Global Journal of Biotechnology & Biochemistry 4 (2): 132-137, 2009
ISSN 2078-466X

Production of Protease in Low-Cost Medium by *Bacillus subtilis* KO Strain

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Abstract: *Bacillus* subtilis KO strain was isolated from molasses that obtained from the industrial products of Kom Ombo sugar factory. Different experiments were performed to optimize the cultivation conditions of *Bacillus* subtilis KO strain and the medium contents to reduce the cost of protease production. Optimum protease production was from 30-32 I.U recorded at 48 hr. incubation period, at 40-45°C, pH ranged from 7-7.5, 0.1% (w/v) gelatin concentration in the presence of ammonium phosphate as inorganic source. From the available present data, it could be recognized that this study may be the unique to use molasses not only to isolate *Bacillus subtilis* KO strain but also to grow and maintain it. Moreover, it used as a medium contain only molasses to produce considerable amount of protease.

Key words: Molasses · *Bacillus subtilis* · Protease production

INTRODUCTON

Molasses is an interesting raw material, it is rich in nutrients and minerals, cheap in price as well as it is present in plenty hence a by-product in sugar industries. Different microorganisms have been isolated from molasses sugar cane e.g. lactic acid bacteria and yeasts, thermophilic alkaliphilic *Bacillus sp* JB-99; *Lactobacillus plantarum* [1-2]. Molasses as nutrient medium can be used as a relatively inexpensive and economic alternative to synthetic medium for the production of gentamicins by *Micromonospora purpurea*, biosurfactant by *Lactococcus lactis* 53 and *Streptococcus thermophilus* A, erythromycin by *Saccharopolyspora erythraea*, uricase by *Bacillus thermocatenulatus*, scenic acid by *Actinobacillus succinogenes*, polyhydroxybutyric acid by *Bacillus megaterium*, acetone, butanol and ethanol by *Clostridium beijerinckii* BA101 [3-5]. Proteolytic enzymes catalyze the peptide bonds in proteins so they are necessary for the life of living organisms, being found in wide diversity such as plants, animals and microorganisms [6]. They are being used in a wide variety of applications such as detergents industry, leather processing industry, dairy and food industry, the manufacture of pharmaceutical products, the industry of proteins hydrolysis and peptide synthesis and texture industry [7].

Protease is the single class of enzymes which occupy an important position with respect to their applications in both physiological and commercial fields, thus, it was the objective of the present study to investigate the production of protease in low-cost medium by *Bacillus subtilis* K.O.

MATERIALS AND METHODS

Organism Used in the Study: KO strain which isolated from molasses that obtained from the industrial products of Kom Ombo sugar factory was used. According to the morphological, physiological and biochemical characteristics and the data of partial sequence of 16S rRNA, the used isolate was identified as a strain of *Bacillus subtilis.*

Media Used: Molasses liquid medium (Molasses 100ml Dist. H_2O up to 1000ml) and molasses agar medium (molasses liquid medium supplemented with 2% agar) were used for *Bacillus* subtilis KO strain isolation, purification and maintenance as well as the production and the optimization of protease.

Determination and Assay of Protease Production: Protease production was determined and assayed using two different methods, gelatin-cup plate clearing zone (GCZ) and spectrophotometric techniques [8].

Determination of Protein Content: The protein content was determined as an indication of protease production [9].

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Factors Affecting Protease Production by *Bacillus subtilis* KO Strain: Tested cultivation conditions of factors affecting protease production by *Bacillus subtilis* KO strain were: incubation period; incubation temperature and pH. Protease production was assayed in the cell free supernatant using GCZ and spectrophotometric techniques and determination of its protein content.

Effect of Different Inorganic Nitrogen Sources: *Bacillus subtilis* KO strain was grown on the production medium supplemented with % (w/v) of various inorganic nitrogen sources: ammonium phosphate, ammonium oxalate, ammonium acetate, sodium nitrate, ammonium chloride and ammonium sulphate.

Effect of Different Substrate Concentration (Gelatin): This was determined by growing *Bacillus subtilis* KO strain using different gelatin concentrations (0.05, 0.1, 0.5, 1, 2, 3, 4 and 5(%, w/v)). For each gelatin concentration, the protease production was assayed.

RESULTS AND DISCUSSION

In the present study different experiments were performed to optimize the cultivation conditions and the medium contents in order to reduce the cost of protease production. Molasses was used to isolate *Bacillus subtilis* KO strain and also for the enzymes production. The two used media were tested regarding their ability to get highest production of protease. Molasses liquid medium showed the highest production. This has the advantage to reduce the production cost not only for protease but also of many economic products. Many workers used the natural sources as one of the medium constituents e.g. rice bran, soybean, wheat flour, wheat bran, corn bran, corn starch orange peels and puples to support growth of different bacteria to produce different enzymes [10-11].

The effect of incubation period, temperature, pH values, substrate concentration and inorganic nitrogen sources on protease production and total protein content were studied in all free supernatant using the clear zone technique at 40ºC. The effect of different factors on enzyme production was performed using the two media; Molasses liquid and molasses agar media.

Factors Affecting Protease Production by *Bacillus subtilis* KO Strain: Data presented in Tables (1 and 2) and Figers (1 and 2) indicate the effect of incubation periods, temprature and pH on protease production and the total protein content in the cell free supernatant.

Incubation Period: It was found that the incubation period required for the maximum protease production was the same to obtain the maximum protein content (Tables 1 and 2; and Figure 1).

The present *Bacillus subtilis* KO strain exhibited its maximum production of protease within 48 hours incubation period (32 I.U.). This was in correlation with the findings of other workers, maximum protease production was observed after 48 hour incubation period for *Bacillus* sp. 103, *Bacillus subtilis* PE-11, *Bacillus subtilis* IKBS-10, *Bacillus* sp. I-312 and *Bacillus licheniformis* LBBl-11 [11-12].

Maximum protein content of the crude protease enzyme was obtained in molasses liquid medium after 48 hour incubation was 989 µg/ml liquid medium.

Fig. 1: Protease production after 48 hr incubation period as showed by CZT (mm diameter) on left and control on right

Fig. 2: Protease production at 40 °C on molasses liquid medium (left) compared with control (right)

Table 1: The effect of incubation periods, temprature and pH on protease production

	Protease production (I.U)		Protease production (I.U)		Protease production (I.U)
Incubation period (hour)	I.U	Temperature (ºC)	I.U	pH	I.U
6	0.0	25	18	4.5	0.0
12	1.75	30	20	5	0.0
24	27	35	24	5.5	25
36	30	40	30	6	27
48	32	45	30	6.5	28
60	28	50	0.0	7	30
72	27	-	-	7.5	30
-	-	-	-	8	25
-	-	-	-	8.5	20

Table 2: The effect of incubation periods, temprature and pH in the total protein content

	TPC		TPC		TPC
Incubation period(hour)	(µg/ml liquid medium)	Temperature (°C)	(µg/ml liquid medium)	pH	(µg/ml liquid medium)
6	121	25	100	4.5	0.0
12	226	30	675	5	1073
24	538	35	991	5.5	1362
36	758	40	1070	6	1756
48	989	45	1140	6.5	1700
60	385	50	0.01	7	1643
72	121	-	-	7.5	686
-	-	-	-	8	168
-	-	-	-	8.5	70

Temperature: There was no doubt that temperature was an important environmental factor that affects the growth of microorganisms as well as production of enzymes. *Bacillus subtilis* KO strain exhibited its maximum production of protease was 30 I.U. between 40 and 45 ºC (Tables 1 and 2 and Fig. 2). This was correlated with the results obtained by other workers, whom found that the maximum protease production occurred at 45 ºC for *Bacillus thermoruber*, thermophilic *Bacillus* sp. and *Bacillus horikoshii* respectively [13].

Table 3: The effect of substrate concentration and inorganic nitrogen source on protease production

Gelatin concentration (%, w/v)	Protease production (I.U)	Inorganic Nitrogen sources	Protease production (I.U)
Control	28	Control	30
0.05	28	Amm. phosphate	30
0.1	30	Amm. acetate	28
0.5	30	Amm. oxalate	27
1	30	Amm. chloride	25
2	30	Sodium nitrate	24
3	30	Amm. sulfate	20
4	27	-	-
5	25	-	-

Table 4: The effect of different gelatin concentration and different inorganic nitrogen source in the total protein content

Gelatin (%, w/v)	TPC(µg/ml liquid medium)	Inorganic nitrogen source	TPC (µg/ml liquid medium)
Control	983	Control	1780
0.05%	981	Amm. phosphate	1819
0.1%	1082	Amm. acetate	1632
0.5%	1122	Amm. oxalate	1486
1%	1121	Amm. chloride	1116
2%	1128	Sodium. nitrate	811
3%	1137	Amm. sulfate	635
4%	1261	-	-
5%	1351	-	-

Total protein content in the supernatant (crude enzyme) was obtained on molasses liquid medium (Table 2 and Fig. 2) at a temperature range between 40 and 45°C were 1070 and 1140 µg/ml liquid medium, respectively .

pH: *Bacillus subtilis* KO strain exhibited its maximum protease production (30 I.U.) and protein content (1756 µg/ml liquid medium) at a pH ranged 7-7.5 (Tables 1 and 2). This is in complete accordance with the findings of many workers. The optimum pH for the maximum protease production were 7.5 by *Bacillus subtilis*, 7.2 by *Bacillus subtilis* k11 and *Bacillus licheniformis*, 6.9 by *Bacillus* sp. SMIA-2 and 7 by *Bacillus* sp. TKU004 [14 -16].

The Effect of Substrate Concentration and Inorganic Nitrogen Source: Data obtained in Tables 3 and 4 was revealed the effect of substrate concentration and inorganic nitrogen source on protease production and total protein content in the cell free supernatant. It was found that gelatin enhanced protease production when was added to at a concentration 0.1% (w/v) as recorded in Table (3). The results were revealed that the maximum protease production was 30 I.U. at 0.1% (w/v). This result was in complete accordance with the finding of other workers whom found that the maximum protease production by *Bacillus* sp. was exhibited with gelatin concentration between 1.5 %, (w/v) and 2 %, (w/v) and 1%, (w/v) for *Bacillus anthracis* S-44 and *Bacillus sp*. K 30 [17] ; 0.5 %, (w/v) for *Bacillus firmus* 7728 [18].

Other workers, used molasses to enhance protease production as one of the medium constituents. Maximum protease production by *Streptomyces* sp. 594 occurred by using molasses (0.3 %) as one of the important medium constituents was observed [19].

Gelatin has nearly no or even slight effect on protease production when molasses liquid medium was used. Maximum protein content was on molasses liquid medium. Moreover, protein content was increased by increasing of substrate concentration and showed the highest protein content (1819 µg/ml liquid medium) in the presence of ammonium phosphate as inorganic nitrogen source.

The importance of the inorganic nitrogen source for the production purpose of bacterial protease by *Bacillus subtilis* KO strain was evaluated by introducing different inorganic nitrogen sources ammonium phosphate, ammonium oxalate, ammonium acetate, ammonium chloride, ammonium sulphate and sodium nitrate into the two production media. Results of Table 3 exhibited the fact that ammonium acetate and ammonium oxalate has a slight inhibitory effect. Ammonium phosphate enhances or even no effect on protease production and total protein

content. Similar effect of ammonium phosphate on protease production was observed when protease was produced by *Bacillus cereus* and *Bacillus anthracis* S-44, thermophilic *Bacillus* sp., *B. sphaericus* B-5, *B. subtitis* B-6 and *B. cereus* B-10 and *Conidiobolus coronatus* [20-22].

Interestingly the fact that *Bacillus subtilis* KO strain succeeded to produce protease in the absence of any of the inorganic nitrogen source supplied to the molasses liquid medium. This was in complete accordance with the result obtained by [23-26].

The results recorded in Table (3) showed that there is no effect on protease production in the presence of ammonium phosphate, while the addition of the other inorganic nitrogen sources were inhibited protease production on molasses liquid medium. Moreover, ammonium sulphate and sodium nitrate inhibited protease production in the used media. From the resulte recorded in Table 4, it was found that the maximum total protein content was obtained in molasses liquid medium when ammonium phosphate added (1819 µg/ml liquid medium), while there is no considerable enhancement of production as a result of adding the other inorganic nitrogen source to molasses liquid medium. From the available present data, it could be recognized that this study may be the unique to use molasses not only to isolate the bacterial *Bacillus subtilis* KO strain , but also to grow and maintain it. Moreover, it used molasses as a natural medium to produce considerable amount of protease.

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