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Silk Degumming Solution as Substrate for Microbial Protease Production

Pilanee Vaithanomsat*, Taweesiri Malapant and Waraporn Apiwattanapiwat

ABSTRACT

As waste solution from the silk degumming process contains high nitrogen levels, wastewater must be treated prior to discharge. In this study, waste solution was prepared and tested as a nutrient substrate for microbial growth and protease production by *Bacillus licheniformis* TISTR 1010 and *Aspergillus flavus* TISTR 3130, TISTR 3366, TISTR 3135 and TISTR 3041. All strains were preliminarily screened for their protease activity by growing on casein-agar plates with *B. licheniformis* TISTR 1010 being chosen as the best producer of protease. Cultivation in a silk degumming solution as the nutrient source demonstrated that the highest protease activity was achieved at an optimum pH of 10 for 36 h. Among the culture media used, the specific activity of released protease was best with a medium containing 6% protein from the silk degumming waste, 1% malt extract, 1% polypeptone and 1% Na_2CO_3. This study was the first to report the use of silk degumming waste as a nitrogen source for microorganism growth and protease production. As such it could suggest an alternative way to convert wastes into more valuable and marketable products.

Key words: silk degumming, alkaline protease, protease production

INTRODUCTION

Silk manufacturing is one of the industrial sectors where intensive water consumption cannot be avoided and therefore a large volume of wastewater is produced. The degumming process is used to remove external sericin prior to dyeing and is a source of waste water. This process generally uses a synthetic soap solution at 95°C for 1 h, with 100 kg of silk producing 22 kg of sericin. Sericin is a globular protein in the form of a tube outside the silk fibroin with its molecular weight ranging between 10 and 300 kDa (Fabian *et al.,* 1996; Zhang *et al.*, 2004). When subjected to the alkaline degumming process, sericin is degraded into sericin peptide or hydrolyzed sericin with a molecular weight of less than 20 kDa (Zhang *et al.*, 2004). Both the peptide and the hydrolysate of sericin have excellent moisture absorption properties and are also involved in a lot of biological activities such as antioxidation, tyrosinase activity inhibition and anticancer activity (Kato *et al.*, 1997; Chang-Kee *et al.*, 2002). As a result, they can be used in many fields including cosmetics, biomaterials, textiles and pharmaceuticals (Zhang, 2002).

High concentrations of BOD, COD and nitrogen in the degumming waste solution make it complicated and costly to treat (Rigoni-Stern *et al.*, 1996; Fabiani *et al.*, 1996). However, the high nitrogen and protein content in this wastewater derved from sericin products 2008 could be

Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: p_vaithanomsat@yahoo.com, oplv@ku.ac.th

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recovered and used. Vaithanomsat *et al*. (2008) applied ultrafiltration to recover sericin from silk degumming waste which was further hydrolysed into sericin hydrolysate of a similar quality to that of commercially-available sericin hydrolysate used for cosmetics purposes. It would also be very advantageous if such a waste solution could be used as a substrate for microbial growth or enzyme production. In most cases, the growth media makes up approximately 40% of the production cost of industrial enzymes. Organic nitrogen substrates such as casein, yeast extract, soy protein or gelatin are widely used in many microbial applications due to their favourable amino acid balance and high protein content. However, no research has been reported on the use of silk protein, especially those derived from degumming waste, as a substrate for microbial enzyme production.

The aim of this work was to prepare a nutrient source from silk degumming waste and to examine the suitability of this substrate as a microbial growth medium for protease production.

MATERIALS AND METHODS

Materials

Degumming waste solution from *Bombyx mori* silk processing was obtained from the silk manufacturer in Thailand and kept at 4°C in a cold room to avoid decomposition until use.

Microorganisms

Four strains of *Aspergillus flavus*: TISTR 3130, TISTR 3366, TISTR 3135, TISTR 3041 and *Bacillus licheniformis*: TISTR 1010 were provided by the Thailand Institute of Scientific and Technological Research (TISTR), Thailand.

Cultivation and media

Fungal inocula were routinely grown in potato dextrose agar (PDA); made up from: potato; 200.0 g/l dextrose 20.0 g/l; and agar 17 g/l. Bacterial inocula were routinely grown in nutrient agar (NA) made up from: beef extract 3.0 g/l; peptone 5.0 g/l; and agar 15 g/l. The silk degumming media used for growth and protease production had silk degumming waste solution as the only source of nitrogen. Concentrations of silk degumming media were prepared in deionized water. All media were autoclaved at 121°C for 20 min. Cultivations were performed on a rotatory shaker (180 rpm) at 37°C, in 250 ml Erlenmeyer flasks. The cultures were centrifuged and the cell-free supernatants were used for the estimation of proteolytic activity. All experiments were repeated at least twice.

Comparative study of protease production by different species

A qualitative screening for the proteolytic activity of strains was based on their growth and the appearance of clear zones on casein hydrolysis media with a pH of 5.4 and containing KH_2PO_4 (1.0 g/l), KCl (0.5 g/l), $MgSO_4•7H_2O$ (0.2 g/l), $CaCl_2•2H_2O$ (0.1 g/l), glucose (10.0 g/l), 1.5% skim milk (25.0 ml/l) and agar (12.0 g/l) (Paterson and Bridge, 1994). The media were autoclaved at 105°C for 30 min. The bacterial culture was spotted onto the casein hydrolysis medium. All fungal cultures on PDA were cut using a 0.5 cm.-cork borer and placed onto the casein hydrolysis medium. After three days incubation at 37°C, clear zone diameters were measured.

Preparation of silk degumming waste solution

The silk degumming waste solution was first passed through ultrafiltration (Amicon model 8400, Amicon Inc., USA) with a membrane (molecular weight cut-off 20-80 kDa, Millipore Co., USA) to remove any sodium salt introduced eariler in the silk degumming process. The resulting desalted degumming solution was then concentrated at 40°C under vacuum or diluted in deionized water to obtain the required concentration of nitrogen in the silk degumming media. The pH of the media was adjusted by the addition of NaOH or HCl solutions, and then stored in glass bottles at 4°C until use.

Optimization studies

The following media were used with the compositions as shown below: Medium 1 (pH 10) (Potumarthi *et al.*, 2007) contained 1% casein, 1% malt extract, 1% polypeptone and 1% Na_2CO_3. Medium 2 (Patel *et al.*, 2005) contained 1.5% yeast extract, 1.5% peptone, 1% Na_2CO_3. Medium 3 contained 6% silk degumming solution, 1% Na_2CO_3. Medium 4 contained 6% silk degumming solution, pH 10. Medium 5 (adapted from Potumarthi *et al.*, 2007) contained 1% casein, 6% silk degumming solution, 1% Na_2CO_3. Medium 6 (adapted from Potumarthi *et al.*, 2007) contained 6% silk degumming solution, 1% malt extract, 1% polypeptone, 1% Na_2CO_3. The pH of all media as adjusted to 10.

For each medium, 50 ml was placed in a separate 250-ml Erlenmeyer flask. Cultivation was carried out for three days at 37°C with shaking at 180 rpm. Incubation periods ranged from one to six days and the incubation temperature was maintained at 37°C. The effects of total protein concentrations in the silk degumming solution (0, 1, 1.5, 3, 4.5, 6, 7.5, 9 and 10.5%) and the pH (5, 7, 9 and 10) of the fermentation medium were evaluated in relation to protein yields and protease activity. The experiments were conducted in duplicate and the results were the a averaged for each experiment. At the end of fermentation period, the culture media were centrifuged to obtain cell-free supernatant as the protease source.

Assay of proteolytic activity (Wang and Hesseltine, 1965)

Protease activity was measured using casein as a substrate. A 1.0 ml aliquot of suitably-diluted culture supernatant was incubated at 40°C for 5 min before mixing with a bicarbonate buffer (pH 10.0) containing 1% casein and incubated at 40°C for a further 10 min. The reaction was stopped by the addition of 3.0 ml 5% trichloroacetic acid (TCA). The mixture was allowed to stand at room temperature for 30 min and then filtered through Whatman No.1 to remove the precipitate. A 1.0 ml aliquot of filtrate was mixed with 5.0 ml 0.4 M sodium carbonate solution, before 0.5 ml 1 N Folin-Ciocalteu reagent was added, and then incubated at 37°C for 30 min. The absorbance of the supernatant was measured at 660 nm against a distilled water blank. Negative control was performed as previously described, except that 5% TCA was added before the 1% casein solution. A standard curve was generated using 0 to 100 µg/L tyrosine solution.

One unit of alkaline protease activity was defined as the amount of enzyme required to liberate 1 mg of tyrosine in 1 min under the experimental conditions.

Analytical method

The waste solution was characterized in terms of total Kjeldahl nitrogen (TKN), crude proteins, chemical oxygen demand (COD) and biological oxygen demand (BOD5). TKN was determined according to AOAC (2000). Crude proteins were estimated by multiplying the total nitrogen content by the factor 6.25. COD and BOD5 were analyzed using the closed reflux methods 5220C and 5210A, respectively, according to APHA-AWWA-WEF (1995). Protein content in the alkaline protease produced was measured using serum albumin (0.05 to 0.3 mg/ml) as a standard according to Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Characteristics of silk degumming waste solution

The characteristics of sericin waste solution are given in Table 1. It demonstrated high amounts of total nitrogen and protein (1,100 mg/L and 6,875 mg/L, respectively), which was considered to perhaps have been enough to be used as a nitrogen source for microbial growth and protease production. This was also reported by Souissi *et al.* (2007) with wastewaters from marine-products processing containing 1,289 mg/L total nitrogen that could be used as a sole

nitrogen source for protease production from several bacterial strains such as: *B. cereus* BG1; *B. subtilis* from the "Centre de Biotechnologie de Sfax-Tunisia", *B. licheniformis* from an activated sludge reactor treating fishing industry wastewaters; and *P. aeruginosa* from tannery wastewater. Analysis of the degumming waste solution also indicated high BOD5 and COD levels of 4,840 mg/L and 8,870 mg/L, respectively. Rigoni-Stern (1996) and Fabiani (1996) also reported similar results with high amounts of nitrogen residues, COD and BOD5, and therefore also considered that treatment was needed for this kind of waste water before discharge.

Preparation of silk degumming waste solution

A flow diagram for microbial growth media preparation from the silk degumming solution is shown in Figure 1. The waste solution was ultrafiltrated, concentrated and pH adjusted to accommodate the growth of microorganisms. Table 1 shows the characteristics of the degumming solution after ultrafiltration. It contained 10 times less nitrogen and protein content than the original solution. However, this might be beneficial for microorganisms as Souissi *et al.* (2007) also reported that 10 times dilution of marine-products processing wastewaters led to a two to eight times higher production of protease

Table 1 Composition of degumming waste solutions before and after filtration.

Parameters	Degumming solution	
	Before filtration	After filtration
TKN (mg/l)	1,100	100
Crude proteins (mg/l)	6,875	625
pH	9.24	7.20
COD (mg/l)	8,870	260
BOD_5 (mg/l)	4,840	158

Average values of three samples.

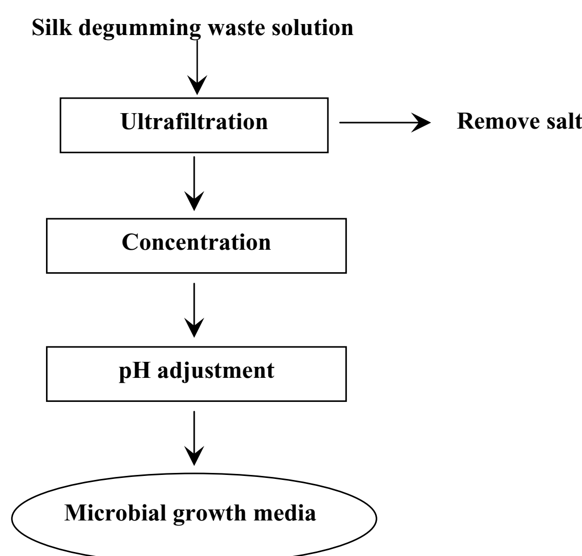

Figure 1 Flow diagram for the preparation of the silk degumming waste solution as a microbial growth media.

from *B. cereus* and *B. licheniformis*, respectively.

Comparison of protease production by different species

A comparative study of protease production by different strains was performed on casein-agar plates, where casein was the only nitrogen source available for microbial growth. The above procedure was qualitative and so was used only for the primary screening of the most efficient strains for further experiments. As a result (Table 2), *B. licheniformis* TISTR 1010 and *A. flavus* TISTR 3130 were selected due to their high values for the ratio of clear zoneto diameter to diameter colony.

Culture conditions for protease production

Effect of incubation period

Figure 2 shows the effect of incubation period on protease production for *A. flavus* TISTR 3130 and *B. licheniformis* TISTR 1010 in silk degumming media. It demonstrates that enzyme production by both strains steadily increased through the production time (1 to 6 days) and the 36 h incubation period gave the highest protein yield and protease activity for *A. flavus*

Table 2 Primary screening of microorganisms.

Strains	Colony diameter (cm)	Clear zone diameter (cm)	Ratio (clear zone/colony)
B. licheniformis TISTR 1010	1.0	1.8	1.8
A. flavus TISTR 3130	1.5	2.8	1.8
A. flavus TISTR 3041	1.6	2.5	1.5
A. flavus TISTR 3135	2.0	2.5	1.3
A. flavus TISTR 3366	2.1	2.7	1.3

Average values of three samples.

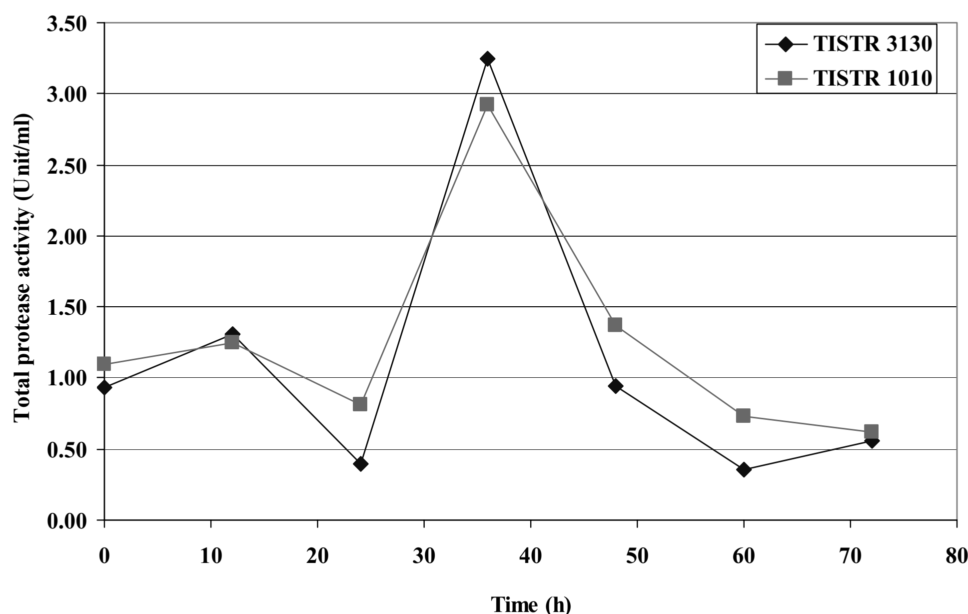

Figure 2 Effect of incubation period on protease production by *A. flavus* TISTR 3130 and *B. licheniformis* TISTR 1010 in medium 6 at 37°C with 180 rpm shaking. Average values of three samples.

TISTR3130 and *B. licheniformis* TISTR 1010 of 3.25 unit/ml and 2.92 unit/ml, respectively. Therefore, 36 h was used as the optimum time period for further experiments.

Effect of incubation pH

Figure 3 illustrates the effect of media pH on protease production by *A. flavus* TISTR 3130 and *B. licheniformis* TISTR 1010 after 36 h. It shows that protease production by the two strains was affected by the pH of the culture media and the optimum pH for protease production by *A. flavus* TISTR 3130 and *B. licheniformis* TISTR 1010 was at 3 and 10, respectively. The highest protease activity by *A. flavus* TISTR 3130 and *B. licheniformis* TISTR 1010 was 5.06 unit/ml and 6.34 unit/ml with protein concentrations at 257.48 mg/ml and 278.52 mg/ml, respectively. A similar response in protease production was also observed by Potumarthi *et al.* (2007), where the optimum pH for the highest protease activity by *B. licheniformis* was in the range between 9.5 and 10. As a result, *B. licheniformis* TISTR 1010 was chosen for the next experiment, as the strain survived and produced the most protease at pH 9.0 in the silk degumming waste, and thus no chemicals were needed for the pH adjustment of the medium.

Effect of culture media

Nitrogen, in both organic and inorganic forms, has been known to have some regulatory effects on enzyme production in microorganisms since it is used in the production of amino acids, nucleic acids and proteins (Patel *et al.*, 2005). Figure 4 illustrates the effect of culture media on protease production by *B. licheniformis* TISTR 1010 after 36 h incubation. It shows that the nitrogen sources used in this study supported growth as well as enzyme production. Among all media used, protease production ranged between 1.33 and 2.56 unit/ml. The optimum activity (2.56 unit/ml) was achieved with Medium 3 followed by 1, 5, 6, 2 and 4 (2.48, 2.35, 2.34, 1.89 and 1.33 unit/ml, respectively). However, the media containing silk degumming waste (media 4, 3, 5

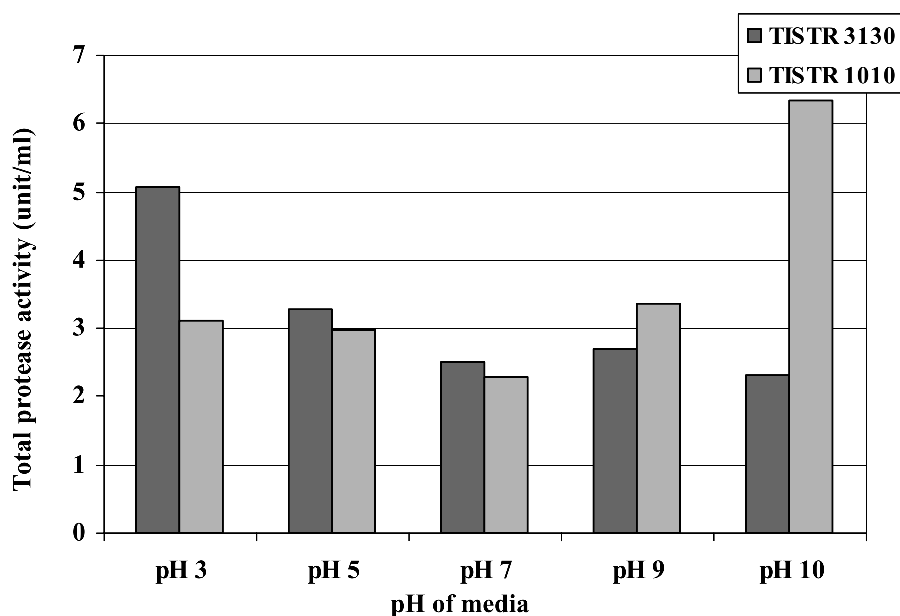

Figure 3 Effect of media pH on protease production by *A. flavus* TISTR 3130 and *B. licheniformis* TISTR 1010 in Medium 6 for three days at 37°C with 180 rpm shaking. Average values of three samples.

and 6) showed a greater influence on the protein production from *B. licheniformis* TISTR 1010 (76.31, 85.39, 55.41 and 52.49 mg/ml, respectively) than those without silk degumming waste (19.79 mg/ml from Medium 1 and 16.15 mg/ml from Medium 2). Therefore, the protein in silk degumming waste seemed to be a good nitrogen source for the microorganisms to promote protein production. The highest specific activity (0.125 unit/mg) was obtained from Medium 1. Specific activity from media 2, 6, 5, 3 and 4 were 0.117, 0.045, 0.042, 0.034 and 0.016 unit/mg, respectively. Thus, this indicated the nitrogen source influenced the protein production and protease activity by the microorganism. The study by Patel *et al.* (2005) also reported that peptone and yeast extract were the best nitrogen sources for maximum protein production and growth of *Bacillus* sp. Ve-1 when compared with other sources such as casamino acid, tryptone and caseitone. In contrast, the highest protease activity of *Bacillus* sp. Ve-1 was achieved when casamino acid was applied as a nitrogen source.

From these results, it could be concluded that the microorganism was able to consume protein in silk degumming waste as growth nutrient. However, this nitrogen source might not be approproiate for protease production. But since the purpose of this study was to utilize silk degumming waste for protease production, the medium containing silk degumming waste that gave the highest protease specific activity was chosen for the next experiment. Medium 6 proved to be the optimum medium for protease production by *B. licheniformis* TISTR 1010 and so it was further investigated at varied nitrogen concentrations in the next experiment.

Effect of nitrogen concentrations

Figure 5 illustrates the effect of silk protein concentration in the fermentation media on protease production by *B. licheniformis* TISTR 1010 after 36 h incubation. It was found that an increase of protein concentration (from 1 to 6%) in the fermentation media raised the enzyme content the from 15.22 to 111.88 mg/ml and its activity from 0.40 to 4.13 unit/ml, respectively.

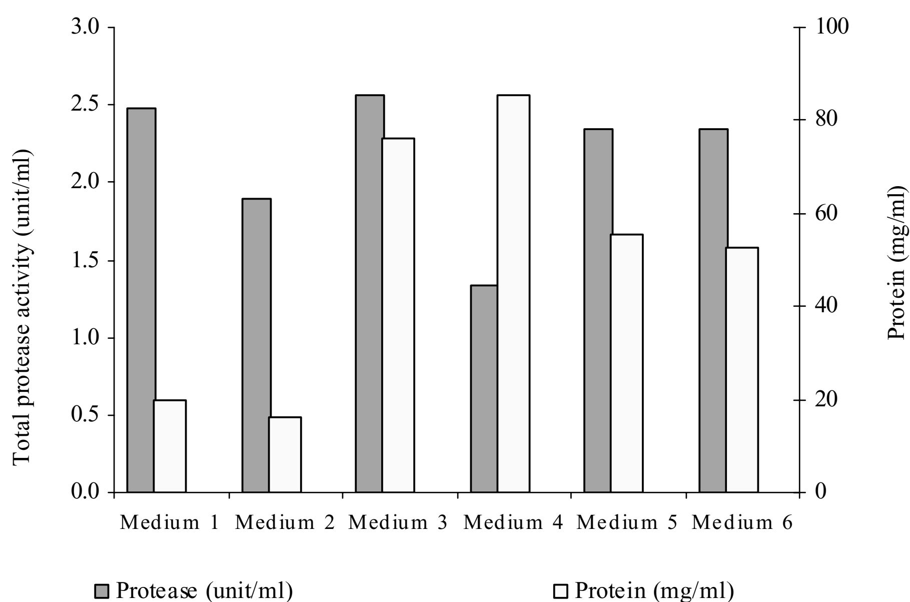

Figure 4 Effect of culture media on protease production by *B. licheniformis* TISTR 1010 for three days at 37°C with 180 rpm shaking. Average values of three samples.

However, when the protein concentration in the fermentation media reached 7.5%, it lowered the content and activity of protease to 63.30 mg/ml and 1.87 unit/ml, respectively and the protease production gradually decreased there after. This was also reported by Chauhan and Gupta (2004) where the production of protease by *Bacillus* sp. RGR-14 was repressed in the presence of high concentrations of a casamino acid protein source. Furthermore, Souissi *et al.* (2007) also demonstrated an induction or repression in protease production from *B. licheniformis*, *B. cereus* and *B. subtilis* by protein concentrations in the fermentation media prepared using waste water marine-products processing. However, when specific activity was calculated, the highest value of 0.044 unit/mg was obtained when 4.5% protein in the fermentation media was used. Thus, the optimum protein concentration using silk degumming waste for protease production by *B. licheniformis* TISTR 1010 was 4.5%.

CONCLUSION

This study reported for the first time on the use of a silk degumming waste solution as a microbial substrate for the protease production by the protease-producing strain *B. licheniformis* TISTR1010. The solution contained the organic and inorganic substances essential for microbial growth and protease production, such as sources of carbon and nitrogen. The addition of more silk degumming waste to the fermentation media decreased protease production. This effect could have been the result of the presence of unknown toxic compounds in the silk degumming waste at high concentrations. Based on this study, it appeared that by-products from the silk degumming process could be used in the fermentation substrate for protease-producing microorganisms. However, this experiment should be extended by investigating the presence optimization of the composition of the

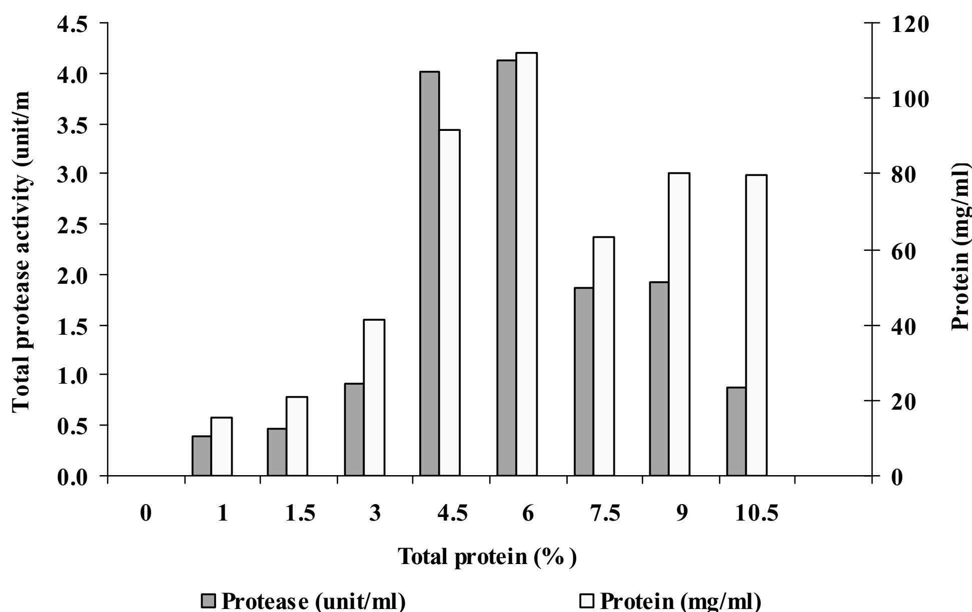

Figure 5 Effect of total protein in fermentation media (medium 6) on protease production by *B. licheniformis* TISTR 1010 for three days at 37°C with 180 rpm shaking. Average values of three samples.

fermentation media and the presence of toxic compounds in the silk degumming waste, to gain a better understanding of and thus improve the process of protease production.

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LITERATURE CITED

AOAC, 2000. **Official Method of Analysis of AOAC International. 17th ed**. Association of Official Analytical Chemists. Gaithersburg, Md.

APHA-AWWA-WEF., 1995. **Standard Methods for the Examination of Water and Wastewater, 20th ed**. American Public. Health Association, Washington, DC, NY.

Chang-Kee, H., J. Yuk-Hyun, L. Sung-Hee, P. Geum-Ju and C. Deock-Hyoung. 2002. Anticancer agents containing antigenotoxic and immunostimulative peptides produced from the hydrolysate of silkworm cocoon. **Patent No. WO02076487**.

Chauhan, B. and R. Gupta. 2004. Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. **Process Biochem**. 39: 2115-2122.

Fabiani, C., M. Pizzichini, M. Spadoni and G. Zeddita. 1996. Treatment of waste water from silk degumming processes for protein recovery and water reuse. **Desalination** 105: 1-9.

Kato, N., S. Sato, A. Yamanaka, H. Yamada, N. Fuwa and M. Nomura. 1997. Silk protein, inhibits lipid peroxidation and tyrosinase activity. **Biosci. Biotechnol. Biochem**. 62(1): 145-147.

Lowry, O.H., N.J. Rasebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with Folin Phenol reagent. **J. Biol. Chem**. 193: 256-275.

Patel, R., M. Dodia and S.P. Singh. 2005. Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and Optimization. **Process Biochem**. 40: 3569-3575.

Potumarthi, R., Ch. Subhakar, and J. Annapurna. 2007 Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: Effect of aeration and agitation regimes. **Biochem. Eng. J**. 34(2): 185-192.

Rigoni-Stern, S., L. Szpyrkowicz and F. Zilio-Grandi. 1996. Treatment of silk and Lycra printing wastewaters with the objective of water reuse. **Water Sci. Tech.** 33(8): 95-104.

Souissi, N., Y. Ellouz-Triki, A. Bougatef, M. Blibech and M. Nasri. 2007. Preparation and use of media for protease-producing bacterial strains based on by-products from Cuttlefish (*Sepia officinalis*) and wastewaters from marine-products processing factories. **Microbiol. Res**. In press.

Vaithanomsat, P. and V. Kitpreechavanich. 2008. Sericin separation from silk degumming waste water. **Sep. Pur. Tech**. 59: 129-133.

Wang, H.L. and C.W. Hesseltine. 1965. Studies on the extracellular proteolytic enzymes of *Rhizopus oligosporus*. **Can. J. Microbiol**. 11: 727-732.

Zhang, Y.Q., 2002. Applications of natural silk protein sericin in biomaterials. **Biotech. Adv**. 20(2): 91-1.

Zhang, Y.Q., M.L. Tao, W.-D. Shen, Y.Z. Zhou, Y. Ding, Y. Ma and W.L. Zhou. 2004. Immobilization of L-asparaginase on the microparticles of the natural silk sericin protein and its characters. **Biomaterials** 25: 3751-3759.