Utilization of Collagen Hydrolysate in Keratinase Production from *Bacillus subtilis* ATCC 6633

by

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Abstract

In this study, chrome shavings from the tanning industry were hydrolyzed with a commercial protease in alkali medium. Some chemical analyses were made and amino acid composition of collagen hydrolysate obtained from chrome shavings determinations. This collagen hydrolysate was used for the production of keratinase from *Bacillus subtilis* ATCC 6633 as a carbon and nitrogen source. The culture grew in fermentation medium containing various proportion of collagen hydrolysate (1-5% w/v) at pH 7.0 and pH 10.0 with agitation rate of 200 rpm. The best keratinase production was obtained from the medium containing 1% hydrolysate (w/v) at pH 7.0 for 36 hours and with 3% hydrolysate (w/v) at pH 10.0 for 24 hours.

Abstracto

IEn este estudio, las virutas de cromo de la industria curtidora fueron hidrolizadas con una proteasa comercial en medio alcalino. Algunos análisis químicos fueron realizados y la composición de aminoácido del hidrolizado del colágeno obtenida de las virutas del cromo determinadas. Este hidrolizado del colágeno fue utilizado para la producción de queratinasa desde el *bacillus subtilis* ATCC 6633 como fuente del carbón y del nitrógeno. El cultivo creció en el medio de la fermentación que contenía variadas proporciones del hidrolizado de colágeno (1-5% w/v) en pH 7.0 y pH 10.0 con un índice de la agitación de 200 rpm. La mejor producción del queratinasa fue obtenida del medio que contenía el hidrolizado del 1% (w/v) a pH 7.0 por 36 horas y con el hidrolizado del 3% (w/v) a pH 10.0 por 24 horas.

Introduction

Approximately 18 billion ft^2 leathers are produced in the world every year and 90% of this volume of leather is tanned with chromium[1]. Each year, the world leather industry generates more than 500 000 metric tons chromed leather wastes from leather processing[2]. These wastes have the possibility to be toxic to plant growth and aquatic life and also pollute ground water. Increased local restrictions on land disposition and the cost of ecological dispositions of these have encouraged the leather industry to find more ecological solutions and ways of utilization. Chrome shavings, splits, trimmings and dust are suitable for processing to recover chrome and collagen based protein hydrolysate[3,4,5,6]. Modified or non-modified forms of this hydrolysate have been investigated for usage in leather processes[7,8,9,10]. In addition to this, utilization of collagen hydrolysate in different fields such as plant fertilizing, animal feeds, micro-encapsulation and biocomposite layers was investigated[11,12,13,14,15].

There are a limited number of studies with regard to utilization of leather and fur wastes from beamhouse process in the production of proteolytic enzymes. Macedo et al.[16] reported that *B. subtilis* produced a novel keratinase that was able to unhair completely bovine skin and inactive upon collagen when growing in a culture medium composed of whey (a dairy by-product) and untreated bovine hair at pH 8.0.[16] However, we are not aware of any reports in literature describing the production of proteolytic enzymes by using a collagen hydrolysate obtained from chrome shavings waste.

In this research, considering that collagen hydrolysate represents a good mixture and a good source of amino acids, it was aimed to determine the protease production profile of *Bacillus subtilis* ATCC 6633 in the medium with collagen hydrolysate as a carbon and nitrogensource, which is a rational biotechnological process for utilization of chromed leather solid wastes.

Materials and Methods

Preparation of Collagen Hydrolysate

Shaving wastes of chrome tanned sheep leathers were obtained from a local tannery in Izmir-TURKEY. They were soaked in water five times their weight and treated with 4 % MgO at 65°C for 30 min. In the following step, the enzyme Rodazym

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ML (Rohm) was added to this solution at a concentration of 1% (w/v) and the mixture was digested for four hours. Then, this mixture was filtered to separate chrome cake and liquid collagen hydrolysate and the liquid collagen hydrolysate was dried to powder form in a spray drier (Niro Atomizer)[17,18].

Determination of Some Chemical Characteristics of Collagen Hydrolysate

In order to evaluate the quality of collagen hydrolysate; pH (IUC 11), moisture content (SLC 113), hide substance (IUC 10) and sulphated total ash (IUC 7) were determined[19].

The chrome content[20,21] of the collagen hydrolysate was measured with a Perkin-Elmer atomic absorption spectrophotometer (model 2380), and its amino acid content[22] was determined using a Eppendorf amino acid analyzer (model LC 3000).

Microorganism and Media

Bacillus subtilis ATCC 6633 was used for keratinase and protease production by submerged fermentation. 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% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2$, supplemented with 1-5% collagen hydrolysate (w/v). pH values were adjusted to 7.0 and 10.0 with 0.1 N HCl and 1% Na_2CO_3, respectively. After, Na_2CO_3 was separately sterilized and cooled; it was added to sterilized media[23]. 5 ml of inoculum cultures, grown in the media at pH 7.0 and 10.0 without collagen hydrolysate at 30°C and 180 rpm for 96 hours, were inoculated to 100 ml of the media prepared in 500 ml flasks. After incubation at 30°C and 180 rpm, the culture broth was centrifuged at 10 000 rpm and 4°C for 10 min. A cell-free culture supernatant was used as enzyme source for the determination of protease and keratinase activities.

Enzymatic Assays

Protease activity was determined by the modified method of Anson[24]. 0.6% Hammerstein casein dissolved completely in buffers (pH 7.0, 20 mM phosphate buffer and pH 10.0, 20 mM glycine buffer) as a substrate. Then, a reaction mixture containing 500 ml enzyme solution and 2 ml of substrate were incubated to measure enzyme activity for 30 min at 40°C. A 2.5 ml of 10% trichloroacetic acid (TCA) solution was added into the mixture as a reaction stopper and stored further for precipitation at room temperature for 30 min. After centrifuging at 10 000 rpm for 5 min, released tyrosine concentration was measured spectrophotometrically at 280 nm. One unit (U) of the enzyme activity was defined as the amount of enzyme released 1 mg of amino acid equivalent to tyrosine per minute under the standard assay conditions[25,26].

Hydrolysis of azo-keratin was measured by increased azo-dye as described previously by Suntornsuk and Suntornsuk[27]. Keratin azure (Sigma) was cut into small pieces and suspended at a concentration of 4 mg/ml in buffers (pH 7.0, 20 mM phosphate and pH 10.0, 20 mM glycine). The substrate suspension was autoclaved at 121 °C for 15 min. 1 ml of substrate and 1 ml of enzyme solution was incubated for an hour at 50°C for reaction with a constant agitation rate of 180 rpm. The reaction was stopped by cooling immediately at -20°C for 5 min in deep freezer and then tubes containing reaction mixture was transferred into a boiling water bath and incubated for 15 min for a complete denaturation of the enzyme. As a control, the enzyme that was denaturized by boiling was added to the substrate solution which has effected the same treatment with the other reactants. After centrifugation at 10,000 rpm for 5 min, the absorbance of the supernatant was measured spectrophotometrically at 595 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. The results were statistically evaluated using Microsoft Excel program.

TABLE I
Amino Acid Contents of Collagen Hydrolysate

Amino Acid Types	Collagen Hydrolysate[a] (%)	Collagen Type I[b] (%)	Collagen Hydrolysate[c] (%)	Fleshing Hydrolysate[d] (%)
Gly	32.34	32.7	33.0	22.8
Hyp	9.23	8.6	10.0	0.0
Pro	13.34	13.0	12.5	10.18
Ala	8.65	11.4	8.4	9.46
Arg	5.01	5.2	4.8	8.62
Asp	5.32	4.6	5.1	7.90
Cys	0.0	0.0	0.0	0.0
Glu	7.47	7.5	7.7	10.99
His	1.03	0.5	0.9	2.13
Ile	1.36	1.2	1.4	2.37
Leu	2.97	2.5	2.6	3.68
Lys	2.73	2.8	2.7	3.92
Met	0.77	0.6	0.2	1.31
Phe	1.81	1.3	1.3	2.50
Ser	2.88	3.1	4.1	5.52
Thr	2.04	1.6	2.1	2.37
Tyr	0.58	0.4	0.5	1.42
Val	2.45	2.3	2.4	3.08
Total	**99.98**	**99.3**	**100**	**99.97**

[a] Our study, [b] Piez and Reddi,[39] [c] Taylor et al.,[33] [d] Kanagaraj et al.[40]

RESULTS AND DISCUSSION

Chemical Properties of Collagen Hydrolysate

The collagen hydrolysate used in this research, was shown by amino acid analysis, to consist of 17 amino acid types with acidic, basic, polar and non-polar side chains.

As seen in Table I, when the amino acid profile of collagen hydrolysate is compared to previous studies, it can be seen that our findings are quite similar to others[22,28,29].

TABLE II
Certain Chemical Characteristics of Collagen Hydrolysate.

Chemical Analyses	Mean
pH	8.76
Moisture (%)	8.17
Chromium (ppm)	4.52
Hide substance (%)	88.00
Ash (%)	11.99

The chemical characteristics of the collagen hydrolysate obtained from chromed sheep leather shavings are shown in Table II.

In recent years, novel methods have been developed to obtain collagen hydrolysate products using different chemicals with various proportions. Hence, the pH of collagen hydrolysate products ranged between 8.6 and 9.6 in general[13,30,31]. Similarly, the pH degree of collagen hydrolysate used in this study was found to be 8.76 (Table II).

The moisture content of spray dried collagen hydrolysate was found to be 8.17% (Table II), a low enough level to prevent microbial growth and enable an effective storage.

The most important factors affecting collagen hydrolysate quality are hide substance, ash and chromium amount[22,30]. Determined hide substance, ash and chromium values in collagen hydrolysate are presented in Table II. Hide substance and ash amount of collagen hydrolysate were determined as 88.00% and 11.99% respectively. These results were found to be within the range of values given by previous studies[5,22,32,33,34]. Chromium content of the hydrolysate determined to be 4.52 ppm is negligible for using in animal feeding, plant fertilizing and nutrient for microbiologic fermentation[11,35,36,37,38,39].

Utilization of Collagen Hydrolysate in Protease Production

Bacillus species are considered to be dominant bacterial workhorses in microbial fermentation and they produce and secrete large quantities (20-25 g/l) of extracellular enzymes including proteases and amylases[40]. Although some microorganisms producing high level proteases such as *Aspergillus fumigatus* and *Dermatophytes* are serious pathogens, *B. subtilis* and *B. licheniformis* are generally regarded as safe[40,41,42]. Consequently, *B. subtilis* ATCC 6633 was selected as proteolytic enzyme producer which is active against insoluble and soluble substrates e.g. azo-keratin and casein.

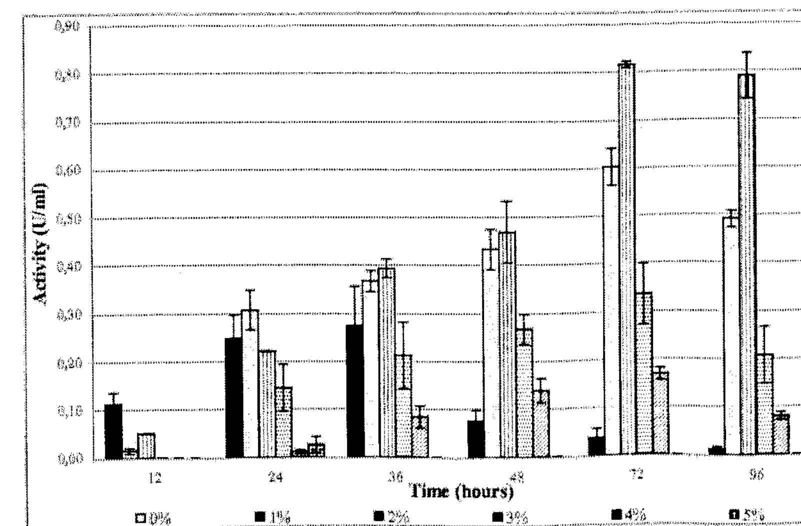

Figure 1. - Protease production of *B. subtilis* ATCC 6633 cultured in the medium (pH 7.0) with various initial levels of collagen hydrolysate.

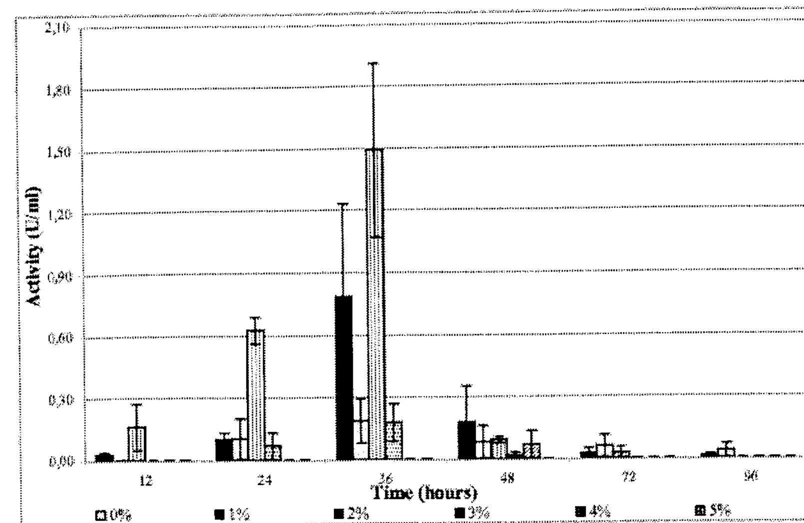

Figure 2. - Protease production of *B. subtilis* ATCC 6633 cultured in the medium (pH 10.0) with various initial levels of collagen hydrolysate.

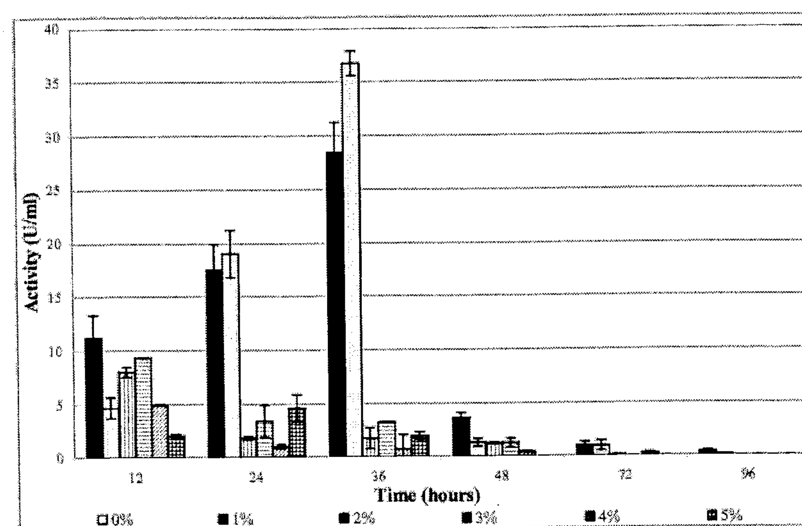

Figure 3. - Keratinase production of *B. subtilis* ATCC 6633 cultured in the medium (pH 7.0) with various initial levels of collagen hydrolysate.

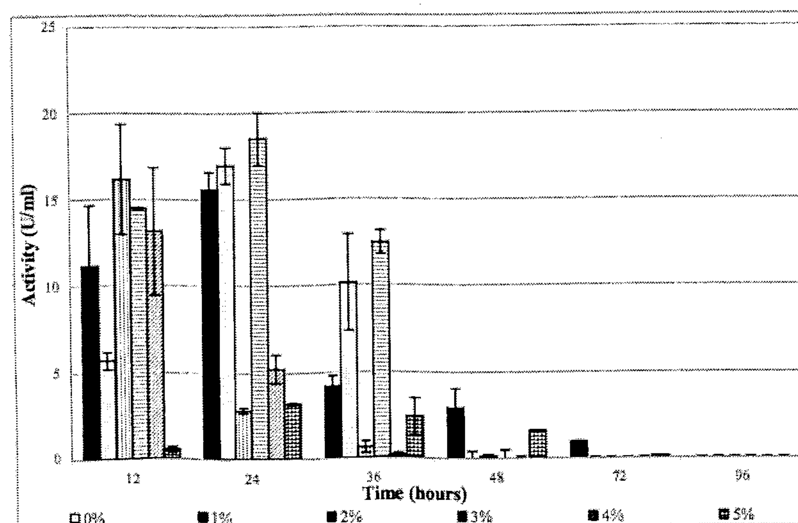

Figure 4. - Keratinase production of *B. subtilis* ATCC 6633 cultured in the medium (pH 10.0) with various initial levels of collagen hydrolysate.

The activities of proteases and keratinase produced by *B. subtilis* ATCC 6633 in the fermentation medium containing different proportion of collagen hydrolysate at pH 7.0 and 10.0 were determined using Hammerstein casein for protease activity and keratin azure for keratinase activity at the pH of fermentation medium. As seen in Figure I and II, protease activity reached the maximum level (0.82 ± 0.01 U/ml) in the medium containing 2% collagen hydrolysate (w/v) at pH 7.0 for 72 hours. In contrast to this, in the same composition when the medium is adjusted to pH 10.0, incubation for 36 hours was enough for the highest protease activity with 1.49 ± 0.42 U/ml (Figure II).

However, as given in Figure III and IV, maximum keratinase production (36.76 ± 1.15 U/ml) in the medium with 1% collagen hydrolysate was observed at pH 7.0 for 36 h. The keratinase production at pH 10.0 was attained at the maximum value (18.48 ± 1.52) in the medium with 3% collagen hydrolysate, after incubation of 24 h, but this accounts for about 50 % of keratinase production at pH 7.0.

Optimum pH for keratinase production is 7.0 of *B. licheniformis* K-508[43] and *B. subtilis* ATCC 6633[44] but 9.0 of *Bacillus* sp. FK 46[27,43,45] which indicated that this phenomenon is specific species and varies with the producing microorganism.

CONCLUSIONS

The collagen hydrolysate used in this study was obtained from tanning industry chrome shavings by an alkali enzymatic method. *Bacillus* ATCC 6633 produced higher protease and keratinase values in the medium containing various level of collagen hydrolysate as compared to the medium without collagen 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 enzyme production cost by utilizing less expensive substrates. Removing chrome leather waste, which is one of the solid wastes from tanning industry, is an eco-friendly approach. These results indicated that collagen hydrolysate obtained by alkali enzymatic method could be used in proteolytic enzyme production from *Bacillus subtilis* ATCC 6633.

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