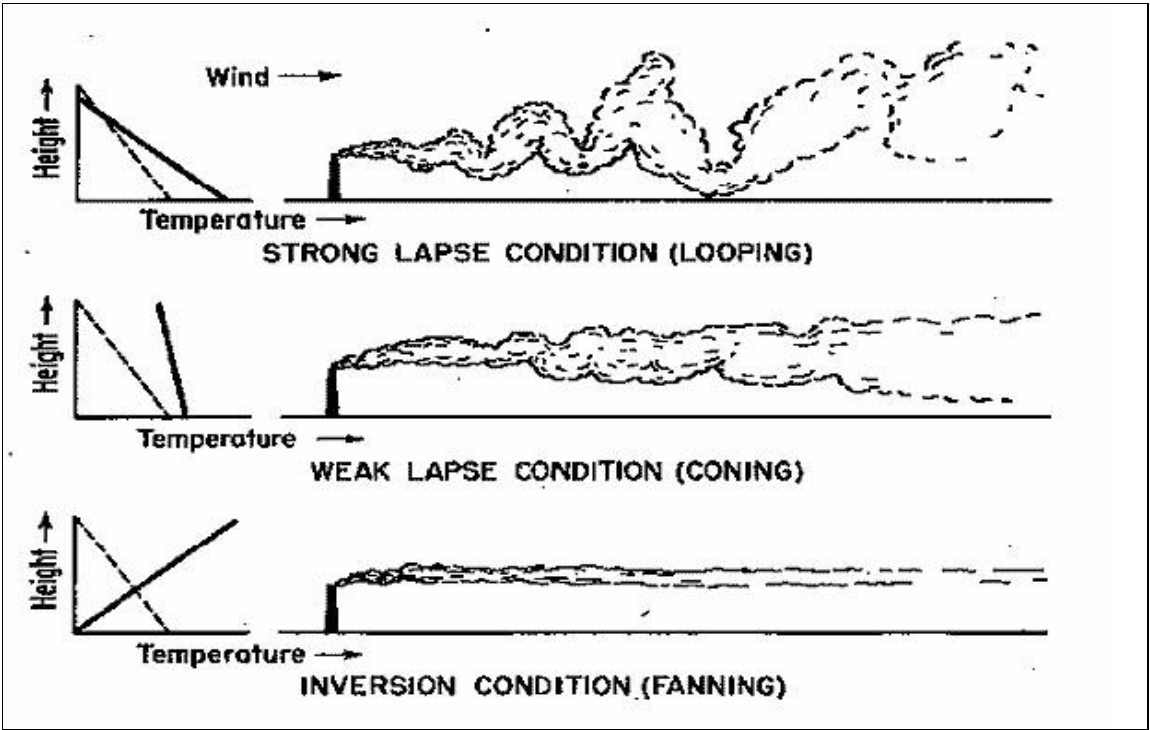
Fig 5.0   A Typical Stack plume dispersion
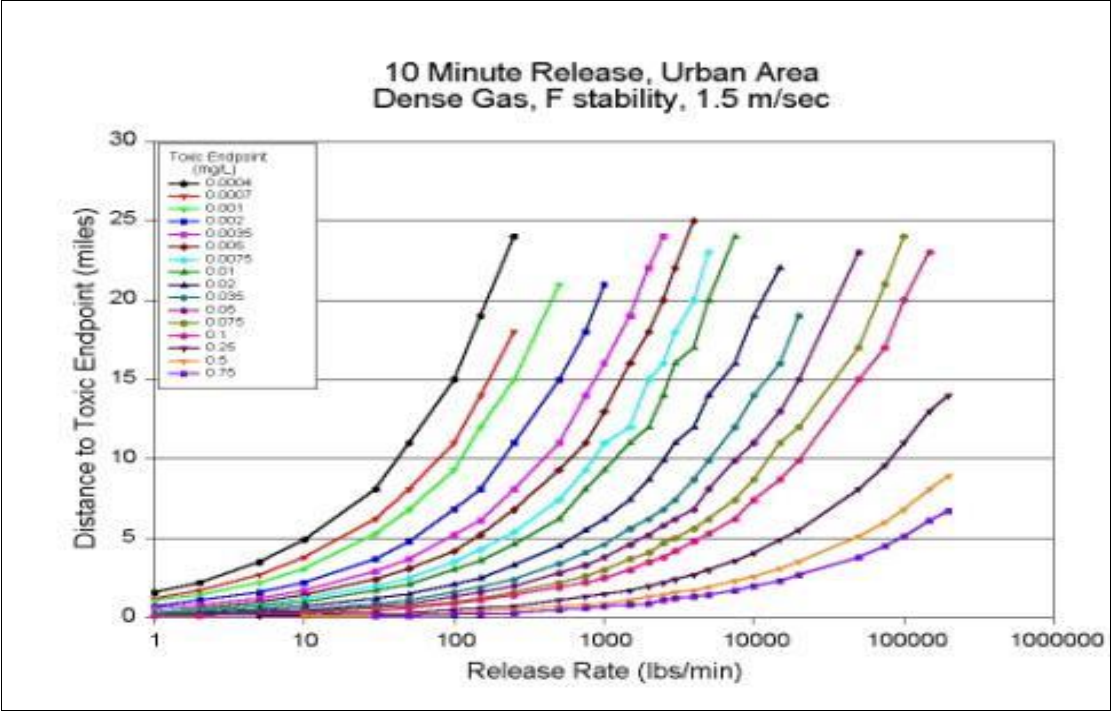
Fig 6.0 Dense gas dispersion model in Urban area
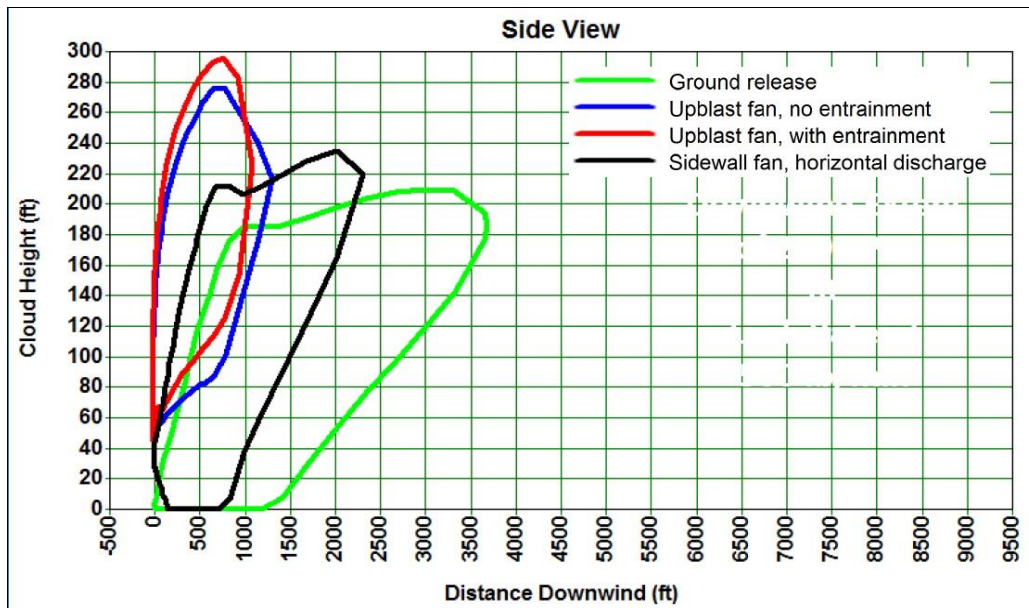
Fig 7.0 Discharge of Ammonia at various locations – a typical diagram

Conclusions

This is a preliminary literature survey work carried out on dispersion modelling. Many models exists to solve certain problems and perform certain functions. The future course of action is to develop and standardize the software to simulate and validate the dispersion model.

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MASSIVE OPEN ONLINE COURSES FOR ENRICHING HUMAN CAPACITY

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Indian higher education system has expanded at a fast face by adding nearly 25,000 colleges and more than 8 million students in a decade from 2001-01 to 2010-11. By 2050, India is projected to have 1 billion employable people (Source: Indian National Academy of Engineering, Vision, Mission and Values – INAE2037). The human resource must be channelized into enhancing national and intellectual property and productivity. Massive Open Online Courses (MOOCS) has caught up in India, and many corporations are using this platform to train people and supply the industry with skilled labor. This paper proposes a framework for MOOCS and eLearning system for higher education. Knowledge management is about creating an environment where information can be readily shared. Creating a learning organization culture is critical. The future of integrated teaching and learning systems provides exciting opportunities to create new interactive learning environments quickly and at a much lower cost.

Keywords: Standards, System Architecture, Global education, MOOCS, eBooks, Lifelong learners, Global Network

1.1 INTRODUCTION

Technology has the power to transform the class room and it can pave new ways of training and ideas. Blended learning refers to learning models that combine traditional classroom practice with e-learning solutions. Taking advantage of the bandwidth to desktops, the capabilities of mobile technologies, and the emerging Web-based standards for delivering instruction creates an environment for collaborative learning. Providing libraries of information that can be used to train employees, students will reduce the duplicate expenses being spent because the learning objects can be used in different formats, for different purposes. With distributed technologies, learning can take place anytime, anywhere. The Web can now integrate learning and mission-critical business applications delivering timely knowledge to each desktop. The end result is a knowledge management structure which includes an inventory of knowledge objects and a system in which these can be shared. Knowledge has long been a tool of innovation and a key driver for economic and social development. The "21st Century Skills," includes digital age literacy consisting of functional literacy, visual literacy, scientific literacy, technological literacy, information literacy, cultural literacy, and global awareness, inventive thinking, higher-order thinking and sound reasoning, effective communication, and high productivity. Various competencies must be developed throughout the educational system for ICT integration to be successful.

1.2 PROPOSAL

The proposed eLearning system (Digital ecosystem)[1] has the following features:

- Integrates traditional lecture based teaching with online learning materials and communication technologies through Video conferencing wherever essential
- Enhances teaching effectiveness using rich multimedia
- EL content, inside the campus, and study centre.
- Enables Subject Matter Experts to rapidly create eLearning content, in – house, using simple technologies and tools saving time, money and effort.
- Provides anytime anywhere learning for large audiences, spanning rural and global students and working professionals.
- Utilizes resource personnel from Industries and Higher learning Institutions.
- Provides Guided practice sessions through Self learning with an intensive online training program.
- Provides eBooks, Digital library, Virtual labs, Video based curriculum, Video conferencing and multi-language support.
- Creates a Knowledge Centre with multiple Knowledge Channels, with a long term vision of creating a Knowledge HUB, connecting to National and Global Knowledge network.

1.3   eLearning

eLearning delivers instruction anytime, at any place and in any combination desired by the learner. Web-based learning, interactive distance learning, Intranet-based learning, CD- ROM etc, are of different types of learning technologies which come under 'e-learning' with different capabilities. The salient features of e_Learning are: Anytime, Anywhere Availability, Efficiency, and Flexibility and Inexpensive worldwide distribution. The objective in such a case is to ensure that people need not re-invent the wheel. The second important factor for a learning organization is to keep the focus on continuous improvement, to expand the coverage of teacher training, to enhance the way teachers are being trained to cater to the students' requirements, to enhance the training component and to reduce the tedious and mechanical aspects of some of the current training methods.

The combination of distance learning, ICT and networked computer capabilities would enable providing education and training cost effectively. e_ Learning is the convergence of learning and the internet. It is learning delivered via a range of technologies such as Mobile, Internet, Television, Videotape, Intelligent tutoring systems, Computer based training etc.,. e-Learning encompasses learning at all levels, both formal and non-formal, that uses an information network—the Internet, an intranet (LAN) or extranet (WAN)—whether wholly or in part, for course delivery, interaction and/or facilitation. This is also referred as online learning. Web-based learning is a subset of elearning and refers to learning using an Internet browser such as Mozilla, Internet Explorer and other latest browsers.

1.4   MOODLE (MODULAR OBJECT-ORIENTED DYNAMIC LEARNING Environment)

OpenSourcee-LearningSoftware

Moodleisa CourseManagementSystem(CMS)-asoftware packagedesignedtohelp educators create quality online courses and manage learner outcomes. Such e-learning systemsare sometimes also calledLearningManagementSystems (LMS),VirtualLearning Environments(VLE) and Learning Content Management Systems (LCMS). Students need only a browser (e.g., IE, Firefox, Safari) to participate in aMoodlecourse. Moodle Package system diagram is represented in Fig 1.0

Moodle is an Open Source software, which means one is free to downloadit, use it, modify it and even distributeit(undertheterms of the GNU General PublicLicense). Moodlerunswithout modification onUNIX,Linux,Windows, MacOSX,Netwareandanyothersystemthatsupports PHP,includingmostweb hostproviders.Dataisstoredina singledatabase:MySQLand PostgreSQL arebest supported,butitcanalsobeusedwithOracle,Access,Interbase,ODBC and others.
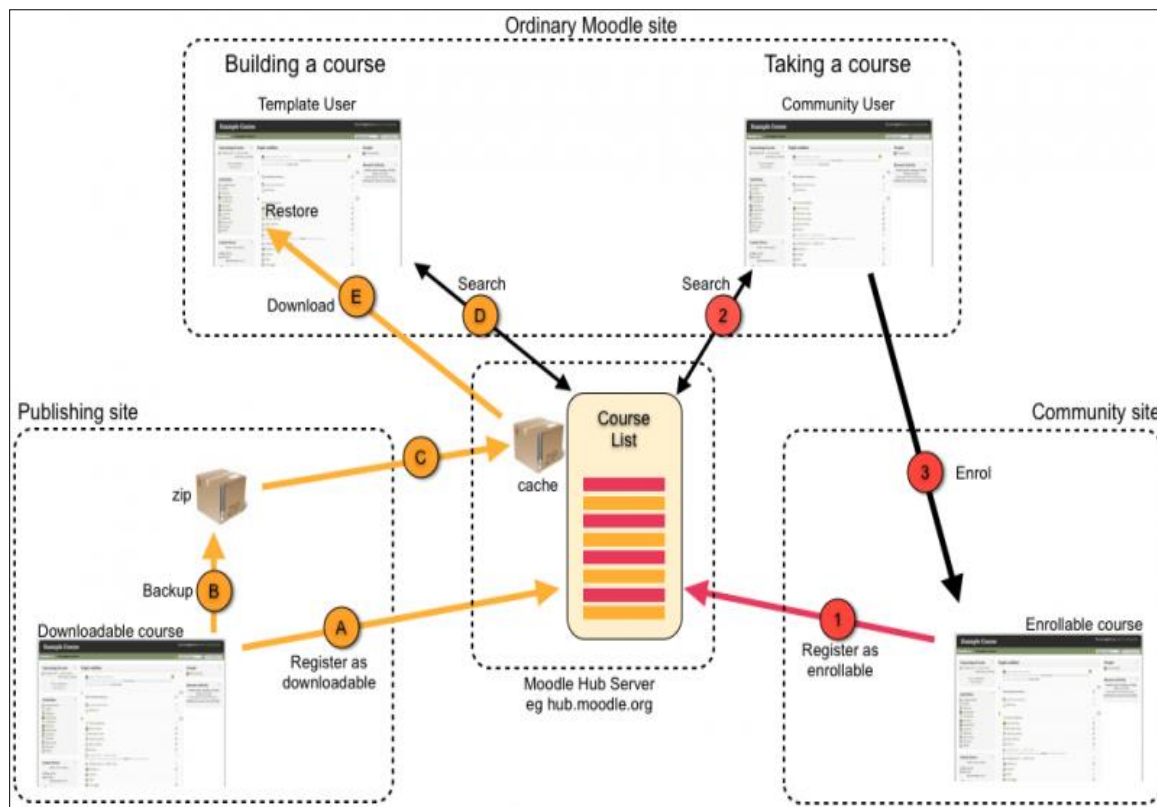
Fig 1. 0 Original MOODLE Package System Diagram
(Source: www.moodle.org - parent website)

1.5   MAJOR STEPS in creating the infrastructure[1]:

• Phase1: Establish an e-learning environment
• Phase 2: Content development
• Phase 3: LMS selection and implementation
• Phase 4: Upload the content
• Phase 5: Tracking, Document management

1.6 DATA CENTRE RUNNING DISTANCE LEARNING PROGRAMME[2]

Withtheseconcepts onecanprovidedistance learning programmeracrossthecontinents andonecanalsoprovide the learningmaterialin multiplelanguages. Fig 2.0describesa Data centresetup runningaDistanceLearningProgramme.Withthe adventofCloudcomputing, thiscanalsobegivenasaSAS modelto providea costeffectivesolution.

Fig 2.0DataCentre

1.7 BIG DATA ADMINISTRATION

BIG DATA represents data sets whose sizes vary from terabytes to zettabytes and it has one or more of the following characteristics – high volume, high velocity, and high variety. Using advanced analytics techniques such as text analytics, machine learning, predictive analytics, data mining, statistics, and natural language processing, businesses can analyze previously untapped data sources independent or together with their existing enterprise data to gain new insights resulting in significantly better and faster decisions. Fig 3.0 represents the BIG DATA Administration sequences.
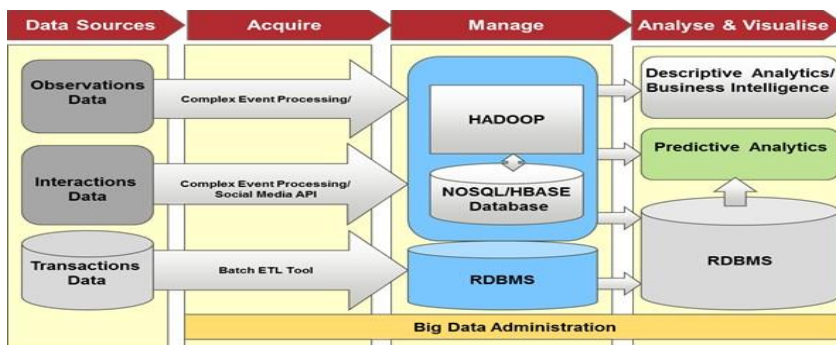
Fig 3.0 BIG DATA Administration

1.8 EBOOKS AND DIGITAL LIBRARY USING MOBILE TECHNOLOGY

Central Leather Research Institute, the World's largest Leather Research Institute, was founded on 24 April, 1948. CLRI made an initiative with foresight to link technology system with both academy and

industry. CLRI, today, is a central hub in Indian leather sector with direct roles in education, research, training, testing, designing, forecasting, planning, social empowerment and leading in science and technology relating to leather. State-of-art facilities in CLRI support innovation in leather processing, creative designing of leather products viz. leather garment, leather goods, footwear and development of novel environmental technologies for leather sector. CLRI being one of the best educational institutes has got a huge collection of books on the leather and allied field. The process of Digital Preservation is given in Fig 4.0
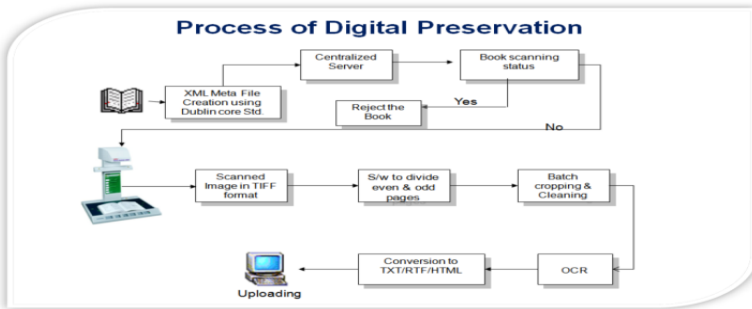
Fig 4.0 Process of Digital Preservation
(Source: A&T video and networks.)

1.9 ENTERPRISE MISSION MANAGEMENT AND KNOWLEDGE PORTAL

The enterprise knowledge portal is the intersection between knowledge management and the enterprise portal. It allows the organization to implement their knowledge management initiatives straight into their business strategies. As a result the enterprise knowledge portal solution brings people, work processes, content and technology into a single solution.

Green Industry promotes sustainable patterns of production and consumption i.e. patterns that are resource and energy efficient, low-carbon and low waste, non-polluting and safe, and which produce products that are responsibly managed throughout their lifecycle.
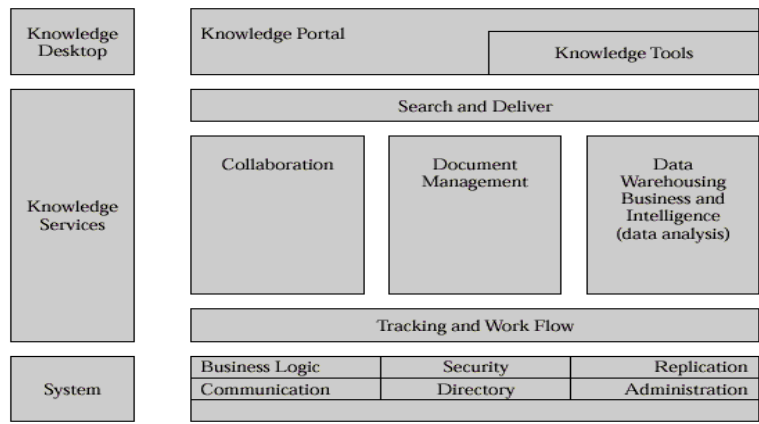
Fig 5.0 Enterprise Mission Management Portal

The Green Industry agenda covers the greening of industries, under which all industries continuously improve their resource productivity and environmental performance. Building an enterprise Mission Management Portal[3] – a Knowledge Park (Fig 5.0) is essential for the success of the Industry.

1.10   eSTEM FOR CURRICULUM

Electronic Governance (eGov) research studies the use of Information and Communication Technologies to improve the governance processes. Sustainable Development (SD) research studies possible development routes that satisfy the needs of the present generation without compromising the ability of the future generations to meet their own needs. Developing an eGovernance system on environmental issues will help an organization to increase its efficiency in natural resource management and diminish wastes and emissions.

A green economy is likely to depend crucially on innovation, in particular eco-innovation. Global carbon trading will be a cost-effective tool to significantly cut greenhouse gases. Carbon credits are a key component of national and international emissions trading schemes. They provide a way to reduce greenhouse effect emissions on an industrial scale by capping total annual emissions and letting the market assign a monetary value to any shortfall through trading.

The following equations are considered for finding the relationship between Gross Domestic product, Carbon credit and Energy Intensity.

Carbon Intensity = Energy Intensity (x) Fuel Mix

Carbon dioxide emissions (CO2) often hypothesized to follow environmental Kuznets curve model: *CO2t = α + δt + β1GDPt+β2GDP2t+ εt*   where CO2t is CO2 emission per capita at time t, GDPt is GDP per capita at time t, t =1,2, ... ,T, t is the time variable and GDP represents the Gross Domestic Product (Fig 6.0). STEM represents the fields of study in the categories of Science, Technology, Engineering, and Mathematics. eSTEM represents environmental STEM.
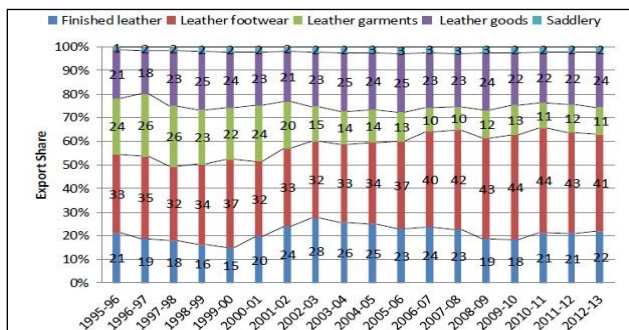
Fig 6.0 Export share of Leather and Leather Products
(Source: DGCI & S.GoI, 2013)
The areas in the figure represent environmental performance efforts [5] at different levels (Fig: 7.0.).

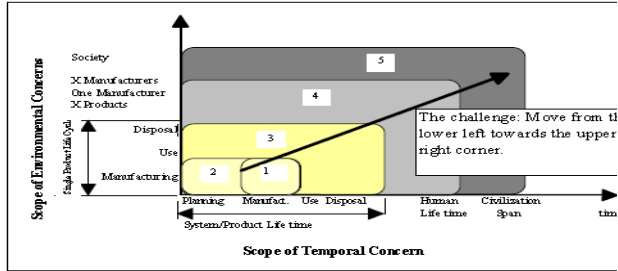|	
1. Environmental Engineering 2. Pollution Prevention 3. Environmental Conscious Design and Manufacturing 4. Industrial Ecology 5. Sustainable Development	 Fig 7.0 Levels of Environmental Performance (Source: EnvironmentalManagement– VijayKulkarni,T.V.Ramachandra)

1.11   RESULTS

The following pictures illustrate how student can login to the LMS on an intranet by giving appropriate password and can access their course material (Fig 8.0). Fig 9.0 illustrates the instructional design on Leather Hand Bag making.

Fig 8.0 Students Authentication system for accessing the course material

Fig 9.0 Instructional design on Leather Hand Bag making

Fig 10.0 Global Reach with eLearning

Fig 11.0. Accessing eBooks through iPad [4]

Fig 12.0 eGov portal for Environmental Management

Fig 10.0 gives the schematic diagram of global reach with eLearning. Fig 11.0 gives accessing of eBooks through iPad and Fig 12.0 depicts the proposed eGovernance portal for tannery.

There are several models and frameworks exist to describe the Green ICT systems for an organization. The digital economy both enables and requires organizations to continually learn new knowledge and systematically deploy it for value creation. A storage area network (SAN) is a dedicated network that provides access to be consolidated, block level data storage. SAN is primarily used to make storage devices, such as disk arrays, tape libraries, and optical jukeboxes, accessible to servers so that the devices appear like locally attached devices to the operating system. Fig 13.0 represents cloud architecture for Digital ecosystem and Fig 14.0 gives the frame work for innovation centre and Business incubators.
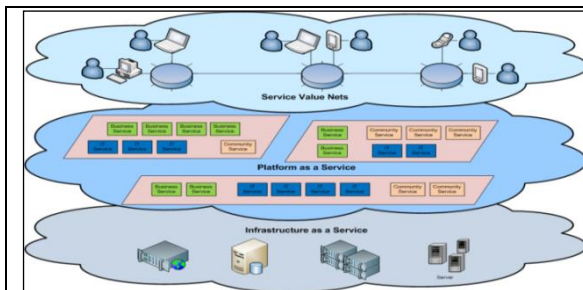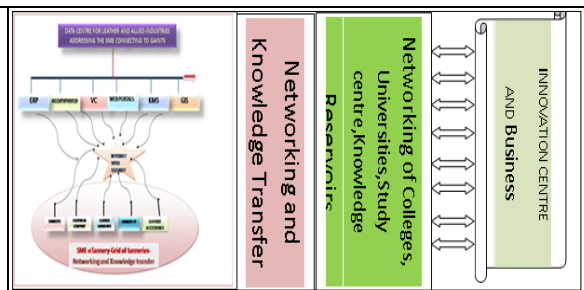
| Fig 13.0 represents cloud architecture for Digitalecosystem | Fig 14.0 A Framework for Innovation centre |

DASHBOARD DEVELOPED BY CISR

Business view provides Mind Map Navigation to the menus. My work page (Fig 15.0) provides details about Trainings, Test and Course Catalog. Employee can also view Task to do, Alerts, Request Status, Announcements and Notifications in the Intelligence panel. Menu helps to navigate to other modules. Fig 16.0 illustrates how one can view Course Name, Duration, Progression, Bookmarks, Supplementary resources, Discussions, Tests and FAQ. Fig 17.0 illustrates how Course Details allows to Participate in Discussions, View Supplementary Resources, Bookmarks and KR Documents. One can also Ask Queries to Experts and View Course Usage Report and Feedback submitted by employees. Fig 18.0 gives typical structure of computer application courses for B.Tech Leather Technology course work.
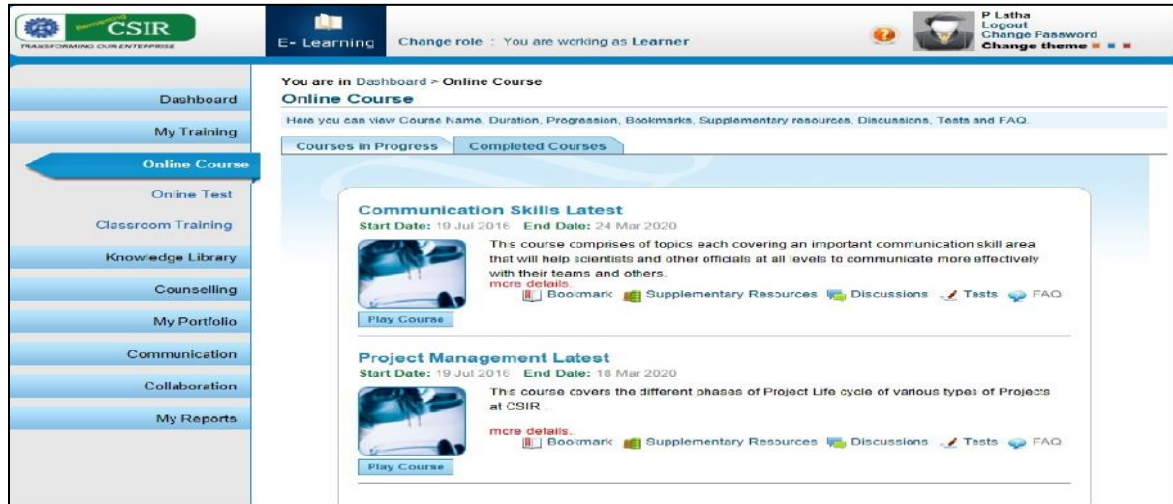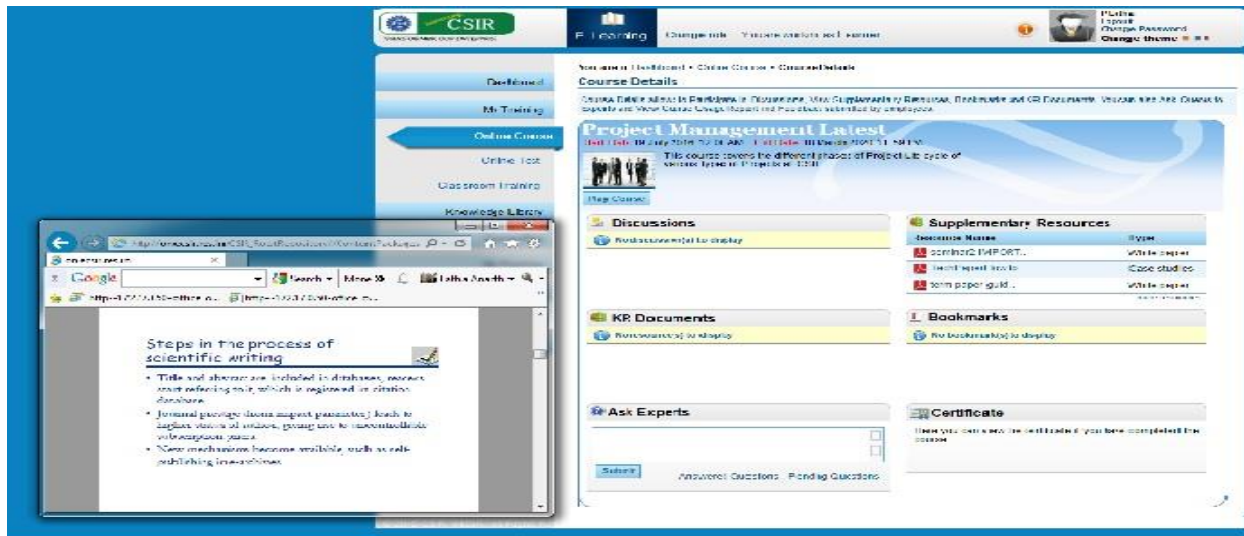
Fig 15.0 Dash board

Fig 16.0 Dash board

Fig 17.0 Course Details

Fig 18.0 Syllabus for Computer courses

1.12 CONCLUSION

The results reported are based on the research carried out in the development and application of Learning Management model for various learning levels such as computer literacy course, certain training material on leather products making.   This Research framework can work very well for higher education. The future direction of this work is to implement it to analyze the learning content, the media and tracking of student's performance. The information posted on this system for any discipline can be connected to National Knowledge Network for wider dissemination and building of a Knowledge Society. Several software tools such as Adobe, Lectora, Articulate and LMS such as Adobe connect pro, Blackboard etc., are evaluated and benchmarked. Cost effective solution was given more priority and hence the open source LMS was selected. Networking of various academic institutes, Integration of LMS

across Internet and Intranet will lead to creation of Knowledge society. Sharing of Knowledge using ICT techniques across the Global network will help the Distance and Lifelong learners immensely.

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A NOVEL METHOD TO PROMOTE OPENING UP FIBER STRUCTURE BY USING XYLANASE IN LEATHER MAKING

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Opening up fiber structure of hide is one of the main purposes in beamhouse, and it is mainly achieved by the liming process with sodium sulfide and lime and the bating process with proteases. However, a great deal of pollutions are produced in the liming process, and the biotechnologies of opening up fiber bundles based on proteases often cause loose and even damaged grain. Hence it is necessary to develop cleaner and more efficient methods to open up fibers. Proteoglycans in skins play an important role in the assembly, formation and connection of collagen fibers and constructing crosslinking network by winding and wrapping with collagen and elastic fibers. It has been widely accepted that there is a close relationship between the content of intradermal proteoglycans and the effect of opening-up fiber. Moreover, much work focusing on destroying intradermal proteoglycans through breaking glucosidic bonds of polysaccharide molecules with carbohydrases, such as amylase, pectinase and hyaluronidase and so on, to accelerate collgen fibers opening-up were reported. However, it is still controversial whether and how these kinds of carbohydrases can effectively destroy intradermal polysaccharides, especially dermatan sulfate, because of the high specificity of glycoside hydrolase toward glucosidic bonds. Hence, firstly, the effects of a serials of carbohydrases on degrading intradermal proteoglycans were investigated through evaluating the contents of proteoglycan, aminopolysaccharide and total sugar in the wastewaters from treating cattle hides with different glycoside hydrolases,and a kind of xylanase was selected. Further, the xylanase was applied to the enzyme unhairing process based on a special protease with low collagenolytic and elastinolytic activities, and its effect on improving unhairing and opening up fibers was investgated. The results showed that the xylanase can promote hairremovaland openning up fibers, and the leather has tighter grain and higher softness. It may be attributed to that the xylanase can efficiently cut off the glycosidic bonds of the stucture unit of galactose-β-1,4-xylose-O-serine/threonine, by which polysaccharide chains connect with core protein, thus polysaccharide chains are released from proteoglycan.

Key words: opening-up; proteoglycan; glycoside hydrolase ; xylanase; leather making

1 Introduction

Opening up fiber structure is one of the most important purposes in beamhouse of leather manufacture, and the degree of opening-up directly affects the penetration of the chemicals in skin and their combination with reactive groups of collagen, thus affecting the properties of finished leather. Fiber opening is mainly achieved in the liming process using sodium sulfide and lime and the bating process using proteases in the conventional leather manufacture process. Liming using lime of oppropriate fiber opening is low-cost and easy-controlled, but produces a large amount of organic matters and sludges because of the soluble epidermis and hair, insoluble lime in the effluent, which accounted for 60%-70% pollutants of leather process(Dettmer, et al., 2013). Liming is gradually eliminating with more and more strict environment protection requirement, and the cleaner unhairing method using enzyme is paid more and more attention. Bating using protease, a kind of biological material, is the only process that can not be replaced by other chemical materials. However, bating need to be controlled strictly to avoid grain loose and grain damaging due to the complexity of the protease components used in the leather industry, which usually hydrolyze all protein components in skin, indiscriminately, such as collagen, elastin and reticulin; moreover, proteasesintensely hydrolyze the proteins in papillary layer of skin, resulting in a bad or loose grain because of difficult penetration in skin of protease with high molecule weight(Yang Qian, 2014).

There are two principal gel-like cement substances in skin to woven with the collagen fiber, one of which is hyaluronan, a long chain glycoaminoglycan not being bound to collagen, so it is removed easily in soaking process(Alexander Ktw, 1986);the other is proteoglycan. Decorin is the most common proteoglycan in skin, which is a kind of dermatan sulfate-proteoglycan (DS-PG)(Uldbjerg and Danielsen, 1988), covalently formed by one or two long chain glycosaminoglycans and core protein through O-glucosidic bond composed by galactose-β-l,3-galactose-β-1,4 -xylose and serine, regularly distributed on the surface of the collagen fibrils. The core protein of the decorin are horseshoe-shaped structures arranged perpendicularly to longer axis of the collagen fibrils and nested with the protrusions on the fibrils, The core protein between the two proteoglycans is linked by the polysaccharide chain (Keene et al. 2000); The side chain dermatan affects the polymerization process of collagen molecule and the existing collagen fibrils, thus affecting the collagen maturation and lateral growth of collagen. Decorin is binding to collagens, including types I, II, III, VI, and XIV to modulate collagen fibrillogenesis by inducing a delayed fibril assembly and a subsequent reduction in the average fibril diameter(Keene, 2000; Raspanti, et al., 2008). Therefore, removal of decorin and other glycosaminoglycan(GAG) is one of the most important step for fiber opening.

Basement membrane, the junction of the epidermis and papillary layer, composed by the VI collagen fibers and perlecan, a kind of macromolecular proteoglycan. Studies showed that the epidermis prevented protease from penetrating into the skin(Jian, et al., 2010). Therefore, opening up fiber by removing the proteoglycans and glycoaminoglycans in the skin before unhairing can make protease permeate more easily, thus unhairing and fiber opening more quick.

The content of proteoglycan in the skin is associated with hair removal and fiber opening(Alexander Ktw, 1986; Aldema-Ramos and Liu, 2010). Therefore, amylase, pectinase, hyaluronidase and other

glycoside hydrolases are applied in combination with protease dehairing(Melville and Deasy, 1977), but there is still controversy whether these carbohydrasescan effectively hydrolyze proteoglycan.Because carbohydrases with different glycosyl and aglycone specificity hydrolyze different kind of glycosidic bonds. (Punitha, et al., 2008; Zeng, et al., 2013).

Xylanase can break the xyloside bond, while the core protein of decorin and other proteoglycan bind to the polysaccharide chain(dermatan sulfate) through galactose-β-1,4-xylose-O-serine / threonine linkages, which may disrupt xylosidic bonds by xylanases and dissociate the macromolecular polysaccharide chains from the core proteins.

Therefore, in this study, firstly several carbohydrases are applied to the soaking process, and the concentration of protein, proteoglycans and aminopolysaccharides and total sugars in the effluent are tested. A kind of carbohydrase is selected. Then, the hydrolysis of intradermal proteoglycan using the carbohydrase and its effect on hair removal and fiber opening are reseached.

2 Materials and Methods
2.1 Materials
Wet salted cattle hide; chondroitin sulfate (BR, Aladdin Industrial Corporation), bovine serum albumin (BR, Biosharp), 1, 9-dimethyl phosphite methyl blue (AR, Sigma - Aldrich), alcine blue 8GX (BR, Amresco), amylase, α-galactosidase, galactosidase, cool, 45053, xylanase and neutral protease with low collagen-hydrolytic and elastin-hydrolytic activity (commercial grade), other reagents are analytically pure.

2.2 Effect of carbohydrases on the hydrolysis of proteoglycans
A piece of wet-salted cattle hide was divided into 6 portions in adjacent part symmetrically and weighted seperately.

250% water of hide weight was added into drum after washed for 3 times, then 0.5% sodium carbonate was added. After running30 min at 30°C, 0.5% enzyme (amylase, α-galactosidase, galactosidase, cool, 45053 and xylanase) was added ,respectively. Repeating 30 min-run and 30 min-pause for 6 times, then drumswere stopped. Nextday, after running30 min, spent liquids were collected. The supernatants were collected by centrifuging waste liquid at a speed of 3500 rpm for 30 min, and the contents of protein, proteoglycan, polysaccharide and total sugar of supernatants were determined according to the reported methods(Lowry, et al., 1951; Mantle, 1978; Farndale, et al., 1986;).

2.3 Effect of xylanase on enzyme unhairing
2.3.1 Unhairing operations
   A piece of wet-salted cattle hide was trimmed and divided into 3 portions in adjacent part symmetrically and weighted seperately. 50% water of hide weight was added into drum after washed for 3 times. Then 0.5% xylanse or cool was added, After running4h, the pH value of liquor was adjusted between 6-7. Then unhairing using protease with low collagen-hydrolytic and elastin-hydrolytic activity. The hides were subjected for conventional shoe leather procedures without bating.

2.3.2 Evaluation of unhairing and opening up

The images of unhairing result after fiber opening 12h, unhairing 2h and 3h and the images of crust leather grain were obtained by digital camera. SEM analysis was done using a Carl Zeiss MA15/EVO18 scanning electron microscope. SEM images of cross sections of crust leather were obtained in the working voltage of 5 kV at 25 $^{\circ}$C.

2.3.3 Histological Staining

Samples of 2cm^2 were cut from the sampling portions of the fiber opening hide at similar positions. Samples were washed thoroughly and were fixed in 10% neutral formalin for histological examination. Section of 20um were obtained using freezing microtome and stained using alcine blue to examine the level of proteoglycan and hematoxylin staining to evaluate the fiber compactness.

2.3.4 Proteoglycan Estimation

The supernatants of fiber opening and unhairing were collected by centrifuging waste liquid at a speed of 3500 rpm for 30 min, and the contents of protein, proteoglycan, polysaccharide and total sugar of supernatants were determined according to methods as mentioned in 2.2.2.

2.3.5 Properties characteristics

Samples for various physical tests from crust leathers were obtained and conditoned as the procedure(IUP 2 JSLTC 84). Tensile strenth and elongation at break and bursting strength were testsed as the procedures(IUP6,IUP9). The softness of the crust leathers was measured using a digital leather softness tester.

3 Result
3.1 Effect of carbohydrases on the hydrolysis of proteoglycans

The contents of proteins, proteoglycans, glycosaminoglycan and total sugars in the effluents are closely related to the effect of opening-up fiber(Madhan, et al., 2010). So these components are tested to characterize the effect of the carbohydrases on the cement substance in skin. The resultsin Fig. 1-4 are deducted the ammount of proteins and carbohydrates in the used enzyme preparations because enzymes of commercial grades contain impurities or carbohydrates in production process.

Figure 1 shows that the protein content in the effluent after xylanase treatment is 1.8 times as the content in the blank group.The reason may be that xylanase can break the O-glycosidic bond between core protein and glycosaminoglycans in decorin or perlecan, which linked through galactose-galactose-xylose-O-serine/threonine and separated the long chain GAG from the protein chains.

All carbohydrases used in the experiment promote the dissolution of proteoglycans(Fig. 2), especially cool and xylanase. Proteoglycan monomers are generally linked to hyaluronic acid via non-covalent linkages to form macromolecular aggregates of molecular weight up to 10^6, which form a network structure with collagen and elastin. Protease is difficult to penetrate into them due to the protection of long chain polysaccharides. These two kinds of carbohydrases are likely to destroy the O-glycosidase between glycosaminoglycans or oligosaccharides and core protein, so that proteoglycan aggregates are fractured and dissolved in the solution, as well as other galactoside enzymes and amylases. But glycosidase may have different effects on proteoglycan with the glycosidic bonds specificity for example one kind of carbohydrase hydrolyze α-glucosidic bond may cannot hydrolyze β-polyglucoside.

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The contents of glycosaminoglycan(Fig. 3) in the waste liquids of hide treated by different carbohydrases are nondistinctive because that the test method of glycosaminoglycan is based on cationic dyestuff combined with acidic groups in macromolecular glycosaminoglycan, so the wavelength of dye absorption peak moved to 525nm, but the long chain glycosaminoglycan is degraded gradually because GAG with terminal saccharide residue is prone to β-elimination reaction in dilute alkali solution, and small molecules can't react with the dyestuff.

These carbohydrases break proteoglycan in skin, so the total sugar in effluent increased(Fig. 4). Xylanase hydrolyzed the most the proteoglycan in the efffulents.

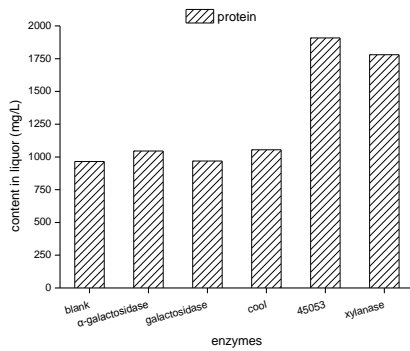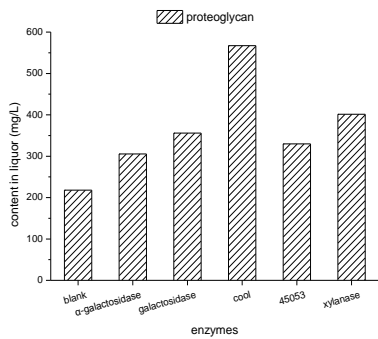Fig. 1 Protein content in soaking liquor
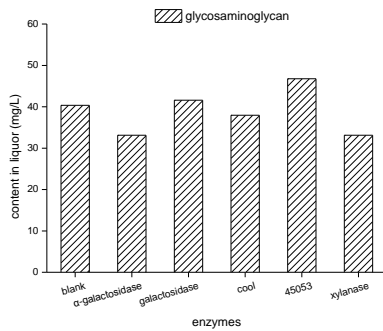

Fig. 2 Proteoglycan content in soaking liquor

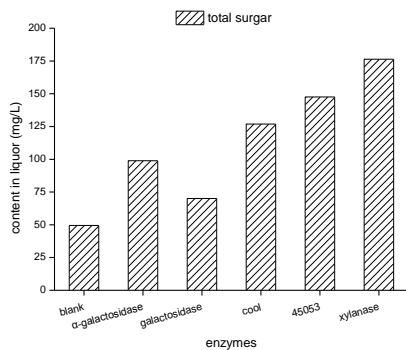Fig. 3Glycosaminoglycan content in soaking liquor

Fig. 4 Total sugar content in soaking liquor

So, several kinds of glycoside hydrolases which may hydrolyze proteoglycan are studied. It is found that these enzymes have certain hydroytic effect and the xylanase of the better hydrolying propertty is chosen for further studying.

3.2 Effect of xylanase on protease depilation
3.2.1 Unhairing effect of glycoside hydrolases in soaking

Unhairing with protease is a cleaner and high efficient method but it is easy to loose grain or damage to grain. One of the main reasons is that protease of high molecular weight is difficult to penetrate into skin, so it is essential to open-up the collagen fiber to accelerate the penetration of protease. Xylanase accelerate the removal of proteoglycan from the skin and thus assist the opening up fiber. So the effect of xylanase on protease dehairing is studied by adding xylanase or cool amylase or nothing before using protease. The results are showed below.

Enzymes are added into drum in pH6.6-6.7after soaking, then protease are added. The pH value of liquor was adjusted to around 8.0 after the drum runing 2h. These skins are the same before soaking. After 12h, the loosening hair roots and removal of hair are observed in the skins treated by LKT xylanase and cool amylase (Fig. 5A-5C). The surface of hide is clean in xylanase treated group. The main reason is that the xylanasewith a little protein hydrolysis activity, hydrolized hair follicles around the keratin and promoted hair removal. In addition, the carbohydrase can also hydrolyze the proteoglycan materials around hair follicles and basement membrane in epidermis, thus loosen the hair root. Almost all hair are removed in hide treated by xylanase while the percentage removed hair of hide of using cool amylase and in blank group are 70% and 50% after dehaing 2h using protease. But 1h later, almost all hair are removed, remaining part of fine hair.
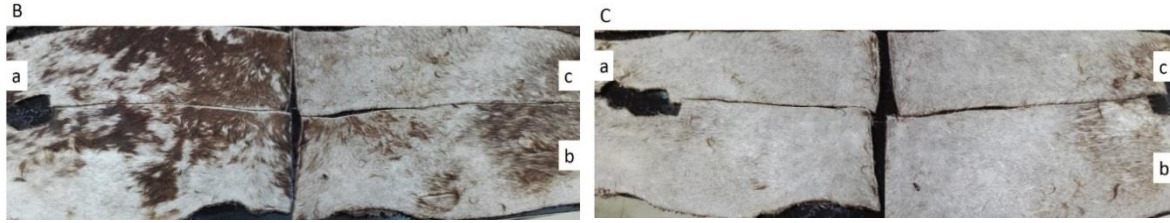
Fig. 5 Images of unhairing result

(A) Opening up 12 h (B) Unhairing 2 h (C)Unhairing 3 h , a stands skin in blank group , b stands skin treated by cool amylase, c stands skin treated by xylanase.

Figure 6-7 show the grain and cross section of crust leather. The grain are fine and in good condition. The cool and xylanase treated leathers show finer fiber bundles than leathers in blank group.

Fig. 6 Images of crust leather grain
a stands skin in blank group , b stands skin treated by cool amylase, c stands skin treated by xylanase
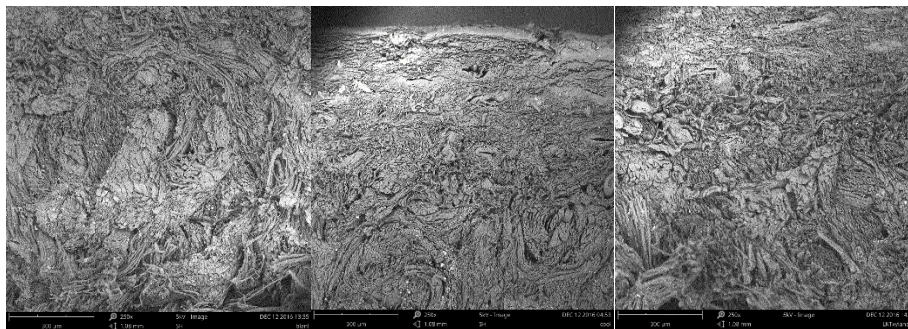
Fig. 7 SEM images of cross section of crust leather

3.2.2 Histochemical studies of proteoglycan and collagen in skin

Histochemical studies on cross section of pelt treated 12h by glycosidase using alcine blue staining (Fig. 8-a) distinguished the proteoglycan around epidermal basal layer and hair follicle, as well as between collagen fibers. Complete absence of the above said constituents is in the section of hide treated by LKT xylanase(Fig. 8-c) and cool amylase(Fig. 8-b).

Features of section of glycosidase treated hide stained by hematoxylin distiguished collagen from other tissues (Fig. 9-a). The epidermis is unbroken when the hide is treated by cool amylase (Fig. 9-b). While the epidermis and dirt are absent in LKT xylanase (Fig. 9-c).

Fig. 8 Proteoglycan staining using alcine blue 8GX
100 magnification, a stands skin in blank group , b stands skin treated by cool amylase, c stands skin treated by xylanase.

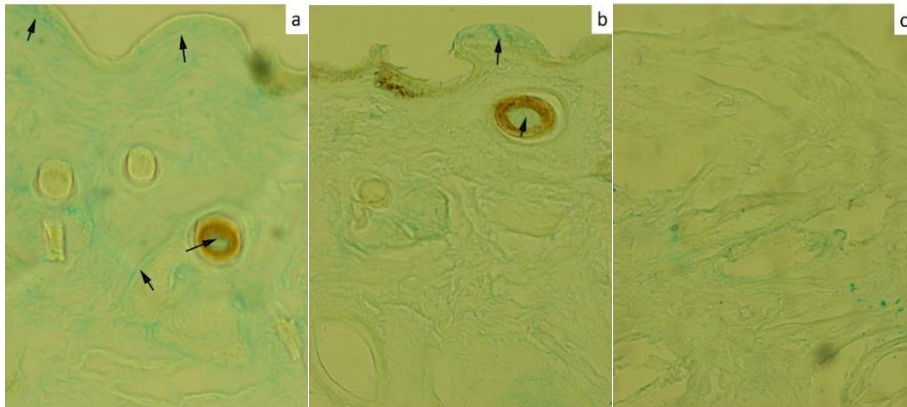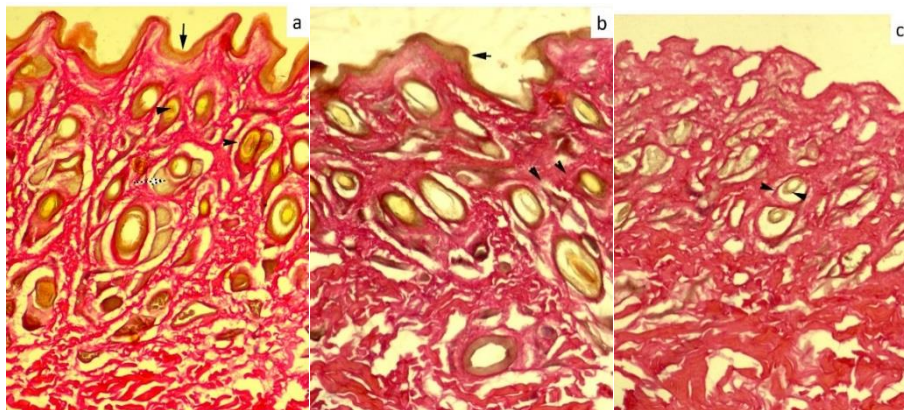Fig. 9 Collgen staining using hematoxylin, 4×10,a:blank, b:cool amylase,c:LKT xylase
Solid arrows indicate epidemis and dirt, dotted arrows indicate adipose glands, triangles stand hair follicle and hair shaft.

3.2.3 Quantitative analysis of protein, PG,GAG and total sugur content in effluent

The content of protein, proteoglycan, GAG and total sugar in soaking effluent addingglycosidase and unhairing effluents using neutral protease with low collagen-hydrolytic and elastin-hydrolytic activity are measured(Fig. 10). The protein content in unhair effluent are much higher than in soaking. The content of protein in effluent of LKT xylanase treated is higher than in cool amylase group and the blank group, but because of adding only 100U/mL of protease in LKT xylanase soaking, the content of protein in unhairing of experiment group is lower than the rest of the groups by adding 120 U/mL of protein, but the speed of unhairing of LKT xylanase treated group is the fastest. The reason may because of xylanase has obvious promoting effect on unhairing of protease by breaking the proteoglycan aroud epidermal

basement membrane and hair follicle and between collagen fiber, which means adding xylanase before unhairing by protease can achieve faster hair removal speed and gain crust leather of full and fine grain with less dosage of protease.

The proteoglycan content after depilation in waste liquid show that more proteoglycan are hrodrolyzed by LKT xylanase than by cool amylase because these proteoglycan aggregates are hydrolized to protein and GAG.

Glycosaminoglycan content in the waste liquid of glycosidase treated increased than untreated group, the xylanase mainly through hydrolyzed O-glucosidic bond, link of polysaccharide chain and the core protein, which was composed by galacto-β-l, 3-galacto-β-l, 4-xyloseryl and serine sugar. And amylase may hydrolyze the O-glucosidic bond linked the oligosaccharide and core protein. The glycosaminoglycan was stable in neutral environment.

The concentrations of total sugar in waste liquid of unhairing are consistent with hair-removal speed and rate, which means the break of cement materials, proteoglycan, contributed to promoted hair removal. After adding protease, the xylanase and amylase further hydrolyze the PGs when some core protein was cut.
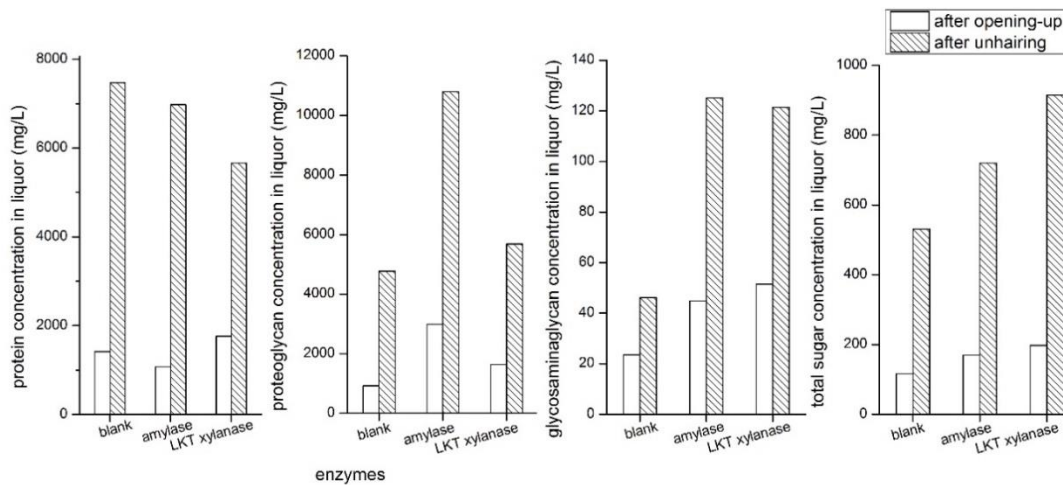
Fig.10 Content of protein, proteoglycan, glycosaminoglycan, total sugur in effluent
After opening up means treated hide by glycosidase for 12h, unhairing means dehairing by protease 3h.

3.2.4 Propertities characteristics

Experiment and blank crust leather were evaluated for physical and mechanical properties(Tab. 1). The tensile strength and elongation was comparable to blank group, and bursting strength and softness and fullness were better than the blank. The propertities of crust leather using cool amylase treated was a little bit lower than the blank group. So xylanase is a proper enzyme for promoting fiber opening and hair removal with good propertities.

Tab. 1 Physical and mechanical properties of crust leathers

Sample	Tensile strength(N/mm^2)		Elongation /%		Bursting strength N/mm^2	Softness/ mm
blank	7.260	7.834	53.452	50.873	214.854	6.02
cool	6.436	9.548	63.492	47.247	208.756	5.82
LKT xylanase	7.576	8.344	42.314	48.090	250.224	6.14

4 Conclusion

This paper studied the effect of different glycoside hydrolase on hydrolysis of intradermal cement materials, especially decorin, the most common proteoglycan in skin. The results show that different glycoside hydrolase having distinct effect on proteoglycan, which consistent with the proteoglycan structure and hydrolysis specificity of these enzymes. Xylanase has a good fiber opening effect by removal of proteoglycan in skin, and promote protease unhairing. But the mechanism of proteoglycan hydrolysis of glycoside hydrolase still needs to further researched.

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AN EFFICIENT AQUEOUS DEGREASING METHOD WITHOUT USING SOLVENT AND SURFACTANT BASED ON THE SYNERGISTIC ACTION OF LIPASE AND ALKALI

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As a cleaner degreasing method, degreasing with lipase has been paid more attention in leather industry. However, at present, most of related studies showed that a significant degreasing effect can not be acquired when using lipase alone at the optimum pH or together with surfactants. In order to solve these problems, firstly, a novel approach of evaluating the performance of lipase was established under simulated leather-making conditions, then the characteristics and the optimum condition of lipase catalyzing the hydrolysis of lipids in skins were invesigated. The results showed that the enzymatic hydrolysis was obviously inhibited by the products, fatty acids, hence the lipid hydrolysis ratio was lower than 65% for most of selected lipases under their optimum conditions. Further, an efficient degreasing method based on the synergistic action lipase and alkali was established. The skins were first treated with lipase and then alkali was added. The produced fatty acids were transformed to soluble soaps and released from the lipid substrate, hence, the product inhibition was greatly reduced. The hydrolysis ratio of lipid is rasied to 90%. Using the novel method of combining lipase with alkali in the degreasing process of pigskins after soaking, the degreasing ratio was 74.8%; then using the method again after bating, the total degreasing ratio reached 96.2%. The efficient aqueous degreasing without solvent and surfactant can be achieved by the synergistic action lipase and alkali.

Keyword: lipase; fatty acid; product inhibition; enzymatic degreasing

1. Introduction

Fat widely exists in animal skins, and the content of fat in animal skins has an inceasing tendency in recent years, due to improved breeding conditon. Natural fat in skin is an useless material in leather making, it will interfere in the uniformity of penetration and distribution of chemicals in skin, thus influence tanning and dying, even cause serious leather quality problems such as "fatty spew", "bad smell" and exceeding standard of hexvalent chromium, which make leather chemists aware of the significance of enhancing degreasing efficiency in leather-making process and devote to finding high-efficient degreasing method(Langridge, et al., 2006; Saran, et al., 2013; Altan and Fatma, 2008).

Common degreasing methods can be classified into physical and chemical methods. Physical method is one of the main methods, removing subcutaneous fat in skin by mechanical operation inluding fleshing or press degreasing. Traditional chemical degreasing methods include solvent degreasing, saponification method, emulsifier degreasing and combined method. Solvent degreasing has a good effect, but it needs to use high cost, toxic, flammable chemicals, such as kerosene and other organic solvents with low operating safety and other issues. Due to the actual temperature of tannery is too low to meet the ideal saponification temperature and uneven distribution of intradermal fat, saponification can only achieve the surface degreasing to some extent. Emulsifier degreasing is the most widely used method, but the fatty substance content varies with animal species, thus the amount of emulsifiers varies greatly, sometimes even more than 10% of skin weight, and degreasing effectiveness is affected by the changes of fat composition in different leather processes, for example, fatty acids reacting with chrome salt to produce chrome soaps in chrome tanning process, which are difficult to remove subsequently and usually aggravate the problems mentioned above(Qu Shasha, 2014); The use of a large number of emulsifiers will cause environmental pollution and impair leather properties by reducing water resistance and increasing fogging value.

It is reported that the enzymatic degreasing process, as a bio-degreasing technology, generally can improve the quality of the final leather such as more uniform colour, cleaner appearance, better waterproof property, and reducing the use of chemicals and can be conducted on a milder condition, so it has been paid more attention(Rasmussen, et al.; Christner, 1992; Kamini, et al., 1999; Muthukumaran and Dhar, 1982).. However, in the actual leather process, there are a few application of lipase as the main degreasing method, generally as an auxiliary degreasing method in a certain range, besides the high cost, the main reason is that the degreasing rate is not ideal when using lipase alone or even together with surfactants, generally lower than 60%, far away from the imaged degreasing effectiveness(Altan, 2008; Palop, et al., 2000). Moreover, the lack of scientific and effective means of characterizing and evaluating lipase properties leads to insufficient practical guidance. The relative lag of the study of high-performance lipase degreasing mechanism limits the further development and innovation of lipase degreasing technology.

To solve the above problems, the high performance bio-degreasing technology of tannery has always been one of the most important subjects in our research group(Liang Liang, 2013). We had established a method to characterize the catalytic hydrolysis properties of lipase toward intradermal fats and degreasing rate in simulated leather-making precoss by using natural animal skin powder rich in fats as the substrate, which is the foundation of further exploring the mechanism of high-performance lipase degreasing. Therefore, firstly we analyzed the critical influencing factors on lipase activity based on the established method, and discussed the mechanism of lipase degreasing, which provided a theoretical basis for the establishment of high-efficient lipase degreasing technology. Then the influnce of the alkali on lipase degreasing effects was investigated. According to the results, an efficient degreasing method based on the synergistic action lipase and alkali was established.

2. Materials and methods

2.1 Materials

Natural pigskin powder (fat content 23.42%, acid value 52.23mgKOH/g, saponification value 198.62mgKOH/g) was prepared in our laboratory; iso-C10, iso-C13 non-ionic sufactants were sourced from BASF Corporation Limited, and iso-C12-C14 non-ionic surfactants were sourced from Shell Chemicals; the chemicals used leather manufacture were of commercial grade and other chemicals were of analytic grade.

2.2 Performance of lipases under simulated leather-making condition
2.2.1 Effect of temperature on lipase activity

Five grams of pigskin powder was incubated with 100 mL 0.05M phosphate buffer (pH7.5) at 40 ^{o}C for 2 h with shaking in 250mL erlenmeyer flasks. Then the suspension kept shaking for 90 minutes after adding 10 U/mL lipase in 20 ^{o}C, 25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40°C,respectively, then terminated the reaction by adding 10g of NaCl and 5mL 6M HCl and vibrating 20 minutes. The skin powder was filtered, dried naturally and pulveried. Extracted oilusing methylene chloride in the skin powder and tested the fatty acid value according to the procedure(ISO4048).

2.2.2 Effect of concentration of enzyme, reacting time and pH on lipase activity

Effect of concentration of enzyme, reacting time and pH on lipase activity was studied in the lipase dosage of 5-50 units /mL, reacting time range of 0-180min and pH value ranging from 4.5 to 10.5, respectively. The other parameters kept constant when changing one parameter. The operation was according to 2.2.1.

2.3 Effect of alkali on fat hydrolysia of lipase
Fifthteen portions of 5g skin powder were divided into three groups. Then incubated with 100mL buffer(pH7.5) in the first and the second groups and 100mL buffer (pH10.5) in the third group at 35 ^{o}C, then all added 10 U/mL LKT lipase and kept shaking, controled pH value to 10.5 with Na$_2$CO$_3$ after 1h in the second group, terminate the reaction by adding 10g of NaCl 10 mL of 6M HCl solution (only 5 mL in the first group) after different reacting time. The skin powder was filtered, dried naturally and pulveried. Extracted oil using methylene chloride in the skin powder and tested the fatty acid value according to the procedure(ISO4048)

Six different portions of 5g skin powder numbered 0#-5# were soaked according to method mentioned in 2.2.1. Then 0# viberated 2h; 1-2# added 10 U/mL LKT lipase, viberated 2h and 4h respectively; 3-4# added 10 U/mL LKT lipase, changed the pH value to 10.5 by adding sodium carbonate after viberated 1.5 hours then viberated 30 minutes and 120 minutes hours respectively. Terminate the reaction by adding 10g of NaCl 10 mL of 6M HCl solution. The skin powder was filtered, dried naturally and pulveried. Extracted oil using methylene chloride in the skin powder and tested the fatty acid value according to the procedure(ISO4048)

Take another six portions of skin powder, treated the same as above mentioned, terminated the reaction by washing 15 min with 100 mL of distilled water twice to replace adding NaCl and HCl, then filtered and dried natually, The skin powder was filtered, dried naturally and pulveried. Tested the fat content by extracting oil using methylene chloride.

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2.4 Pratical application of lipase - alkali synergetic degreasing technology

A Soaking and fleshed pigskin was cut into two equal halves. One half of the skin was treated according to the conventional soda ash-surfactant degreasing(Tab. 1) and the other half was treated with lipase-alkali synergistic degreasing(Tab. 2). The fat content and acid value was tested according to the procedure (ISO4048).

Tab. 1 Conventional soda ash-surfactant degreasing process in skin

Process	Product	Amount(%)	Duration	Special note
Degreasing	Water	100		40 ^{o}C
	Na$_2$CO$_3$	1.5		
	Surfactant	1	2h	
Washing	40 ^{o}C water	200	30 min	Repeat degreasing and washing

Tab. 2 Lipase-alkali degreasing process in skin

Process	Product	Amount(%)	Duration	Special note
Degreasing	water	100		40 ^{o}C
	LKT	20 U /mL	2h	40 ^{o}C
	Na$_2$CO$_3$	X	3h	Run 3h at pH10-10.5 and stop overnight, run 1h nexday.
Washing	Na$_2$CO$_3$	1	30 min	

3. Results and discussion

3.1 Performance of lipases under simulated leather-making condition

The mechanism of enzymatic degreasing is that lipase catalyzes hydrolyzing triacylglycerols to form fatty acids and alcohols. The established method to estimate the activity of lipase is based on evaluating the acid value of fats remained in the skin powder substrate after treated with lipases on varying conditions.

Temperature is one of the key factors influencing the chemical reaction. Generally, the higher the temperauture, the faster the reaction speed. The temperuture in beamhouse is usually lower than 40°C to avoid to damaging the collagen, thus the effect of temperature, ranging from 20°C to 40°C, on lipase catalyzing hydrolysis of fat using natural pigskin powder as the subtrate was investigated. Figure 1 shows that the activities of three commercial lipases rises with the increasing of temperature. The activity increased with the evaluated temperature. Hence the reacting temperature was chosen at 40°C in the following experiments.
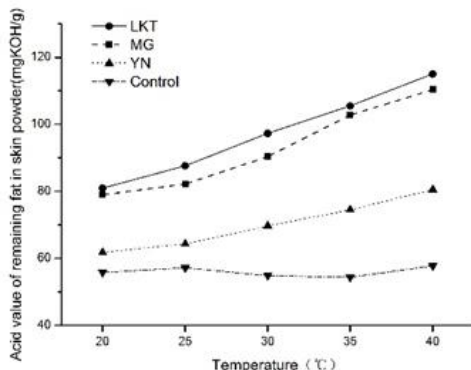
Fig. 1 Effect of temperature on lipase activity

Phosphate buffer pH7.5 (0.05mol/L), lipase concentration 10U /mL, reacting time: 90min.

Figure2 shows that the tendencys of the influences of the three selected lipases concentrations on their activity are similar, i.e. elevated enzyme concentration significancely affects hydrolysis speed of intradermal grease in the skin powder substrate when it is lower than 10 U/mL; the increasing effect of oil hydrolysis with concentration changing becomes weakerwhen it is higher than 10 U/ml, the effect of the concentration of lipase on fat hydrolysis was weaker.
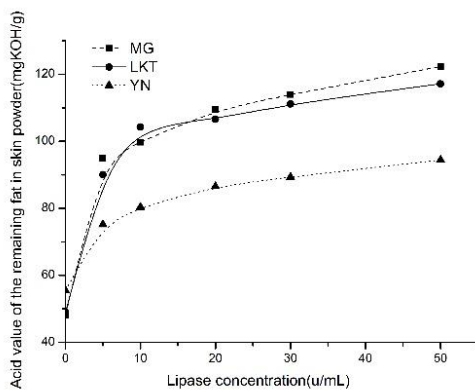

Fig.2 Effect of lipase concentration on on lipase activity

Phosphate buffer pH7.5 (0.05mol/L), temperature: 40 ^{o}C, time: 90min

The effects of reaction time on lipase catalyzed hydrolysis are illustrated on Figure 3. It can be seen that the catalytic efficiencies of the three lipases with time are consistent, although the activity of YN is lower than others. The reaction speed is faster within the beginning 60 minutes, thus the acid values of fat in substrate rise remarkably, and then change slowly. Even when the reacting time was prolonged to 7h, the acid value only arrived at 150 mgKOH/g, which is the much lower than the theoretical value, the saponification value of 200 mgKOH/g.

Figure.2 and Figure 3 reveal that no matter how to increase the lipase concentration and prolong the reacting time, it is difficult to completely hydrolyze fat, which means that the hydrolytic reation is inhibited by the product, fatty acid. When the acid value is increased to above 110 mgKOH/g, the lipase catalyzed acidolysis is obviously inhibited, and the maximum hydrolytic rate of fat is about 60% eventually. The main reason can be attributed to that the produced fatty acid enriched on the surfaces of oil drops hinders the contact of lipase with oil molecules. Hence, when the amount of fatty acid rises to a rather high value,the contaction is completely blocked, and the catalytic reaction can not happen.
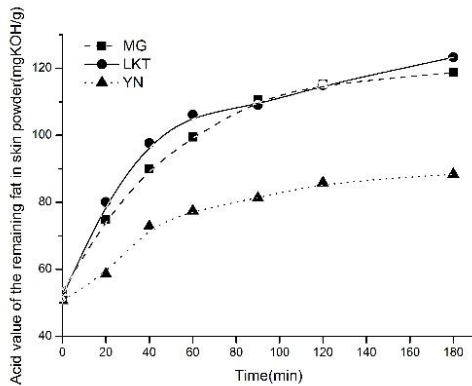

Fig.3 Effect of reacting time on lipase activity

Phosphate buffer：pH7.5(0.05mol/L), temperature: 40°C, lipase concentration 10U/mL

The effect of pH on lipase activity in simulated leather-making condition was further investigated. As the result showed in Figure 4, it is worthnotingthat there are two optimum pH peaks pH 6.5 and 10.0 for lipase LKT, and pH 7.5 and 10.0 for lipase MG. It is widely accepted that most of enzyme have only one optimum pH peak, but the performaces of the two lipases with pH change are unexpected. As mentioned before, the hydrosis of oil catalyzed by liapase strongly is inhibited by the product, fatty acid, when pH is raised to above 10, the formed fatty acids are transformed to soluble salts, thus migrated to aqueous medium form the surfaces of oil drops in skin powder, and the product inhibition is weakened and even eliminated, hence, lipases MG and LKT show "super active" at pH around 10.0.
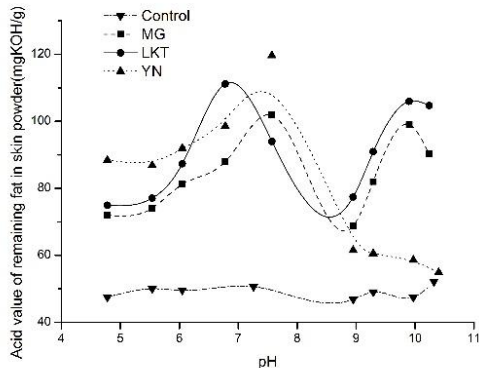
Fig.4 Effect of pH on lipase activity

Lipase concentration 10U/mL, temperature: 40 $^{\circ}$C, time: 90min

3.2 Synergistic action of lipase and alkali on fat hydrolysis

As mentioned above, the produced fatty acids will inhibit lipase action through obstructing the contaction of enzyme and oil melecules, thus the hydrolysis rate is quite low, which is below 65%. Accordingly, one of the keys improving lipase degreasing efficiency is increasing the fat enzymatic hydrolysis degree; obviously, prompt removal of formed fatty acids is necessary. As shown in Figure 4, raising pH to above 10 to make fatty acid be transformed to soluble salts is an effective method to remove it and promote the enzymatic hydrolysis of fat.

Sodium carbonate is a common base in degreasing, so the synergistic action of lipase and it on fat hydrolysis was further investigated. Figure.5 shows that the fat hydrolytic reaction gradually comes into equilibrium after two hours, and the acid value of fat in skin powder approaches to 120 mgKOH/g at the constant pH of 7.5 (the first optimum pH peak in Fig.4); when the fat hydrolytic reaction is conducted the constant pH of 10.5 (the second optimum pH peak in Fig.4), although the acid value increases more slowly than the first case at the initial stage within 2 hours, it continuously increases with reacting time; for the third case, the skin powder substrate is treated with lipase at pH7.5 for one hour, and then adding sodium carbonate to rise pH to 10.5, the the hydrolysis rate reaches to 90%. The synergistic action of lipase and alkali on fat hydrolysis can be arributed to the prompt removal of formed fatty acids.
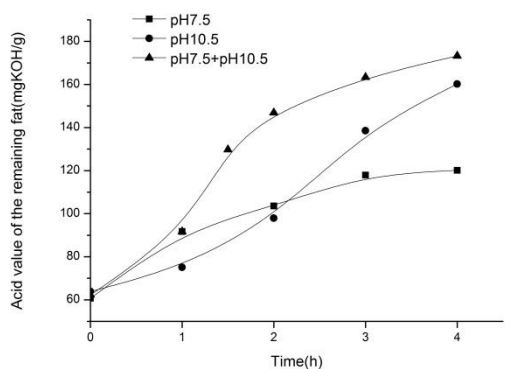

Fig.5 Synergistic action of lipase and alkali on fat hydrolysisin hide powder
Lipase concentration 10U/mL, 35 $^{\circ}$C; pH7.5+pH10.5: pH7.5 for 1h, then ineasing pH to 10.5.

The skin powder enriched fat was treated by different methods, and the degreasing results are shown in Tab.3.

Tab.3 Degreasing effects of skin powder treated with lipase and alkali

No.	Treated time (h)	Degreasing ratio[**]

	Lipase[*]	Soda ash	（%）
0	pH7.5 buffer, 4h		31.36
1	2	0	46.33
2	4	0	50.78
3[***]	1.5	0.5	89.68
4	1.5	2	88.89

[*]Lipase concentration: 10U/mL, 35 $^{\circ}$C; [**]Evaluated after washing with water for twice;

[***] first treating with lipase for 1.5h, then adjusting pH to 10 with soda ash for another 0.5h.

It can be seen that the degreasing ratio is only 31.36% just treated with lipase, and the combination of lipase and soda ash can make it reach to about 89%.

3.3 Degreasing effect of the method based on the synergistic action of lipase and alkali

Animal skin structure is a densely three-dimensional network woved by collagen fiber, elastin and reticulin with other tissues, which is different from skin powder. Animal skin fat is not only concentrated in subcutaneous tissue, but also scattered in the hair follicle around the adipose glands, which will reduce the contact chance of lipase and fat and affect the penetration of enzyme in animal skin. Therefore, the degreasing effectiveness of lipase to skin powder substrate may differ with the actual leather process.

Based on the above results, a high-efficiency lipase degreasing method was designed. Lipase was used in soaking and bating processes of pigskins, and the results are shown in Table 4.

Tab.4 Degreasing for pigskin after soakingby lipase with alkali

Degreasingmethod	Degreasing Process	Remained fat content （%）	Degreasing rate （%）
After Soaking			
Soda ash-surfactant degreasing	before	36.9	-
	after	14.7	60.2
novel lipase degreasing	before	36.5	-
	after	9.2	74.8
After Bating			
surfactant degreasing	before	7.5	-
	after	4.1	45.3
novel lipase degreasing	before	3.5	-
	after	1.4	60.0

Comprehensive degreasing effect	
surfactant degreasing	88.9
novel lipase degreasing	96.2

Compared with conventional alkali-surfactant degreasing method, lipase-alkali synergistic degreasing method had better degreasing effect, and the degreasing rate increased from 60.2% to 74.8% in soaking process. Moreover, during liming, deliming and bating processes, the grease in the skins treated with lipase and alkali after soaking was more easily removed, and the residual fat content was 3.5%, while it was 7.5% when degreasing with the convnetional gegreasing method. After the second degreasing was conducted after bating, the degreasing rate of the lipase-alkali method was about 96.2%, which was higher than that of the conventional degreasing method, 79.7%. At this stage, the residual fat content in the pigskin was further reduced to 1.4%, while the conventional degreasing pigskin was 4.1%. Hence, the lipase-alkali synergetic method not only significantly improves the degreasing efficiency, but also completely avoid the use of surfactants, and the cleaner and high efficient degreasing process is achieved.

4. Conclusion

(1) The enzymatic hydrolysis is strongly inhibited by the product, fatty acid. It is difficult to completely hydrolyze fat through increasing the amount of lipase and prolonging the reacting time under neutral conditions.

(2) The activity of lipase significantly affected by pH, especially, most of so-called neutral lipase also exhibit "superactivity" at pH 10 because of the prompt removal of fat acids.

(3)The established lipase-alkali synergetic degreasing method have an excellent degreasing effectiveness in pigskin processing. After two-step degreasing, soaking and bating respectively, the degreasing rate can reach to 96.2%, and the residual fat content is as low as 1.4% . The synergistic method achieves an efficient aqueous degreasing method without using solvent and surfactant.

Acknowledgements

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