Paper B12

Evaluation of Lipases Properties and Degreasing Effects in Leather Manufacture

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Abstract

Enzymatic degreasing with lipases in leather manufacture has been accepted and it is thought as a high-efficacy and green method. But there are lacks of reports about evaluating the real effects of lipase degreasing. The performances of two common-used commercial lipases and their degreasing effectiveness were estimated based on a new method using sheepskin powder rich in fats as the substrate in this paper.

Fleshed wet-salted sheepskin was pasted an unhairing protease solution to remove wool, and then the unhaired skins were dried and milled into powder. The sheepskin powder contained about 20% fats without any chemical modification. Two so-called commercial alkaline lipase, were selected to react with the sheepskin powder under different conditions. The effects of lipase hydrolysis and degreasing were estimated according to the change of the amount of dichloromethane extract and its acid value. The impact of pH, reaction time and enzyme concentration on the fats hydrolysis and removal were revealed. The results showed that lipases can efficiently hydrolyze fat in skin, but the lipase activity was greatly inhibited by the fatty acids with a high concentration formed from fat hydrolysis, and fatty acids cannot be washed out at neutral and acidic without surfactants. So degreasing just with lipase cannot get satisfied result. It is recommended that the use-pattern of lipase in leather degreasing should be with a relative low amount and several times; the two commonly-used commercial lipases behave the highest activity at around pH8.0, and the activity almost lose at pH over 10.0, so the lipase treatment should be carried out at their optimum pH, and avoid to being used in strong alkaline processes, such as liming, etc.; the lipase hydrolysis products can not be removed at the lipolytic conditions, it needs further be released from the substrate, and the effective method is that the lipase hydrolysis substrates was then washed with alkali solutions to transfer fatty acids to salts and appropriate amount surfactant. Finally, a high-efficiency aqueous degreasing method based lipases without organic solvents is established, and the degreasing ratio reaches to 80%.

Keywords: degreasing; lipase; sheepskin powder

1. Introduction

Recently animal fat content in the skin has an increasing trend with the changes in animal husbandry conditions. A variety of problems will be caused by the insufficient removal of fat in the skin for the leather products, such as spew, bad odor, excessive Cr^{6+} content and so on. Thus, the highly effective degreasing methods of leathers have been actively studied. Degreasing of skins and hides is currently accomplished by using of organic solvents and surfactants. The saponification degreasing with ash soda is also applied in pigskin processing. But solvent degreasing presents health and safety risks and degreasing using surfactants probably presents pollution risks and negative effects on the waterproofing and fogging level of leather products. Recently the use of lipolytic enzymes has been suggested and paid more and more attention in order to enhance degreasing of hides and skins, improve the quality of the final leather, reduce the use of chemicals and replace chemicals with adverse-environmental effect[1-7]. Especially with the development of biotechnology, the mass industrial production of lipase products offers the possibility of application of lipase on an industrial scale.

Although there are many study reports on enzyme degreasing, most of the results show that the efficiency of the degreasing with lipases is not so high as expected, generally only about 50% lipids removed[8-10], and skin or skin pieces commonly were used as the substrate in these researches[11-19]. Thus, the experimental data based on this system is much volatile and the comparability of data is also poor due to the unlevel distribution of fats in skins. Additionally, the hydrolysis level of grease in the skin by lipases was seldom mentioned. Aiming at the above-mentioned problems, a uniformity sheepskin powder rich in fats was prepared as the substrate of the lipolytic degreasing in order to greatly improve the reproducibility of the results. On this basis, the effects of various conditions in leather process on the lipase hydrolysis of fat in skin and the ideal lipase degreasing method were studied.

2. Materials and methods

2.1 Materials

Commercial lipases were supplied by Novozymes and Dowell Technology Co., Ltd., etc. Other chemicals were of analytical grade, unless otherwise stated.

2.2 Methods

2.2.1 Preparation of sheepskin powder rich in fats

Fleshed wet-salted sheepskins were washed and soaked with plenty of water and preservative. The sheepskins were then pasted an unhairing protease paste on flesh side. After piling for about 48h, the wool was removed from the skins by hand. The unhaired skins were then thoroughly washed, and air dried at room temperature. Alkaline chemicals and surfactants were prohibited to avoid the loss of fats. The dried skins were cut into some pieces and milled into powder.

2.2.2 Lipolytic and degreasing effect of Lipases on grease in sheepskin powder

3g of sheepskin powder was soaked in 60 ml buffer solution of a certain pH value in an Erlenmeyer flask and stirred for 10min and then stood overnight. Different amounts of lipase were added to the Erlenmeyer flask, the mixtures were stirred for a period, from 10min to 2h, at 30°C and adjusted pH

value to 6.5 at the end of the lipolytic reaction when the reaction pH was over 8.0. Then the hide powder suspensions were filtered and washed with 100 ml distilled water, 0.5% soda ash solution or surfactant solution. The collected sheepskin powder was air dried at room temperature. Finally, the grease in sheepskin powder was extracted by Soxhlet extraction with dichloromethane, and the acid value (AV) and the saponification value (SV) of the extracted grease were also measured, respectively.

2.2.3 Analysis of fatty acid liberated by lipases with GC/MS

The sheepskin powder was treated with lipases according to procedure as described, and the lipase concentration of the suspension was 20U/ml suspension. The fatty acids liberated by lipase were separated from the grease extract of the treated sheepskin powders. The separated fatty acids mixture was methylated with HCl-methanol. The methyl esters were then analyzed with the Trace GC Ultra DSQ II (Thermoestqu-Finnigan, US).

GC-MS parameters: Gas chromatograph-quadrupole mass selective detector (EI) operated at 70 eV. The column used for all analyses was a TR-MS (30m×2.5mm×0.25mm) quartz capillary column. The MS was operated in SIM mode. The oven temperature was initially held at 120 ^{o}C for 3 min. Then the temperature was raised with a gradient of 5°C/min until 220°C. This temperature, 220°C, was held for 5.0 min. The flow through the column was held constant at 1.0 mL He/min. Injection volume was 0.2μL and split ratio was 1:10. Temperature of the inlet was 250°C. Interface temperature was 250°C. Quadrupole temperature was 200°C.

2.2.4 Inhibition of products for lipases

A certain amount of oleic acid and 12.5 ml olive oil were mixed together and slightly heated for 15 min. After cooling, 37.5 ml PVA (0.4%w/v) was added, followed by the emulsifying operation under 14000 rpm for 3min with three times. The activities of lipases were detected in the above systems with different oleic acid concentrations, and the other operations were the same as the standard lipase activity assaying procedure [20]. The blank test was without oleic acid.

2.2.5 Impact of surfactants on Lipolytic activity and degreasing effect of lipases

The activity of lipases in different surfactant solution with different concentration was analyzing based on the above operating procedure.

3. Results and Discussion

3.1 Parameters of the prepared sheepskin powder

In order to improve the reproducibility of the results of degreasing trial, a kind of sheepskin powder rich in fats was used as the substrate to evaluate the lipolytic activity and degreasing effect of lipases. Hence the uniformity of fat content in hide powder was the key. According to the procedures illustrated in part 2.2.1, the required sheepskin powder rich in fats was prepared and its basic parameters are shown in Table1 and Table 2.

Tab.1 Fat content and distribution uniformity in the sheepskin powder (%)

	Extracted Fat content*1				Average value	RSD
SAMPLE	1	2	3	4		
Dry sheepskin powder	19.47	19.53	19.42	-	19.47	0.29
Soaked sheepskin powder*2	17.71	17.66	17.73	17.60	17.68	0.33

*1: Extracted fat from the sheepskin powder by Soxhlet extraction at 85°C for 6 hour at the extraction rate of one recycle per 8-12min ; the same as the follows.

*2: Sheepskin powder samples were soaked with 0.05mol/L pH 7.5 buffer solutions overnight.

Table1 shows that the average fat contents of the sheepskin powder before and after soaking are 19.47% and 17.68%, respectively, and the corresponding RSD is 0.29% and 0.33%. This indicates that the sheepskin powder has better homogeneity and higher fat content.

Tab.2 Essential parameters of the extracted fats from the sheepskin powder

Project	Initial sheepskin powder	Soaked Sheepskin powder	Sheepskin powder treated with lipase*1
Extracted Fat content (%)	19.40	17.60	18.12
AV*2 of the extract(mg KOH/g fat)	24.63	25.91	150.00
SV*3 (mg KOH/g fat)	186.99	202.02	209.95
EV*4 (mg KOH/g fat)	162.36	176.11	59.95

*1: Lipase treating method: 20:1 of bath ratio; 30°C of temperature; pH7.5 of buffer; 30U/ml of lipase concentration, UG lipase; 2hours of time.

*2: AV means Acid value; *3: SV means Saponification value;

*3: EV means Ester value; EV=SV-AV

Table 2 shows that the prepared sheepskin powder has a relatively high fat content (17.60%, after soaked in a buffer of pH 7.5), and the content of free fatty acid in the extract is rather lower. It is found that the lipase treatment can effectively decrease the EV, but increase the AV of the fats in sheepskin powder, apparently, which indicates that the enzymatic hydrolysis of fat in skin powder is remarkable. And the hydrolysis degree is approximately 2/3. Thus, the AV of the fat extracted from skin can represent the lipolytic degree of the fats in skin. In addition, the fat content of sheepskin powder after lipase treatment is still 18.12%, corresponding to that of no lipase treatment, since the free fatty acids produced by lipase hydrolysis can not be washed out from the skin by water at neutral or weak alkaline pH. In short, the prepared sheepskin powder, which has good homogeneity, high fat content and low AV, are appropriate for being as the substrate to evaluate the degreasing effect.

3.2 Effect of lipase concentration on the lipolytic degree of sheepskin powder

The prepared sheepskin powder was treated with two kinds of lipases (UG and G20) under different enzyme concentrations. The effect of the dosage of lipase G20 and UG on the hydrolysis level of fat in skin powder was illustrated in Figure 1.

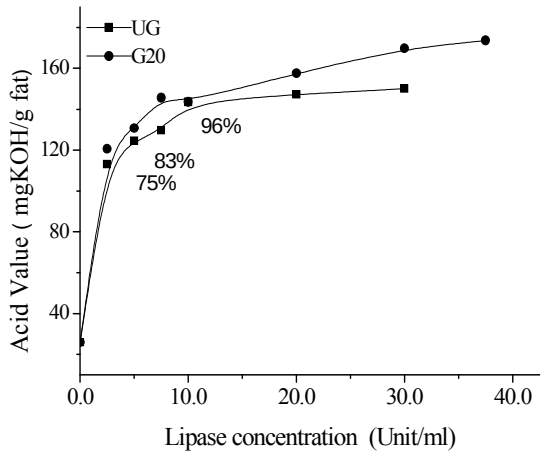

Fig. 1 The effect of the lipase dosage on the acid value of fat

(Lipase treating conditions: pH7.5, temperature 30°C, time 2h)

As shown in Figure 1, with the increase of the amount of lipase, the AV of dichloromethane extract, i.e. the hydrolysis levels of fat in the skin powder for the both lipases, dramatically increase when the lipase concentration is lower than 7.5U/ml, and the enzymolysis degree keeps stable when the concentration is higher than 10 U/ml. So it is seems that the critical concentration of lipase is about 10U/ml. The maximum hydrolysis degree of fat is about 70.50% and 83.90% corresponding to lipase UG and G20. It is worth to be mentioned that the fat content in skin powder doesn't significantly reduce when washed by water, although the AV of the fat in sheepskin powder increases apparently after lipase treatment. This indicates that the performance of lipase degreasing not only depends on the degree of fats hydrolysis, but also the effective removal of the hydrolysis products, i.e. formed fatty acids, which can not be washed out by water in neutral or acidic condition.

If the fat in 3 gram of the above-mentioned sheepskin powder (skin powder moisture, fat content and EA are 13.20%, 17.60% and 176.11 mg KOH / g fat, respectively) was completely hydrolyzed, 1438μmol of free fatty acids could be produced. And in theory, 18000μmol of fatty acids will be produced in 2 hour when the lipase concentration was 2.5u/ml (bath ratio 20:1), but in the actual process only 688μmol and 745μmol of free fatty acid were produced, corresponding to lipase UG and G20. This means the actual hydrolysis degree of fat is around 4% of the theoretical lipolytic capacity and the hydrolysis level of the fat in skin powder was only 47.8% or 51.8% at the concentration level of 2.5u/ml, corresponding to lipase UG or G20. According to our early research on the thermal stability of these two enzymes, both of them behave very high thermal stability and their activities almost have no change in 2 hour under this experimental condition. Thus, there are two reasons why the hydrolysis of fats tends to balance with the increase of the amount of lipase. First, the selected lipases have positional specificity to triglyceride molecules. Second, the inhibition of product occurs during the lipase hydrolysis process of fat.

3.3 Effect of reaction time on on the lipolytic degree of sheepskin powder

The enzymolysis of fats in skin powder is fast during the first 20 min, and then tends to stop, especially after 60min under the experimental conditions, as shown in Figure2. The tendency of the lipase hydrolysis degree of fat with the increase of function time is similar to that with lipase dosage. Why will the enzymolysis of fats not continue with increasing lipase dosage and extending reacting time? The following experiments were designed to find the possible reasons.

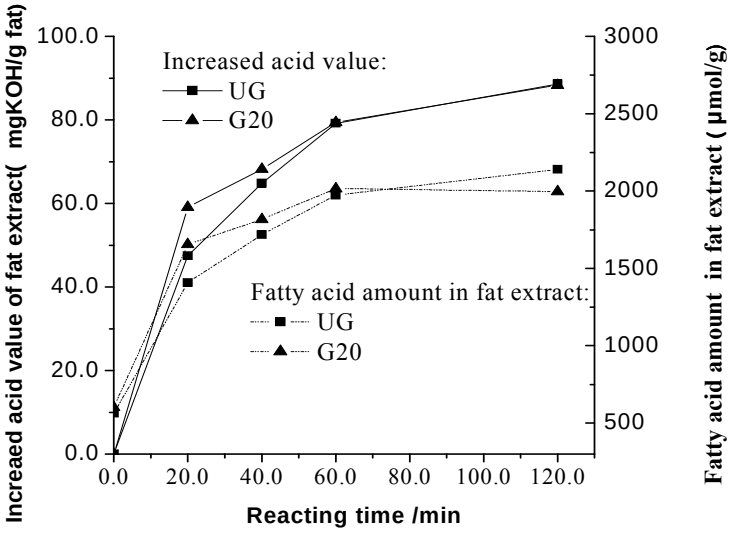

Fig. 2 The curve of time vs. acid value and the amount of fatty acid in the fat extract

(Enzymolysis conditions: bath ratio 20:1, pH7.5, 5U/ml lipase concentration, 30°C)

3.4 Analyzing the enzymolysis products of fats with GC/MS

The free fatty acids were separated from the fats extracted from the sheepskin powder with lipase treatment, and methyl esterification of these was performed, followed by the analysis of GC/MS. The results are given in Table 3.

As shown in Table 3, the main fatty acid components of the fat in sheepskin are myristic acid, palmitic acid, oleic acid and stearic acid. The free fatty acid composition liberated by lipases is almost the same as that without lipase treatment and little difference in the proportion, so it's concluded that the selected lipases has not clear positional specificities, Unless the fatty acid distribution of sheep skin fat molecules in the position is completely random

3.4 The inhibition of products of lipases hydrolysis

According to the results obtained by the GC/MS analysis of fatty acid components, the selected lipases have not clear positional specificities; furthermore, the research on the product inhibition for the selected lipases was performed, in order to find the reason why the lipase hydrolysis tend to stop with the increase of the lipase concentration and reaction time.

Tab.3 The fatty acids components of fat enzymolysis products by lipases

Treatment methods / projects		control▲	Lipase UG■	Lipase G20■
RT/min	Fatty acid	Relative content%	Relative content%	Relative content%
6.59	Capric acid	0.48	0.25	-
11.74	Lauric acid	0.21	0.18	0.20
16.52	Myristic acid	7.23	6.18	8.57
20.35	palmitoleic acid	1.25	1.45	1.85
20.79	Palmitic acid	55.69	50.79	50.72
24.16	Oleic acid	13.71	16.89	12.50
24.63	Stearic acid	15.76	19.22	20.86

▲: Control test: the fat was saponated and then methylated with HCl-methanol

■: Lipase treatment conditions: bath ratio 20:1, pH7.5, lipase concentration 20U/ml, temperature 30°C, time 2h

Using the olive oil emulsion containing different amounts of oleic acid as substrates, the influence of the fatty acid concentration on the selected lipase activities was determined. A certain amount of oleic acid was mixed well with olive oil, and then PVA was added to emulsify the mixture to prepare the substrate. The activity of lipase was measured at pH 7.5 according to the method as previously described [20]. The results are given in table 4.

Tab.4 The effect of the oleic acid concentration on the lipase activity

Concentration of oleic acid in reaction solution (w/v) %	0.00	0.50	1.00
Oleic acid/olive oil(μmol/g)	0.00	194	388
The relative residual activity of lipase UG (%)	100.00	21.27	2.08
The relative residual activity of lipase G20 (%)	100.00	20.56	5.93

As shown in Table 4, the high concentration of oleic acid presented in the system shows high inhibition to the selected lipases. As for the mechanism of the inhibition, it was thought that the accumulation of monoglyceride, diglyceride and fatty acid soap in the oil-water interface has a greater inhibiting effect on the lipase activity [21], and the result of Reis's research[22] showed that lipolytic interface reactions exhibit a self-limiting effect due to simultaneous changes in interface composition and accumulation of highly interfacial active components such as the products of lipase hydrolysis at the interface. These indicate that the inhibiting effect of fatty acids on the selected lipases is not due to changes in enzyme structure itself, but because of the change in the characterization of oil-water interface, thus hindering the enzyme and substrate contact with each other.

According to the above results, it can be concluded that the inhibiting effect of fatty acids on the lipase activity is responsible for the lipase hydrolysis of fat tending to stop with lipase concentration and time. Therefore, it's recommended that using lipase in leather process should be a small amount of lipase and

be used in several processes, and enhancing the removal of fatty acids at the latter stage of enzymatic degreasing, in order to reduce the inhibition.

3.6 The effect of pH value on the enzymolysis of fats in sheepskin powder

Two lipases were used to hydrolyze fats in sheepskin powder at varying pH, and the quantity of fatty acids produced by lipases and the total quantity of fatty acids formed from both enzymolysis and alkaline hydrolysis with pH change are showed in Fig.3.

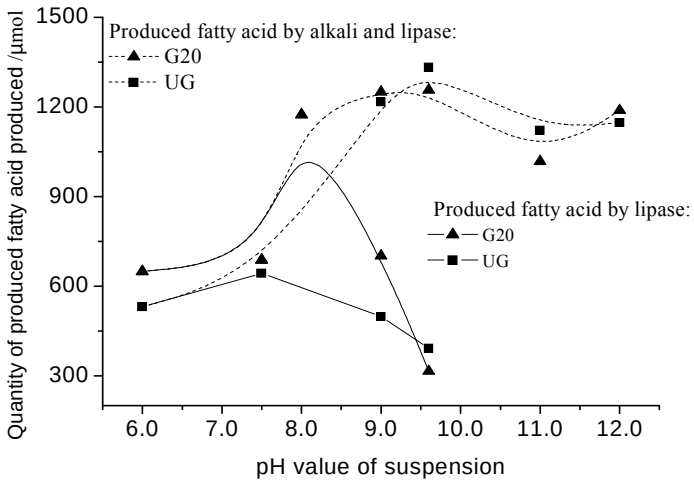

Fig.3 The influence of pH value on the lipolysis of fat in the sheepskin

(Enzymolysis conditions: bath ratio 20:1, pH7.5, lipase concentration 20U/ml, temperature 30°C, time 2h)

As shown in Fig.3, the performance of lipase hydrolysis of fat first increase and then decrease with the increase of the pH. The optimum pH of lipase G20 is 8.0, and that of lipase UG is 7.5 for the fats in the sheepskin powder.

It deserves to be noticed that the alkaline hydrolysis of fats begins when pH value is beyond 8.0. Although the activities of lipases drop down dramatically with pH increase from 8.5 to 10.0, the total quantity of formed fatty acids still rises, which means the alkaline hydrolysis gradually gets better of the enzymolysis. The enzymolysis almost stop when pH rises to 10, so the total quantity of formed fatty acids has a little decrease.

The impact of pH on the enzyme degreasing performance in two ways, on the one hand, the pH value of solution affects the activity of lipase, so lipase hydrolysis should be carried out on its optimum pH range; on the other hand, at high pH, although the alkaline hydrolysis will happen and the fatty acids will be changed to soaps which can easily disperse in water and can emulsify fats to facilitate the removal of fats in skins, lipase enzymolysis will greatly decrease. Consequently, it's recommended that in the enzymatic degreasing process, the pH of solution should be adjusted to the optimum pH of the selected lipase at the early stage and then alkaline materials are added to further hydrolyze fats and remove the formed fatty acids effectively.

3.7 Removal of products of lipase enzymolysis using surfactant and alkali

As previously mentioned, the main products of lipase enzymolysis are fatty acids, monoglyceride, and diglyceride, which can not be efficiently removed from skins under the optimum pH of liapses (8.0). Only lipase enzymolysis cannot obtain desirable degreasing effect. Therefore, it needs to take measurements to further remove the hydrolysis products from skins. The ideal way is combining lipase treatment with alkali and/or surfactant washing.

An anion surfactant, LAS and a commercial non-ionic degreasing agent BE/C were selected to remove the hydrolysis products in the sheepskin powder after lipase treatment. Moreover, the effect of surfactant, alkali and their combination on the fats and fatty acid removal were investigated.

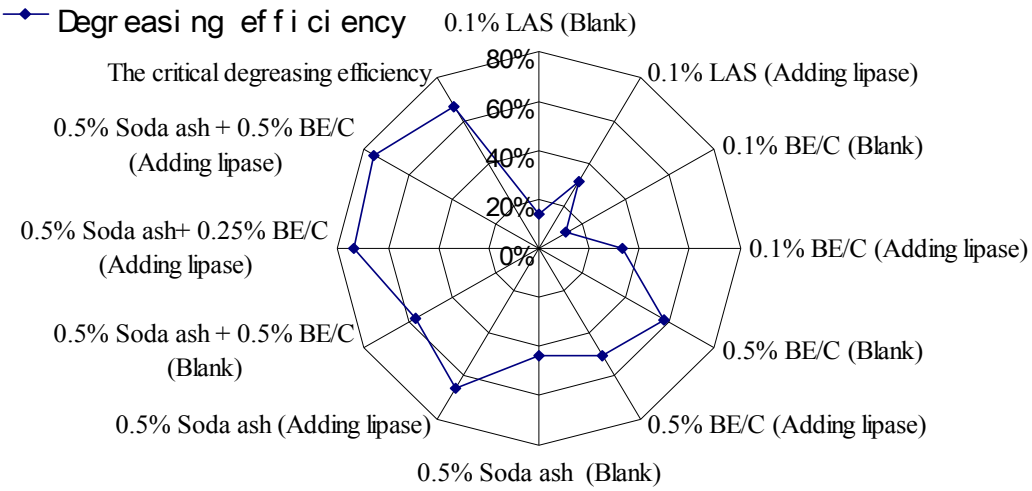

Fig. 4 The effect of fat removal with surfactant, alkali and their combination

(Sheepskin powder was enzymolyzed with lipase UG solution of 20u/ml concentration at 20:1 liquor ratio, pH 7.5 and 30°C for 2h. Then was washed with alkaline or and surfactant solution at 20:1 liquor ratio for 30min. The blank test is just with wash without lipase treatment.)

It has been mentioned that the hydrolysis degree of fats in sheepskin powder was about 70% (see Part 3.2) under given conditions, so the critical degreasing efficiency of lipase UG was regarded as 70% when all lipase hydrolysis was removed completely. Fig 4 shows that surfactant washing without lipase treatment just remove the original fats in skin powder, and the degreasing ratio of BE/C of 0.5%(w/v) concentration reaches to 57.4%. Comparing with the blank, the degreasing ratio of lipase hydrolysis combining with BE/C of 0.5% (w/v) washing, it decreases to 50%. The reason is that the degreasing agent BE/C has lower emulsifying ability to fatty acids, the lipolysis products, than to the original fats. Washing the lipase-treating sheepskin powder with 0.5% (w/v) Na_2CO_3 solution, the fat removal ratio reaches to the 65.5% and near to the critical degreasing efficiency of lipase UG, 70%, which means most of the formed fatty acids are removed. So, it is effective and necessary to combine lipase treatment with alkali washing. Moreover the treated sheepskin powder with lipase was further washed with Na_2CO_3 and a degreasing agent solution, and the degreasing ratio reaches to 80%. A

high-efficiency aqueous degreasing method based lipases without organic solvents is established.

4. Conclusions

A new research model of degreasing was developed using a uniform sheepskin powder rich in fats as the substrate, which can evaluate the lipolytic performance of lipases and the impact of lipases on degreasing. The results shows that the product inhibition phenomenon in lipase hydrolysis process exists and is remarkable, so it's recommended that the use-pattern of lipase in leather degreasing should be with a relative low amount and several times; the two commonly-used commercial lipases behave the highest activity at around pH8.0, and the activity almost lose at pH over 10.0, so the lipase treatment should be carried out at their optimum pH, and avoid to being used in strong alkaline processes, such as liming, etc.; the lipase hydrolysis products can not be removed at the lipolytic conditions, it needs further be released from the substrate, and the effective method is that the lipase hydrolysis substrates was then washed with alkali solutions to transfer fatty acids to salts and appropriate amount surfactant. Finally, a high-efficiency aqueous degreasing method based lipases without organic solvents is established, and the degreasing ratio reaches to 80%.

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