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DEGUMMING OF SILK FIBERS BY PROTEASE FROM *Bacillus subtilis*

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ABSTRACT

Four strains of *Bacillus subtilis* (M2, M14, M35 and M54) were selected for production of protease enzyme and degumming of silk fibers. The data revealed that, maximum protease activity and specific activity were obtained by *B. subtilis*(M14) followed by *B. subtilis*(M54) when grown in Chopra and Mathur medium at 30°C for 5 days under shake culture conditions. When the strains individually incubated in the medium supported with silk fiber, the loss of the gum might amount to 30.56% with high total activity of protease in the culture supernatants. Culture supernatants of four strains with high protease activity were most effective in reducing the gum content of silk fibers within 4 hr and slightly increase within 8 and 16 hr. Four grams silk fiber per 100 ml culture filtrate was the best ratio for the highest degumming by *B. subtilis* M14, M35 and M54 strains while 3:100 (w/v) was the best ratio for degumming by *B. subtilis* (M2). Degumming of silk fibers increased with the using of crude enzyme of *B. subtilis* (M14) followed by (M54) and (M2) strains at 30 °C, while *B. subtilis* (M35) gave the highest silk degumming at 50°C. Results showed that, maximum degumming and total activity of protease have been observed at pH 7.0 to all filtrates of the four strains. These results indicated that, protease of *B. subtilis* plays an active role in the degumming of silk fibers.

INTRODUCTION

Microorganisms have been continuously investigated for their protease activity. These enzymes have been found to be very widely distributed in bacteria. Neutral and alkaline proteinases are secreted as free enzymes outside the cell by many bacterial genera and species (Law, 1980).

Proteolytic enzymes produced by *Bacillus* species find a wide variety of applications, brewing, food processing, meat tenderization, pharmaceutical industries and in treatment of waste materials (Nakanishi *et al.*, 1974 and Gerhatz, 1990).

Silk is a fine continuous strand of protein. Silk fibers are lustrous and have high tensile strength, good draping properties, warmth, softness and durability. Silk fibers are composed mainly of an outer layer of amorphous protein called sericin (22-30%) and crystalline inner layer of protein called fibroin (62.5-67%), together with water and mineral salts. The removal of sericin (outer layer) from raw silk fiber is known as degumming. Degumming improves the luster and sheen of silk and improves the acceptance of silk for dyeing. The methods of degumming are classified according to the degumming agents employed. Traditionally, degumming may be carried out by boiling in soap or alkali solutions, boiling in dilute mineral or organic acids...etc. (Gulrajani, 1992). Degumming by chemical processes may lead to the degradation of silk filaments (Haque and Sharma, 2002).

On the other hand, much better results are obtained with the use of protease enzyme in the degumming by enzymatic processes. Protease

breaks down the sericin protein into water-soluble amino acids (Seves *et al.*, 1998, Forlani *et al.*, 2000 and Li *et al.*, 2003).

Therefore, *Bacillus* strains, which have been shown to produce an extracellular protease (in an earlier investigation by the authors), were used in this study and examined for silk degumming at much lower temperature that user in the traditional methods, which might not cause degradation of fibroin and keep the tensile force of the fiber.

MATERIALS AND METHODS

Silk fibers:

Fibers of silk used in this experiment were imported from china and purchased from local market (El-Azhar, Cairo).

Organisms:

Four protease producing *Bacillus subtilis* strains (M2, M14, M35 and M54) were kindly obtained from the Agric. Botany Dept. (Microbiology branch), Fac. Agric. Moshtohor, Zagazig Univ., Egypt and maintained on nutrient agar slants at 5°C and transferred monthly.

Growth medium:

The bacterial strains were grown on Chopra and mathur medium (1983), which was recommended for protease production and has the following composition: 1% tryptone, 0.25% yeast extract, pH 7.0.

Enzyme production:

The four bacterial strains were individually inoculated on 50 ml Chopra and Mathur medium in 250 ml Erlenmeyer flasks. After incubated at 30°C for 5 days, it was centrifuged for 30 min at 10,000 r.p.m., the supernatant was Seitz filtered and used as the crude enzyme solution for degumming of silk fiber. The filtrates containing the extracellular protease were also examined for protease activity and protein content.

Degumming:

Silk fibers were incubated at different temperatures (30, 40, 50, 60 and 70 °C) with 100 ml filtrate (as crude enzyme) at different pH levels namely, 4, 5, 6, 7, 8 and 9 with potassium phosphate buffer for different periods (30 min, 1 hr, 2 hr, 4 hr, 8 hr and 16 hr). Various substrate (silk fibers) concentrations (0.5, 1.0, 2.0,3.0, 4.0 and 5.0 gm) were incubated with 100 ml filtrate of each of the four strains of *Bacillus subtilis* (M2, M14, M35 and M54) to obtain the optimum conditions which cause the maximum degumming of silk fibers. Samples were taken and centrifuged for 1 min at 14,000 r.p.m. then protease activity was determined.

Enzyme assay:

A diluted crude enzyme solution (0.2 ml) was mixed with 2.5 ml of 1% casein in phosphate buffer pH 7.0 and incubated for 10 min at 30°C. The reaction was terminated by adding 5 ml of 0.19 M trichloroacetic acid. The

reaction mixture was centrifuged. The amount of trichloroacetic acid-soluble casein breakdown fragments using the method of Hindazlothink *et al.* (1983). One unit of protease activity was defined as the amount of enzyme required to release trichloroacetic acid soluble fragments giving blue colour equivalent to 1.0 μg of tyrosine under conditions of the assay. Protein assay was determined according the method of Lowry *et al.* (1951). Always blank assay without the substrate was performed and its absorption was subtracted from the enzyme assay figures.

- The pH was adjusted by the addition of acid phosphate and alkali phosphate and estimated by pH meter model "WHEATON 100".

Calculations:

-Degumming was estimated as follows:

$$\frac{\text{Dry weight of silk fiber before treat - Dry weight of silk fiber after treat}}{\text{Dry weight of silk fiber before treat}} \times 100$$

-Protease activity = units/ ml culture medium.

-Enzyme protein content = mg enzyme protein / ml culture medium (mg/ml).

$$\text{-Total activity of protease} = \frac{\text{Protease activity}}{\text{Enzyme protein content}} \text{ (Unit/mg)}$$

RESULTS AND DISCUSSION

Protease production and degumming:

Four strains of *B. subtilis* namely, M2, M14, M35 and M54 were tested for their proteolytic activity using Chopra and Mathur medium at 30°C for 5 days. As shown in Table (1), *B. subtilis* (M14) gave the maximal total activity of protease after 5 days followed by *B. subtilis* (M54) and (M35). While the lowest total activity of protease was obtained by *B. subtilis*(M2).

Degumming of silk fibers was illustrated in Table (2). Four strains of *Bacillus* were selected for the degumming of silk fibers and production of protease enzyme when the medium was supported with silk fiber as substrate. After 5 days of incubation, the weight loss of the gum reached to 30.56% by *B. subtilis* (M14). This strain gave the maximal total activity and protease activity. The lowest silk degumming (11.93%) was obtained by *B. subtilis* (M2). These results are in agreement with Cortez *et al.* (2002) who reported that the fibroin filaments of cocoon silk are naturally gummed together with the protein sericin. The latter comprises about 30% of the weight of the cocoon. The removal of sericin from raw silk is known as degumming. Enzyme degumming involves proteolytic degradation of sericin using a protease activity, which attack the peptide bonds of certain amino acids found in large amounts in sericin and found in small amounts in fibroin. Hence attack sericin and does not attack fibroin The filtrates of these cultures were used as the crude enzyme to complete this investigation.

Table (1): Proteolytic activity of four strains of *B. subtilis* grown on Chopra and Mathur medium after 5 days incubation.

Microorganisms	Protease activity (U/ml)	Protein content (mg/ml)	Total protease activity (U/mg)
B. subtilis (M2)	43.289	0.1899	227.95
B. subtilis (M14)	49.762	0.1048	474.82
B. subtilis (M35)	57.138	0.1812	315.33
B. subtilis (M54)	41.576	0.1014	410.01

Table (2): Effect of extracellular protease of *B. subtilis* strains grown on Chopra and Mathur medium on degumming of silk fiber and proteolytic activity.

Microorganisms	Protease activity (U/ml)	Protein content (mg/ml)	Total protease activity (U/mg)	Degumming of silk fiber (%)
B. subtilis (M2)	40.941	0.1278	320.35	11.93
B. subtilis (M14)	56.992	0.1324	430.33	30.56
B. subtilis (M35)	48.335	0.1229	393.38	18.57
B. subtilis (M54)	49.941	0.1220	409.28	20.58

Effect of time on the degumming:

The enzyme activity and the degumming by filtrate of four strains of *B. subtilis* were estimated during different periods (30 min, 1, 2, 4, 8 and 16 hr). The data shown in Table (3) indicate that, the weight loss of silk increased as well as total protease activity with increasing of incubation period up to 4 hrs for all filtrates of the four strains. Even when the incubation period was extended to 8 and 16 hrs degumming of silk and total activity of protease were still slightly increasing. Maximum silk degumming and total protease activity were obtained by filtrate of *B. subtilis* (M14) followed by filtrate of *B. subtilis* (M54). These results are in agreement with those obtained by Zheng *et al.* (2001) who found that the residual gum of the fibers decreased after 5 hr of enzymatic degumming.

Effect of substrate concentrations (g/100 ml filtrate) on degumming:

It is clear from the data presented in Table (4) that, silk fiber concentration has a profound effect on silk degumming and total protease activity. The weight loss of silk gum increased with the increase of silk fiber (substrate) level in 100 ml culture filtrate of the four strains up to certain level and decreased thereafter.

Three grams of silk fiber per 100 ml filtrate was the best substrate level for *B. subtilis* M2 while *B. subtilis* M14, M35 and M54 gave the highest degumming when substrate concentration was 4 g /100 ml filtrate, then a slight drop in the degumming was noticed with the increase in substrate concentration. Maximum degumming was obtained with using filtrate of *B. subtilis* (M14). However, active enzyme concentration increased with increasing of substrate concentration for all filtrates.

Table(3): Effect of culture filtrate of *Bacillus subtilis* strains grown on Chopra and Mathur medium for 5 days at various periods on silk degumming and total protease activity.

Degumming periods	*B. subtilis* (M2)		*B. subtilis* (M14)		*B. subtilis* (M35)		*B. subtilis* (M54)	
	Degumming of silk fiber (%)	Total protease activity (U/mg)	Degumming of silk fiber (%)	Total protease activity (U/mg	Degumming of silk fiber (%)	Total protease activity (U/mg	Degumming of silk fiber (%)	Total protease activity (U/mg
30 min	4.21	75.38	6.42	78.35	3.93	58.7	4.62	59.94
1 hour	5.76	94.23	7.83	101.98	6.00	69.34	5.69	72.57
2 hour	8.15	114.54	22.52	136.87	12.35	110.64	17.81	137.41
4 hour	12.45	121.37	24.15	158.31	14.74	124.35	20.82	142.31
8 hour	13.08	129.62	24.65	170.63	15.26	129.17	21.50	152.63
16 hour	13.75	134.86	26.87	181.15	17.98	140.26	23.71	167.46

Table (4): Effect of culture filtrate of *Bacillus subtilis* grown on Chopra and Mathur medium for 5 days on silk degumming and total protease activity with different levels of substrate.

Substrate/ 100ml filtrate* (gm)	*B. subtilis* (M2)		*B. subtilis* (M14)		*B. subtilis* (M35)		*B. subtilis* (M54)	
	Degumming of silk fiber (%)	Total protease activity (U/mg	Degumming of silk fiber (%)	Total protease activity (U/mg)	Degumming of silk fiber (%)	Total protease activity (U/mg	Degumming of silk fiber (%)	Total protease activity (U/mg
0.5 gm	6.11	62.09	7.08	91.57	5.54	65.28	6.54	69.57
1.0 gm	8.50	74.28	14.29	124.37	6.91	79.46	9.07	118.07
2.0 gm	13.14	131.23	20.04	197.18	15.23	129.04	16.65	148.72
3.0 gm	19.38	167.91	21.01	226.01	18.93	228.49	20.09	151.30
4.0 gm	16.05	179.62	29.80	321.78	22.71	239.78	26.31	276.52
5.0 gm	16.24	185.58	28.27	345.39	20.16	243.11	26.77	298.75

* 1ml filtrate of strain (M2) = 320.33 U/mg protein.
* 1ml filtrate of strain (M35) = 393.38 U/mg protein.
* 1 ml filtrate of strain (M14) = 430.33 U/mg protein.
* 1 ml filtrate of strain (M54) = 409.28 U/mg protein.

Effect of temperature on silk fiber degumming by crude protease enzyme (culture filtrate):

The reaction mixtures containing filtrates of each of the four strains as crude enzyme and silk fiber were incubated at different temperatures ranging between 30°C and 70°C.

Results illustrated in Table (5) show that the enzyme exhibited maximum silk degumming and enzyme activity when incubation was at 30°C for filtrates of *B. subtilis* M2, M14 and M54. At higher temperature, the degumming of silk fiber decreased as well as total protease activity. Schinner *et al.* (1991) reported that the optimum temperature for protease activity was 30°C. Maximum silk degumming and total enzyme activity were obtained by *B. subtilis* (M35) filtrate at incubation temperature of 50°C for 4 hrs. Yang *et al.* (2000) reported that optimum temperature for the protease of *B. subtilis* was found to be 50°C. This variation may possibly be due to variations in strains characteristics.

Effect of pH on the protease activity and degumming:

The effect of pH on the catalytic activity of protease and the loss of silk gum under the standard assay condition by using silk fiber as the substrate are presented in Table (6).

It could be seen that, silk degumming and total protease activity increased as the pH increased, reaching the maximum at pH 7.0 for all filtrates of *B. subtilis*. Further increase in pH caused a sharp decline in the degumming percentage of silk fiber and the total activity of protease enzyme. Filtrate of *B. subtilis* (M14) gave the highest value of degumming followed by *B. subtilis* (M54) filtrate. These results are similar to those reported by Stepaniak *et al.* (1982), Choorit and Parsertsan (1992) and Shady and Abdel-Razik (1996). They found that pH 7.0 was the optimum pH for protease activity by *B. subtilis*.

In this study, the authors have demonstrated that the crude protease produced by *Bacillus subtilis* can be utilized for degumming of silk fiber as enzymatic process that does not attack the fibroin unlike traditional degumming methods in which soap, alkali and acids are used

Table(5): Effect of culture filtrate of *Bacillus subtilis* grown on Chopra and Mathur medium for 5 days on silk degumming and total protease activity when incubated at different temperatures.

Temperature	*B. subtilis* (M2)		*B. subtilis* (M14)		*B. subtilis* (M35)		*B. subtilis* (M54)	
	Degumming of silk fiber (%)	Total proteaas activity (U/mg)	Degumming of silk fiber (%)	Total protease activity (U/mg)	Degumming of silk fiber (%)	Total protease activity (U/mg)	Degumming of silk fiber (%)	Total protease activity (U/mg)
30 °C	18.25	131.04	25.34	200.38	14.42	132.80	23.18	190.54
40 °C	18.17	125.51	20.41	190.93	16.98	172.94	18.79	181.38
50 °C	10.57	112.34	15.00	122.53	30.98	185.41	13.48	120.74
60 °C	9.36	109.79	15.12	111.42	11.65	107.12	13.17	113.58
70 °C	5.20	69.22	8.43	89.92	5.27	66.35	6.08	74.18

Table(6): Effect of culture filtrate of *Bacillus subtilis* grown on Chopra and Mathur medium for 5 days on silk degumming and total activity of proteases, at different pH levels.

pH	*B. subtilis* (M2)		*B. subtilis* (M14)		*B. subtilis* (M35)		*B. subtilis* (M54)	
	Degumming of silk fiber (%)	Total protease activity (U/mg)	Degumming of silk fiber (%)	Total protease activity (U/mg)	Degumming of silk fiber (%)	Total protease activity (U/mg)	Degumming of silk fiber (%)	Total protease activity (U/mg)
4	8.24	81.02	12.59	120.31	7.05	84.76	9.27	97.64
5	11.18	100.84	16.22	134.85	10.19	119.68	12.61	128.19
6	18.38	122.53	20.64	169.84	15.28	157.11	17.95	167.13
7	20.73	161.06	26.75	276.70	23.93	195.36	25.46	204.24
8	19.74	138.41	23.52	217.14	18.71	185.05	20.51	198.88
9	11.00	93.31	12.28	130.90	9.48	106.79	11.18	124.68

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التخلص من صمغ خيوط الحرير بواسطة إنزيمات البروتييز المنتجة من بكتيريـــا *Bacillus subtilis*

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استخدمت فى هذا البحث أربعة سلالات من بكتيريا *B. subtilis* وهى *B. subtilis* (M2) و *B. subtilis* (M14) و *B. subtilis* (M35) و *B. subtilis* (M54) فى إنتاج انزيـــم البروتيـــيزوكذلك التخلص من صمغ خيوط الحرير. وأثبتت النتائج أن:

- بتنمية هذه السلالات على بيئة Chopra and Mathur على درجة حرارة ٣٠ °م لمـــدة ٥ أيام تم الحصول على أقصى نشاط لإنزيم البروتييز بواسطة السلالة *B. subtilis* (M14) ثـــم تلى ذلك السلالة *B. subtilis* (M54) .
- بعد خمسة أيام من تحضين السلالات في بيئة الإنتاج والمزودة بخيوط الحرير، كـــان الفقـد فـــي صمغ الحرير قد وصل إلى ٣٠,٥٦% لراشح بكتيريا *B. subtilis* (M14) مع نشـــاط عـــالى لإنزيم البروتييز فى راشح المزرعة.
- استخدم راشح المزارع الأربعة كل على حده فى إزالة صمغ الحرير على فترات مختلفـــة وكـــان التحضين لمدة ٤ ساعات قد أعطى زيادة فى معدل نشاط انزيم البروتييز وكذلك فى معدل ازالـــة الصمغ لخيوط الحرير. ثم كانت هناك زيادة طفيفة بعد ٨ ، ١٦ ساعة.
- كانت أفضل نسبة بين خيوط الحرير (مادة التفاعل) و راشح البكتيريا فى نشاط الانزيـــم وإزالـــة الصمغ هى ٤جم: ١٠٠مل راشـــح بالنســـبة لســـلالات *B. subtilis* (M14) و (M35) و(M54) أما السلالة (M2) فكان أفضل نسبة هى ٣جم : ١٠٠مل راشح.
- أعطى راشح السلالة *B. subtilis* (M14) زيادة فى معدل النقص فى صمغ الحرير تلى ذلـــك السلالة (M54) ثم السلالة (M2) وذلك على درجة حرارة تحضين ٣٠°م أما الســـلالة (M35) فاعطت أعلى معدلات الإزالة لصمغ الحرير على درجة حرارة تحضين ٥٠°م.
- أوضحت النتائج أيضا أن معدلات نشاط انزيم البروتييز وكذلك معدل التخلص من صمغ الحريـــر كانت واضحة عند تركيز أيون الهيدروجين ٧ لراشح السلالات الأربعة.
- وعموما فقد أوضحت النتائج أن البروتييز الناتج من بكتيريا *B. subtilis* يمكن أن يلعـــب دورا نشطا ومهما فى التخلص من صمغ الحرير ليعطى اللمعة والنعومة والكفاءة العالية فى الصباغة.