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

Mohamed S.A. Shabeb, Magdi A.M. Younis, Francis F. Hezayen and Moustafa A. Nour-Eldein

Department of Botany, Faculty of Science, South Valley University, 81528 Aswan, Egypt

Abstract: Different factors were tested for cellulase productivity by *Bacillus subtilis* KO strain using Carboxymethyl cellulose clear zone (CMCZ) and dinitro salicylic acid (DNSA) techniques. Cellulase enzyme revealed its best production; 32 I.U. by CMCZ technique and 228 μg/ml broth medium by DNSA technique as well as 525 μg/ml broth medium of protein content after 24 h. incubation period. At 45°C, cellulase productivity was 36 I.U. by CMCZ and 344 μg/ml broth medium by DNSA with 301 μg/ml broth medium of protein content. Moreover, maximum cellulase productivity was 35 I.U. by CMCZ and 420 μg/ml broth medium by DNSA with 601 μg/ml broth medium protein content when molasses broth medium was supplemented with cellulose and trypton. Our findings make the advantage of molasses to reduce the production cost not only for cellulase but also to shed a new light on the economic value of the yielded product.

Key words: Molasses · Production of cellulase · *Bacillus subtilis* KO strain

INTRODUCTON

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*, succinic acid by *Actinobacillus succinogenes*, polyhydroxybutyric acid by *Bacillus megaterium*, acetone, butanol and ethanol by *Clostridium beijerinckii* BA101 [1, 2]. Synthesis of bacterial extracellular enzymes has been of interest to many scientists [3]. Cellulose is the primary product of photosynthesis in plant biomass and the most abundant renewable bioresource produced in the biosphere [4, 5]. Cellulose is a linear polymer consisting of D-anhydrogluco pyranose molecules joined together by β-1,4 glycosidic bonds with a degree of polymerization [6, 7]. Cellulose degrading organisms have been used to convert cellulose materials into soluble sugars or solutions that have several biotechnological and industrial applications. Most commercial cellulose is of fungal origin and produced by *Trichoderma* species and *Aspergillus* species. However, pure cellulases from bacteria have not yet been commercially produced and standardized enzyme preparations are not available [8, 9]. Recently some bacteria, *Clostridium thermocellum*, *Clostridium cellulolyticum*, *Clostridium cellulovorans*, *Clostridium josui*, *Ruminococcus albus* and the actinomycetes, *Thermoactinomycetes* sp., *Thermomonospora curvata* and *Streptomyces* sp. have also been reported as cellulose producers using different substrates e.g. cellulose, carboxymethylcellulose, starch and glucose as carbon sources [10-12]. Cellulases are relatively costly enzymes and a significant reduction in cost will be important for their commercial use in biorefineres. Most commercial cellulases including β-glucosidases are produced by *Trichoderma* species and *Aspergillus* species [8]. The aim of the present study was to investigate production of cellulase in low - cost medium by *Bacillus subtilis* KO.

MATERIALS AND METHODS

Organism Used in the Study: *Bacillus subtilis* KO strain which isolated from molasses that obtained from the industrial products of Kom Ombo sugar factory was used [13].

Media Used: Molasses agar medium, molasses cellulose minerals broth medium and carboxy methyl cellulose minerals agar medium were used for the production and optimization of cellulase productivity.

Corresponding Author: Dr. Mohamed S.A. Shabeb, Department of Botany, Faculty of Science, South Valley University, 81528 Aswan, Egypt

Cellulase Production: Cellulase was produced by growing *Bacillus* subtilis KO strain on molasses cellulose minerals broth medium for 24 h at 40°C. Then the free cell supernatant was obtained and cellulase was assayed.

Assay of Cellulase Productivity: Cellulase in the cell free supernatant was assayed by the following two techniques: Carboxymethyl cellulose clear zone (CMCZ) technique according to [14] and dinitro salicylic acid (DNSA) technique according to [15].

Factors Affecting Cellulase Productivity: Cellulase productivity was assayed in the cell free supernatant using CMCZ and DNSA techniques as well as measurement of protein content under different growth factors. The tested factors that affecting cellulase productivity by *Bacillus subtilis* KO strain were: incubation period, incubation temperature, different carbon sources and different nitrogen sources.

Determination of Protein Content in the Enzymatic Preparations: The protein content was determined as an indication of cellulase productivity according to [16].

Effect of Incubation Period: Different incubation periods were applied i.e., 6, 12, 24, 48, 72, 96 and 120 hours.

Effect of Temperature: Different temperatures were applied i.e., 25, 30, 35, 40, 45 and 50°C.

Effect of Carbon Source: Different carbon sources; molasses, cellulose, glucose, mannose, lactose, starch and maltose (1g/l, w/v) was added separately as a sole carbon source.

Effect of Nitrogen Source: Molasses cellulose minerals broth medium supplemented with (2 g/l) peptone, alanine, albumin, sodium nitrate or ammonium nitrate separately as a sole nitrogen source.

RESULTS AND DISCUSSION

In the present study, different experiments were performed to optimize the cultivation conditions and the medium contents to reduce the cost of cellulase production. Molasses agar medium, molasses cellulose minerals broth medium and carboxy methyl cellulose minerals agar medium were used for the production and optimization of cellulase productivity by *Bacillus subtilis* KO strain.

The used media were tested regarding their ability to get high productivity of cellulase. Molasses media showed the high productivity. This had the advantage to reduce the production cost not only for cellulase but also of its economic value. 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 [17, 18].

Most of cellulase studies were on fungal cellulase e.g. *Trichoderma harzianum* Rut-C 8230 and *Trichoderma reesei* [19, 20], *Acremonium cellulolyticus* [21]. There are limited studies on bacteria that reported as cellulase producers e.g. *Clostridium thermocellum* [22] *Clostridium cellulolyticum* [23] *Clostridium cellulovorans* [24] *Clostridium josui* [25] *Ruminococcus albus* [10] *Actinomycetes*[11] *Thermoactinomycetes* sp. [26] a mutant of *Bacillus pumilus* BpCRI6 and *Bacillus pumilus* EB3 [27]. It has been demonstrated that bacterial cellulase can be applied to a great extent in different scopes e.g. economical production of ethanol and other chemical industries [28, 29] food and pharmaceutical industries [30].

Factors Affecting Cellulase Productivity: Different factors affecting cellulose productivity by *Bacillus subtilis* KO strain e.g. incubation period, temperature, molasses concentration, carbon sources and nitrogen sources in accordance with protein content were tested. Productivity was tested in the cell free supernatant after 48 h incubation at 45°C. Cell free supernatant (crude enzyme) was harvested and was incubated for 12 hours in test plates. Cellulase productivity was determined by applying the two methods CMCZ and DNSA techniques. The protein content was also determined as an indication of cellulase productivity.

Effect of Incubation Period: Data presented in Table 1 and figures 1 showed the effect of different incubation periods on cellulase productivity by *Bacillus subtilis* KO strain. From the recorded results, it was found that cellulose enzyme revealed its best production after 24 h of incubation period either by CMCZ technique (32 I.U.) or by DNSA technique (228 μg/ml broth medium). On the other hand, The protein content also showed its high amount after 24 h of incubation period (525 μg/ml broth medium). These results were in correlation with the findings of other workers, whom recorded maximum cellulase productivity after 24 h incubation by

Fig. 1: Cellulase productivity by *Bacillus subtilis* KO strain in mm within 48 hours as shown by CMCZ technique

Fig. 2: Cellulase productivity by *Bacillus subtilis* KO strain at 45°C as shown by CMCZ technique

Pseudomonas flourescens NCIB [31]. However, this was in contrast with the finding of many other workers, whom recorded maximum cellulase productivity after 36 h by *Arachnoitus* sp. [32], 72 hours by *Bacillus* spp. B21, *Bacillus pumilus* and *Bacillus subtilis* [33, 34], 120 h by *Bacillus pumilus* [35] and 142 h incubation by *Clostridium cellulolyticum* [36].

Effect of Temperature: Table 2 and Fig. 2 showed the effect of different temperatures on the cellulase productivity. *Bacillus subtilis* KO strain exhibited its maximum cellulase productivity of 36 I.U. by CMCZ technique and 344 µg/ml broth medium by DNSA technique and 301 µg/ml broth medium of protein content at 45°C (Table 2 and Figure 2). These results were approximately in correlation with the finding of many other workers. Optimum temperature recorded for maximum cellulase productivity by the Lactic acid bacteria, *Bacillus amyloliquefaciens* UMAS 1002, *Arachnoitus* sp. and *Bacillus* strain DLG was at 40°C [23, 32, 37, 38]. However, this results were in contrast with the data recorded by many other workers. Optimum temperature recorded for maximum cellulase productivity was 25°C for mutant *Bacillus pumilus* BpCRI 6 [9], 30°C for *Cellulomonas* sp. and *Bacillus pumilus* [35, 39], 35°C for *Bacillus subtilis* CBTK 106; *Bacillus* spp.B21 [33, 40] and 37°C for *Bacillus* sp., *Clostridium cellulolyticum* Ce19M and *Pseudomonas flourescens* [34, 41].

Table 1: Effect of different incubation periods on cellulose productivity by *Bacillus subtilis* KO strain

	Cellulase productivity		
	CMCZ	DNSA technique	Total protein
	Cellulase productivity	Cellulase productivity (µg/ml broth medium)	Con. µg/ml broth medium
Incubation period (hours)	I.U		
12	27	131	219
18	30	135	230
24	32	228	525
36	32	180	492
48	32	172	460
72	32	164	416
96	31	137	335
120	27	128	222

Table 2: Effect of different temperatures on cellulase productivity by *Bacillus subtilis* KO strain

	CMCZ	DNSA technique	Total protein
	Cellulase Productivity	Cellulase productivity (µg/ml broth medium)	Con (µg/ml broth medium)
Temperature (°C)	I.U		
25	22	120	135
30	28	189	200
35	30	220	210
40	35	310	260
45	36	344	301
50	16	0.001	55

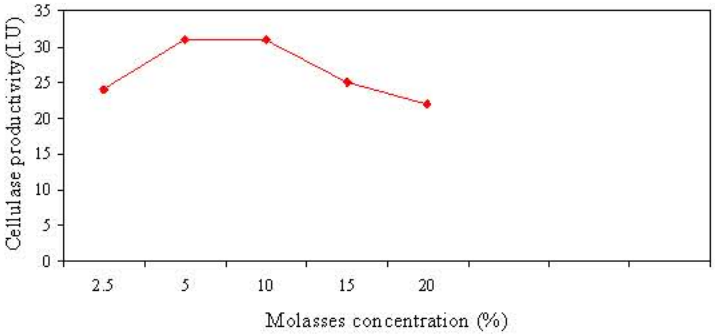

Fig. 3: Effect of different molasses concentrations on cellulase productivity by *Bacillus subtilis* KO strain using CMCZ technique

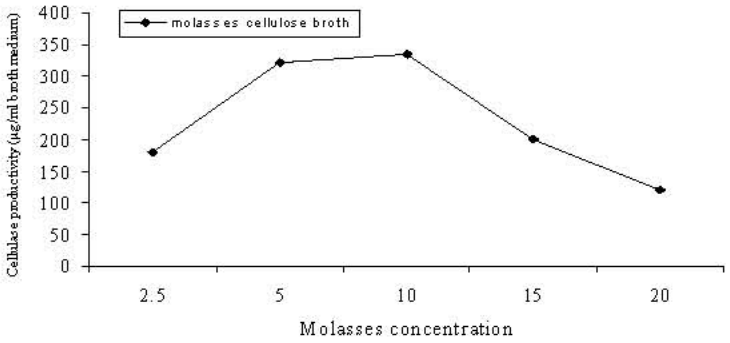

Fig. 4: Effect of different molasses concentrations on cellulase productivity by *Bacillus subtilis* KO strain using DNSA technique

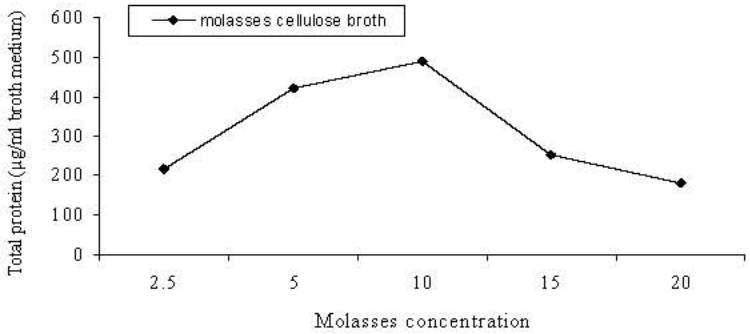

Fig. 5: Total protein content in the supernatant at different molasses concentrations.

Effect of Molasses Concentration: Fig. (3, 4 and 5) showed the effect of different molasses concentrations on cellulase productivity. Maximum cellulase productivity was obtained at 10% (w/v) molasses when using CMCZ technique (32 I.U.) and DNSA technique (335 µg/ml broth medium), while the total protein content recorded 490 µg/ml broth medium.

Effect of Carbon Sources: Figures (6- 9) show the effect of different carbon sources on the cellulase productivity as well as protein content of the crude enzyme.

Fig. 6: Effect of molasses + cellulose combination on cellulase productivity by *Bacillus subtilis* KO strain as shown by CMCZ

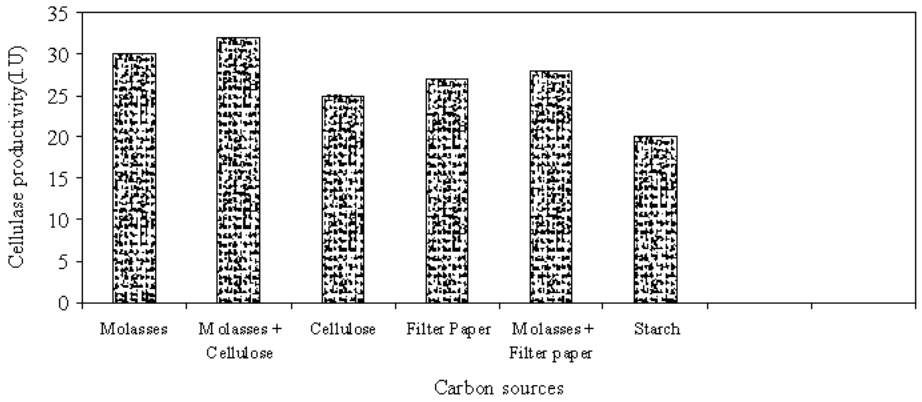

Fig. 7: Effect of different substrates on cellulase productivity (CMCZ)

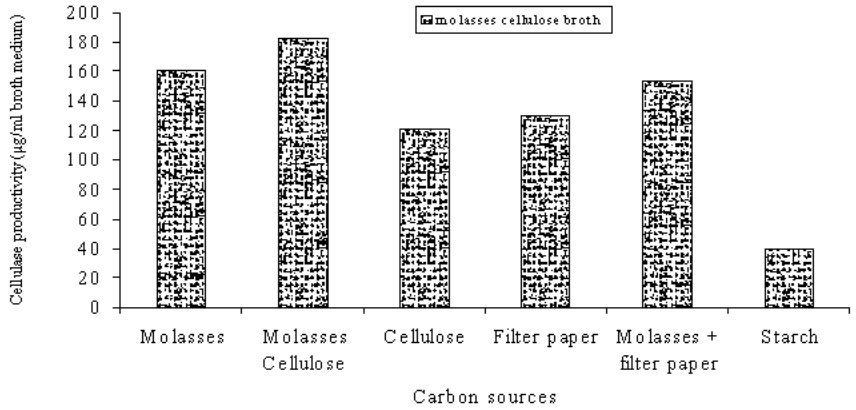

Fig. 8: Effect of different substrates on cellulase productivity (DNSA)

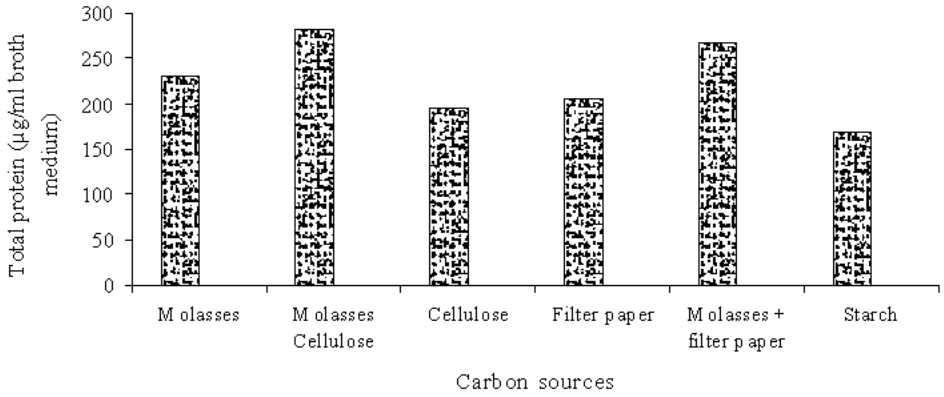

Fig. 9: Total protein content in the supernatant with different substrates

Bacillus subtilis KO strain was able to produce cellulase using different substrates, e.g. cellulose, filter paper or starch as a substrate added to the molasses cellulose mineral broth medium. *Bacillus subtilis* KO strain has the advantage that it doesn't only grows on molasses cellulose broth medium but it also use it for cellulase production (Fig. 6- 9).

Maximum cellulase productivity was exhibited with molasses broth medium when supplemented with cellulose. It was revealed 32 I.U. using CMCZ technique and 182 µg/ml broth medium using DNSA technique. Moreover, the total protein content was 281 µg/ml broth medium.

Table 3: Effect of different nitrogen sources on cellulase productivity

	Clear zone CMCZ	DNSA technique	Total protein
	Cellulase productivity	Cellulase productivity (μg/ml broth medium)	Con. (μg/ml broth medium)
Nitrogen source (0.2%, w/v)	I.U		
Molasses	28	201	260
Molasses+$(NH_4)2PO_4$	32	290	350
Molasses + sulphate	27	180	220
Molasses+ $NaNO_3$	24	150	193
Molasses+ trypton	32	270	380
Molasses + cellulose + $NaNO_3$	27	193	261
Molasses+ cellulose+ $(NH_4)_2PO_4$	35	400	550
Molasses + cellulose + Ammonium sulphate	30	296	334
Molasses+ cellulose + trypton	35	420	601

Our findings stated that molasses and cellulose support each other for higher cellulase productivity. These were in correlation with the findings of many other workers whom found that the addition of filter paper, carboxymethyl cellulose, starch, cellobiose or cellulose into the production medium as substrates enhanced cellulase production by *cellulomonas* sp. NT3060, *Cellulomonas* sp. ATCC 21399, *Bacillus* sp. B21, *Clostridium cellulolyticum*, *Bacillus pumilus* EB3 [23, 30, 42].

Some investigators showed that agro-industrial residues such as rice bran, rice straw, sugar cane bagasse and wheat bran could be used as substrates for cellulase production i.e *Bacillus subtilis* CBTK 106, *Bacillus subtilis* BL62 and *Bacillus pumillus* exhibited their maximum cellulase productivity when wheat bran, banana fruit stalk and soybean were added to the production media respectavily [34, 35].

Effect of Nitrogen Source: Table 3. showed the effect of different nitrogen sources on cellulase productivity.

A wide range of nitrogenous compounds, either organic or inorganic can affect the productivity of cellulase. The results recorded in table 3 revealed that the addition of $(NH_4)_2PO_4$ or trypton to molasses medium enhanced cellulase productivity.

Maximum cellulase productivity as shown by CMCZ technique (35 I.U.), DNSA technique (400 and 420 μg/ml broth medium) and protein content (550 and 601 μg/ml broth medium) of the cell free supernatant was exhibited when molasses broth medium was supplemented with cellulose as a carbon source and $(NH_4)_2PO_4$ or trypton as a nitrogen source (Table 3).

This was in correlation with the findings of many other workers whom found that maximum cellulase productivity was obtained by *Bacillus pumilus* BpCRI 6, *Pseudomonas flourescens*, *Monascus purpureus* and *Streptomyces* sp. BRC2 when tryptone was added as an organic source to the production medium [41, 43, 44]. Moreover, this result was also in correlation with the findings of many other workers, whom found that maximum cellulase productivity was obtained when ammonium phosphate was added to the production media by *Bacillus pumillus*, *Ruminococcus albus*, *Bacillus sp.*, *Bacillus* spp. B21. *Streptomyces* sp. BRC2 respectively [9, 22, 34, 35, 43]. From all the above data, the obtained results suggested that this study may be the unique to use molasses not only to isolate the *Bacillus subtilis* KO strain but also to grow and maintain it. Moreover, it used a medium contain molasses to produce considerable amount of cellulase. Our findings make the advantage of molasses to reduce the production cost not only for cellulase but also to shed a new light on the economic value of the yielded product.

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