Evaluation of Protein Hydrolysate from Chrome-Tanned Leather Waste in Keratinase Production by Bacillus Subtilis Atcc 6633

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Chrome-tanned leather waste from tanning industry was hydrolyzed in a commercial proteolytic preparation (Rhodozyme, ML-RHOM, Deutschland) with stirring at a temperature of 65°C for 4 hours. The chemical analysis of the powder protein hydrolysate indicated that it has a small portion of ash (3%), low content of chromic compounds and balanced composition of amino acids. This enzymatic hydrolysate as a carbon and nitrogen source was used to production of keratinase from *Bacillus subtilis* ATCC 6633. The culture was grown in fermentation medium containing 0-5% (w/v) of protein hydrolysate at pH 7.0 and pH 10.0 with agitation (180 rpm) at 30°C for 96 hours. The best keratinase production was obtained in the medium containing 1%(w/v) hydrolysate powder on 36 h at pH 7.0 and with 3% (w/v) hydrolysate powders on 24 h at pH 10.0.

Keywords: *Bacillus subtilis*, leather waste hydrolysate, keratinase, protease

INTRODUCTION

Keratins are the largest and most complex family of cytoskeletal intermediate filament proteins of animal cells, particularly epithelia (Scott and Untereinen, 2004). Keratins are grouped into hard keratins (feather, hair, hoof and nail) and soft keratins (skin and callus) according to the sulfur content (Gupta and Ramnani, 2006). Hard keratins are insoluble and resistant to degradation by common proteolytic enzymes, such as trypsin, pepsin and papain because of their high degree of cross-linking by disulfide bonds, hydrogen bonding and hydrophobic interactions (Farag and Hassan, 2004) Keratinous wastes are increasingly accumulating in the environment generated from various industries. To recycle of such wastes, biotechnological alternatives are developing (Gupta and Ramnani, 2006).

Keratinases [EC 3.4.21/24/99.11] are serine or metallo proteases degrading keratinous proteins. They use in detergent, medicine, cosmetics, leather and feed industries and in newer fields like prion degradation for treatment of dreaded mad cow disease, biodegradable plastic manufacture and feather meal production. Most of the reports on keratinases, group them as inducible enzymes; however few constitutive keratinases have also been reported (Gupta and Ramnani, 2006). Keratinolytic enzymes are widespread in nature, especially in microbial world. A vast variety of bacteria, actinomycetes and fungi are known to be keratin degraders (Wang et al., 2003; Letourneau et al., 1998; Santos et al., 1996).

In this research, an evaluation of collagen hydrolysate for production of protease and keratinase by *Bacillus subtilis* ATCC 6633 is reported with a comparison of two substrates; hammerstein casein and azo-keratin.

MATERIALS AND METHODS

Preperation of collagen hydrolysate

Shaving wastes of sheep leather, that had been tanned with chromium with a pH of 3.8 at the end of basification were obtained from commercial tannery, and were soaked in water five times their weight. They were treated with 4 % MgO (Merck) at 65°C for 30 min. In the following step, the enzyme Rodazym ML (obtained from Rohm, Darmstadt) was added to the solution at 1%, and the mixture was shaken for four hours. The solution obtained was then filtered to separate chromium cake and liquid collagen hydrolysate (Taylor, 1994, Crispm and Mota, 2003). The pH of the separated liquid collagen hydrolysate was adjusted to 7 by adding 0.1 N HCI, and the solution was dried to powder form in a spray drier.

Determination of Some Chemical Characteristics of Collagen Hydrolysates

The chromium content of the collagen hydrolysates were measured with a Perkin-Elmer 2380 model atomic absorption device, and their amino acid content were measured with Eppendorf LC 3000 Amino acid Analyzer.

Among other chemical analysis, pH was measured according to SLC 13; the ratio of humidity, according to SLC 113; hide substance, according to IUC/10; the amount of total sulphated ash, according to IUC/7 and substance soluble in dichloromethane was measured according to IUC/4 (Anonymous, 1996)

Microorganism and Media

Bacillus subtilis ATCC 6633 was used for keratinase and protease production by submerged cultivation. This strain was routinely maintained on nutrient agar at 4°C. The medium used for enzyme production was composed of 0.5 % glucose, 0.1 K_2HPO_4, 0.02 % $MgSO_4.7H_2O$,

0.01 % $CaCl_2$, supplemented with collagen hydrolysate (1-5 % w/v). pH were adjusted to 7.0 with 0.1 N HCl and to 10 with 1% Na_2CO_3. Na_2CO_3 was sterilized separately and added to sterilized media after cooling (Gessesse, 1997). 5 ml of inoculum cultures, grown in the media with pH 7.0 and pH 10.0 without collagen hydrolysate at 30°C and 180 rpm for 96 h, inoculated to 100 ml of the media prepared in 500 ml flasks. After incubation at 30°C and 180 rpm, culture broth was centrifuged at 10000 rpm and 4 °C for 10 min. The cell-free culture supernatant was used as enzyme source for the determination of protease and keratinase activities.

Enzymatic assays

Protease activity was assayed by using the following assays by a method of Nakiboğlu et al. (2001); (1) preparation of substrate; 0.6% Hammerstein casein dissolved completely in buffers (pH 7.0, 20 mM phosphate buffer and pH 10.0, 20 mM Glisin buffer). (2) Activity measurement procedure; reaction mixture containing 500 µl enzyme solution and 2 ml of substrate were incubated for 30 min at 40°C. To stop the enzymatic reaction, 2.5 ml of 10% TCA solution was added into the mixture and stored for precipitation at room temperature for 30 min. After centrifuging at 10000 rpm for 5 min to remove the precipitate, absorbance of released tyrosine was measured at 280 nm. 1 Unit enzyme activity was defined as the amount of enzyme which released 1 µg amino acid equivalent to tyrosine per minute under above assay conditions.

Hydrolysis of azo-keratin was measured by increased azo-dye as described previously by Suntornsuk et al. (2003). 4 mg/ml keratin azure (sigma) in buffers (pH 7.0, 20 mM phosphate and pH 10.0, 20 mM Glisin) was cut up and autoclaved at 121°C for 15 min. 1 ml of substrate and 1 ml of enzyme solution was incubated for 1 h at 50°C for reaction with constant agitation (180 rpm). The reaction was stopped by abrupt cooling at -20°C and following boiling for 15 min. As a control, denaturized enzyme by boiling was added to substrate solution which has effected the same treatment with the other reactants. After centrifugation at 10000 rpm for 5 min, absorbance was measured spectrophotometrically at 585 nm. One unit (U) of keratinase was defined as the amount of enzyme causing 0.01 absorbance increase between sample and control at reaction conditions.

RESULTS AND DISCUSSION

Amino acid contents of collagen hydrolysates, used as the study material, are given in Table1 and some other chemical characteristics are given in Table 2.

Amino acid	Collagen hydrolysate (%)	Collagen Type1[b] (%)	Amino acid	Collagen hydrolysate (%)	Collagen Type1[b] (%)
Gly	32.34	32.7	Ile	1.36	1.2
Hyp	9.23	8.6	Leu	2.97	2.5
Pro	13.34	13.0	Lys	2.73	2.8
Ala	8.65	11.4	Met	0.77	0.6
Arg	5.01	5.2	Phe	1.81	1.3
Asp	5.32	4.6	Ser	2.88	3.1
Cys	0.0	0.0	Thr	2.04	1.6
Glu	7.47	7.5	Tyr	0.58	0.4
His	1.03	0.5	Val	2.45	2.3
			Toplam	99.98	99.3

[a]Our study, [b]Piez and Reddi (1984)

Table 1. Amino acid contents of collagen hydrolysates

Chemical analyses	Mean
pH	6,98
Moisture (%)	8.17
Chromium (ppm)	4.52
Hide substance (%)	88.00
Ash (%)	11.99
Substances soluble in dichloromethane (%)	0.05

Table 2. Certain chemical characteristics of collagen hydrolysates

The values of keratinolytic and proteolytic activities measured at different pH were given in graphs. As can be seen in Table 3, the best protease production at pH 7.0 was gained as 0.82 U/ml at 72 h with 2 % of supplemented hydrolysate. In contrast to this, at pH 10.0, 36 h was enough for the highest activity with 1.49 U/ml (Table 4).

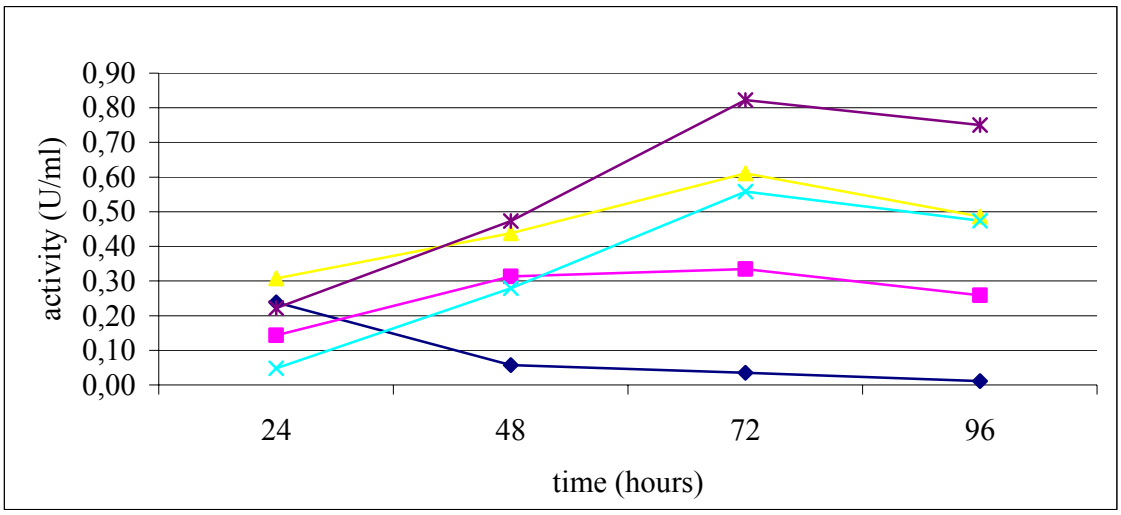

Table 3. Protease production of *B. subtilis* ATCC 6633 cultured in the medium (pH 7.0) with various initial levels of collagen hydrolisates: (◊) 0.0 %, (□) 0.5 %, (Δ) 1.0 %, (x) 1.5 %, (*) 2.0 %.

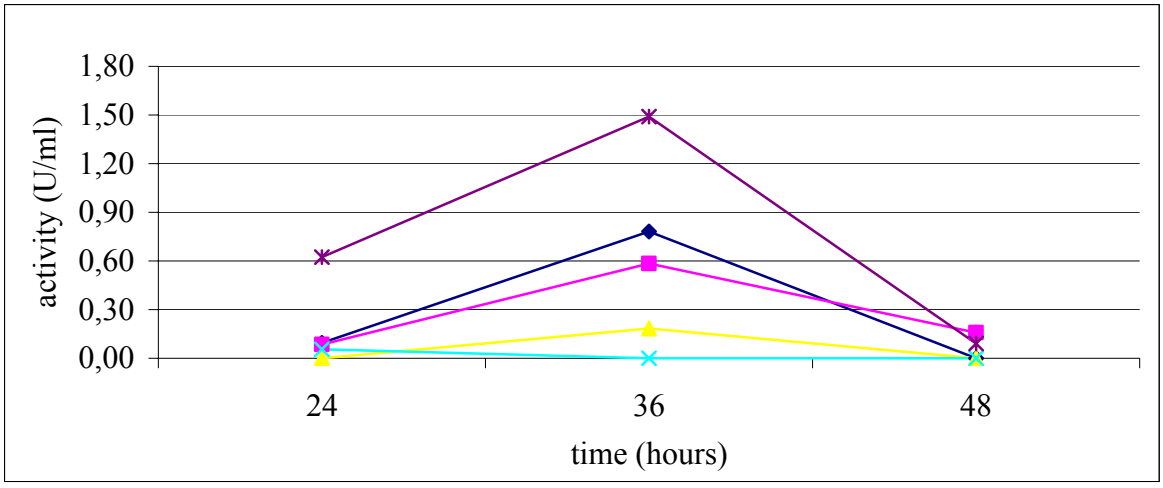

Table 4. Protease production of *B. subtilis* ATCC 6633 cultured in the medium (pH 10.0) with various initial levels of collagen hydrolisates: (◊) 0.0 %, (□) 0.5 %, (Δ) 1.0 %, (x) 1.5 %, (∗) 2.0 %.

In Table 5 and 6 keratinase production was shown. In comparing the keratinase activities at pH 7.0 and pH 10.0, the highest activity was at pH 7.0 with 36.76 U in 1% supplemented medium.

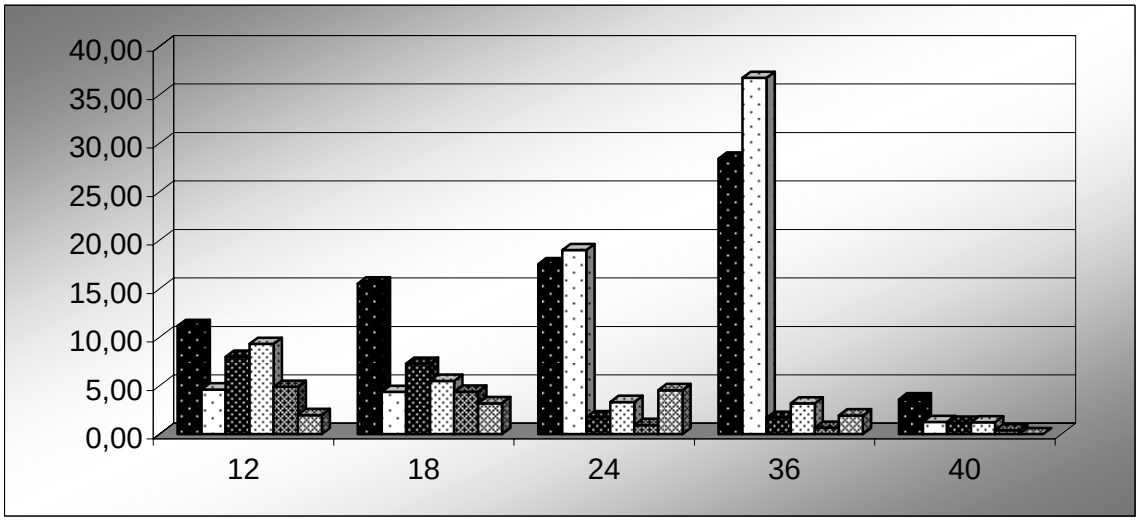

Table 5. Keratinase production of *B. subtilis* ATCC 6633 cultured in the medium (pH 7.0) with various initial levels of collagen hydrolisates: (■) 0.0 %, (▨) 1.0 %, (▩) 2.0 %, (▨) 3.0 %, (▩) 4.0 %, (▩) 5.0 %.

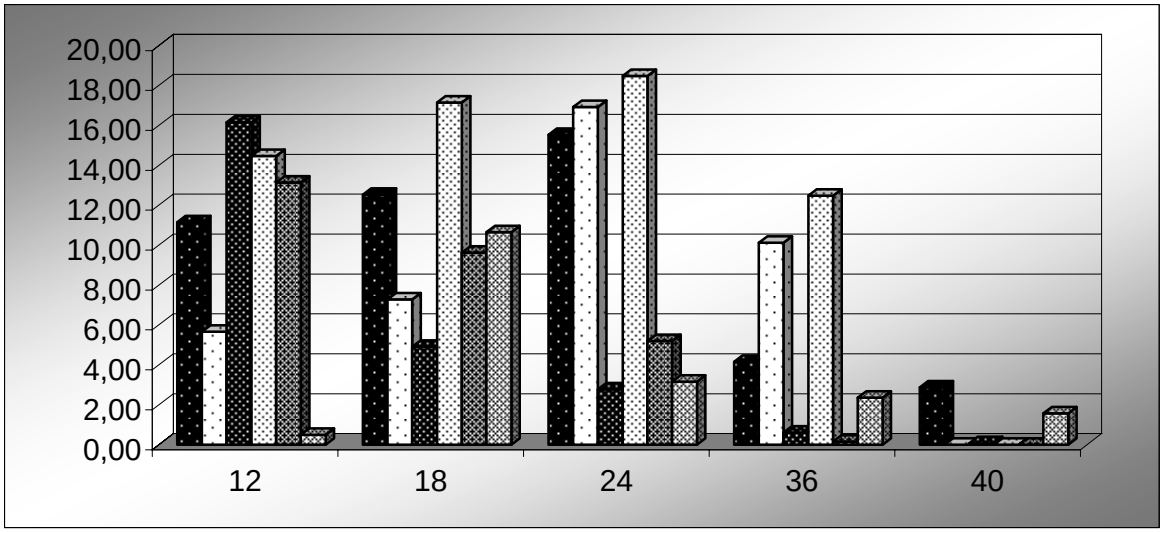

Table 6. Keratinase production of *B. subtilis* ATCC 6633 cultured in the medium (pH 10.0) with various initial levels of collagen hydrolisates: (■) 0.0 %, (⁘) 1.0 %, (▩) 2.0 %, (▨) 3.0 %, (▩) 4.0 %, (▩) 5.0 %.

During growth, keratinases are produced in late exponential or stationary phase. But keratinase degradation takes from 24 h to several days according to complex mechanism of keratinolysis of microorganisms (Gupta and Ramnani, 2006).

Microbial keratinases have pH optima in neutral to alkaline range. In this investigation protease activity determinated by hammersten casein was better at alkaline pH, whereas keratinolytic activity by azo-keratin was good at neutral pH. This can be probably explained by formation of inhitor substances in alkaline pH in the production medium.

The collagen hydrolysate used in this study as inducer was hydrolyzed from chrome-tanned leather waste from tanning industry. *Bacillus* ATCC 6633 produced higher protease and keratinase in the medium containing various level of kollagen hydrolysate than basal medium without kollagen hydrolysate.

In consideration of the advantages of microbial enzyme in leather tanning, a detergent additive for low temperature washing etc., it is important to lower the cost by cheap substrates. Removing leather wastes from tanning industry is an eco-friendly approach. Keeping in mind, collagen hydrolysate can be used as an ingredient of medium for protease production.

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