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Optimization of Cultivation Medium and Growth Conditions for *Bacillus subtilis* KO Strain Isolated from Sugar Cane Molasses

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Abstract: The maximum growth of *Bacillus subtilis* KO strain was from 0.608 to 0.780 g/100 ml broth medium recorded at molasses concentration of 10%, w/v at 45°C and 24 h. incubation period when molasses broth medium supplemented with 3%, w/v of gelatin. *Bacillus subtilis* KO strain has a slightly effect of growth (0.741 g/ 100 ml broth medium), when molasses broth medium supplemented with 0.1%, w/v of ammonium sulphate as a nitrogen source. Our findings confirm the fact that *Bacillus subtilis* KO strain succeeded to growth strongly in the absence of any of the inorganic nitrogen source supplied to the molasses broth medium, considering molasses as the sole carbon and nitrogen source.

Key words: Molasses · Optimization growth conditions · *Bacillus subtilis* KO Strain

INTRODUCTON

There is an increasing interest, in the last few years based, on working on molasses due to the possibility to u se it as a cheap carbon and energy source, not only to cultivate microorganisms on it, but also to obtain valuable products, which have different applications with economic importance [1, 2].

Different microorganisms have been isolated from molasses sugar cane e.g. lactic acid bacteria and yeasts, thermophilic alkaliphilic *Bacillus sp* JB-99; *Lactobacillus plantarum* [3, 4]. Molasses as nutrient medium can be used as a relatively inexpensive and economic alternative to synthetic medium for the production of some bio-product [5, 6].

Synthesis of bacterial extracellular enzymes has been of interest to many scientists [7, 8]. Incubation period has also great effect on mass production. It was found that the effect of different factors affecting growth as incubation period, temperature, pH, aeration, addition of surfactants, the constituents of the production medium used especially carbon sources, nitrogen sources and minerals depends greatly on the microbial strain and its growth rate [9, 10]. Moreover, temperature for maximum protease productivity can be varied even from a strain to another within the same species under different growth conditions e.g. it was 36°C, 45°C and 39.5°C for *Bacillus licheniformis* [11, 12]. Alkaline protease production parameters by *Bacillus* sp. were investigated using Taguchi methodology and pH of the medium was found to be the most significant factor [13]. Two bacterial strains, *Bacillus* sp. AL-20 and AL-89 used feather as the sole carbon and nitrogen source for both growth and alkaline protease production [14]. Glucose was used as the sole carbon source for protease production by *Bacillus Sp.* PE-11 and by *Bacillus sphaericus* [15, 16]. Each of lactoglobulin, bovine serum albumin and immunoglobulins was used as the sole carbon and nitrogen source for growth and protease production by *Serratia marcescens* [17]. The objective of the present work was to optimize the cultivation medium and growth conditions for *Bacillus subtilis* KO strain isolated from sugar cane molasses collected from Kom Ombo sugar factory for industrial products in Aswan governorate as well as the investigation of the physical and chemical characters of molasses, through the replacement of carbon and nitrogen sources to cheaper one in order to reduce the cost of raw materials.

MATERIALS AND METHODS

Sampling and Locations: The samples of molasses were collected from different sources at Kom Ombo sugar factory for industrial products.

Physical Analysis: Moisture content was determined and calculated by drying a certain weight of fresh molasses till a constant weight was obtained.

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$$\text{Moisture \% (w/w)} = \frac{\text{Fresh weight of molasses - dry weight of molasses}}{\text{Fresh weight of molasses}} \times 100 = ?\ \%\ (w/w)$$

A known weight of fresh molasses sample was diluted and filtered; the non dissolved particles on the filter paper were dried and calculated. The supernatant was evaporated and the dissolved substances were calculated.

Chemical Analysis: To determine the different chemical ions present in molasses, a filtrate extraction of molasses / distilled water (1:5) was used. The pH of molasses was determined in molasses water extraction (1:5) by digital pH-meter Hanna instrument model HI8418 with glass electrode. Chlorides were determined basically according to Moher method as described by the [18]. Na^+ was determined according to [19], using Jenway flame photometer model PEP77. Carbonate and bicarbonate were determined basically according to [19]. Calcium and magnesium ions were determined by titration of molasses water extract against 0.01 N versenate solutions [20]. Sulphate ion concentration was determined spectrophotometerically at λ = 420 nm in molasses water extract with sodium chloride/ HCl-glycerol-ethanol reagent and barium chloride according to the method described by [18]. The phosphate ion concentration in molasses water extraction was determined spectrophotometrically at λ= 690 nm by the molybdate blue method as described by [21].

Microbiological Analysis

Organism Used in the Study: *Bacillus subtilis* KO strain, which isolated from that obtained from the industrial products of Kom Ombo sugar factory was used [22].

Media Used in the Study: The following media were used for cultivation of *Bacillus subtilis* KO strain: Molasses broth medium; molasses, 100 ml, Dist. H_2O up to 1000 ml and pH was not adjusted; Molasses agar medium; the above medium was supplemented with 2% agar, pH was not adjusted; Gelatin yeast extract mineral broth medium (g/l); gelatin, 10, Yeast extract, 1, $(NH_4)_2$ PO_4, 0.5, K_2PO_4, 0.4, $CaCl_2$, 0.1, $MgCl_2$, 0.1, $MgSO_4$, 0.05, dist. H_2O up to 1000 ml, pH was adjusted to 7; Beef extract peptone broth medium (g/l), Beef extract, 3, Peptone, 5, NaCl, 3, dist. H_2O up to 1000ml, pH was adjusted to7; Gelatin agar medium (g/l); Gelatin, 10 , Agar, 20 , phosphate buffer up to 1000 ml, pH was adjusted to 7.

Determination of Dry Cell Mass (DCM): This was performed by growing *Bacillus subtilis* KO strain in molasses broth under the tested factors. Cells were harvested by centrifugation at 9000 rpm for 20 minutes. The cell pellet was washed with phosphate buffer and recentrifuged. After that the cell pellet was dried until constant weight was obtained and the DCM was calculated. DCM was the mean for determination of optimum growth conditions.

Effect of Molasses Concentrations on Growth: Effect of different molasses concentrations was tested on molasses broth serial dilutions (%, w/v); 0.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 in water.

Effect of Temperature on Growth: This was performed at different temperatures (20, 25, 30, 35, 40, 45 and 50°C).

Response to pH: Response to pH was performed on molasses broth medium using different pH's (4.5, 5, 5.5, 6, 6.5 7, 7.5, 8 and 8.5).

Effect of Incubation Period on Growth: This was also determined on molasses broth medium through determination of DCM under different incubation periods (6, 12, 24, 36, 48, 60 and 72 hours).

Effect of Different Gelatin Concentrations: 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).

Effect of Different Inorganic Nitrogen Sources: *Bacillus subtilis* KO strain was grown on the production media supplemented with 0.1 % (w/v) of various inorganic nitrogen sources such as ammonium phosphate, ammonium oxalate, ammonium acetate, sodium nitrate, ammonium chloride and ammonium sulphate.

RESULTS AND DISCUSSION

In the present study, different experiments were performed to optimize ideal medium and ideal conditions for *Bacillus subtilis* KO strain growth and used to further studies. Molasses broth medium, molasses agar medium, gelatin yeast extract mineral broth medium, beef extract peptone broth medium and gelatin agar medium were used for cultivation, enumeration, maintenance and preservation of *Bacillus subtilis* KO Strain. The physical

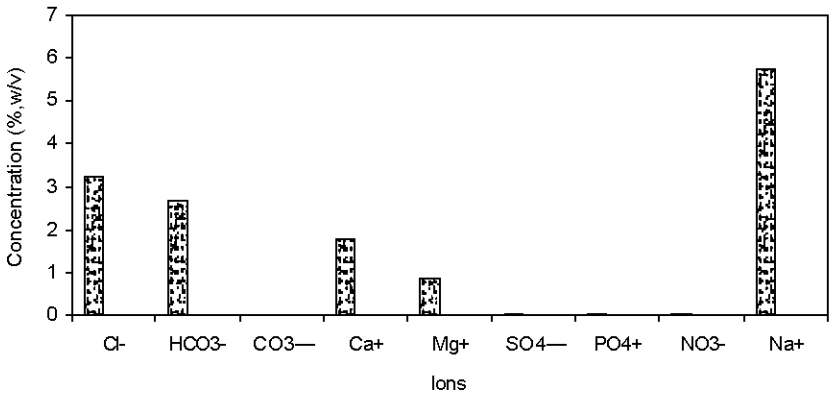

Fig. 1: Ions concentration in molasses sample

and chemical characters of molasses were investigated. Molasses media were used to reduce the cost of the cell mass production. Many workers used the natural sources as one of the medium constituents e.g. rice bran, soybean, wheat flour, wheat bran, corn bran and corn starch orange peels to support growth of different bacteria to produce different enzymes [23, 24].

Physical Analysis: Molasses was obtained as a by-product in sugar industry from sugar cane or beet. In sugar industry, when the juice was obtained, it was purified and then concentrated by heating until it becomes sticky mass in which sugar was crystallized; the residue of this process was the molasses. The composition of molasses was also variable depending on its grade. It was more or less consisted of (%, w/v); water, 20-30; organic solids, 70; inorganic substances, 10-15. Organic substances include sugars 35-55 and non sugar substances 15-25 from the total molasses structure. The non sugar substances include protein, starch, gum, organic acids, waxes, coloring substance and vitamins [25, 26]. Molasses sample in this study was sticky-thick and dark syrup with a strong sweet flavor. Molasses was very rich in energy. Physical analysis of molasses sample showed that it contains 34 % (w/v) water (moisture content) and 66 % (w/v) solids, in which 4.4 % (w/v) non dissolved solids (impurities) and 61.6 % (w/v) dissolved solids.

Chemical Analysis

Ions Concentration: Results which represented by Figure 1 showed that Na^+ and Cl^- exhibited the highest concentration (5.748 & 3.26 w/v) while PO_4^+ and NO_3^- were the lowest (0.023 & 0.025 w/v).

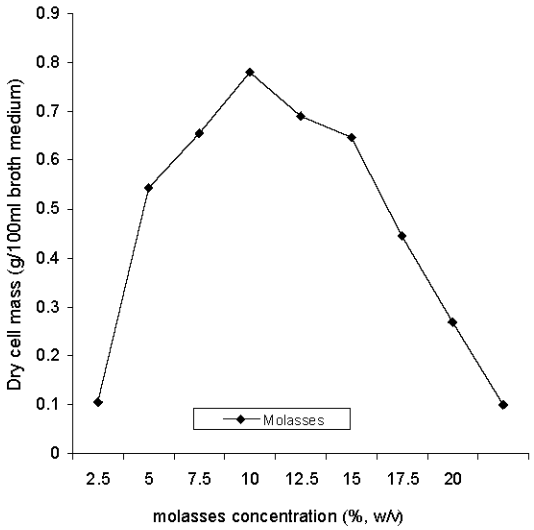

Fig. 2: Dry cell mass at different molasses concentrations

The molasses sample was rich in its constituents not only from sugars but also its content from ions was also high and represented 14.14 %, (w/v) from the sample. Amongst analyzed ions, Na^+, Cl^-, HCO_3^- and Ca^+ were the highest i.e. 5.748, 3.26, 2.684 and 1.76 (%, w/v), respectively. CO_3^{--} was not detected, while PO_4^{+++}, Mg^{++} and SO_4^{--} were the lowest, i.e. 0.023, 0.025 and 0.05 (%, w/v), respectively. The recorded available data by other workers showed that each 100 g molasses give 265 calories, 68.5 g carbohydrates, 0.1 g total fat and 0.242 g magnesium (w/v), molasses also contains elements such as sodium, potassium, iron, magnesium, manganese, calcium, cupper and zinc [27, 28].

The molasses samples were slightly acidic and the pH ranged between 5.9 and 6.2. The chemical analysis of the present molasses sample showed a variety of minerals, Na^+ and Cl^- gave the highest concentration. This was in

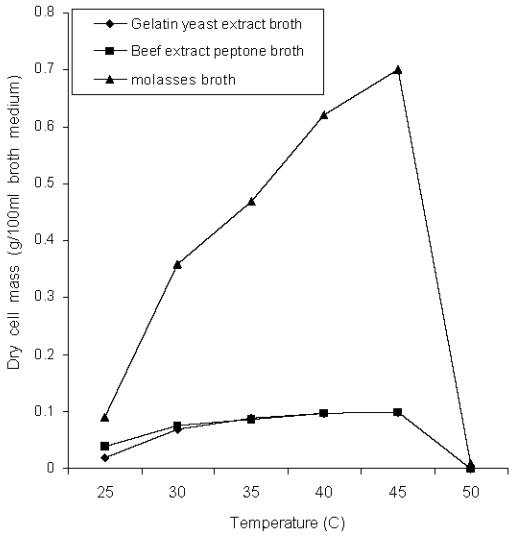

Fig. 3: Dry cell mass at different incubation temperatures

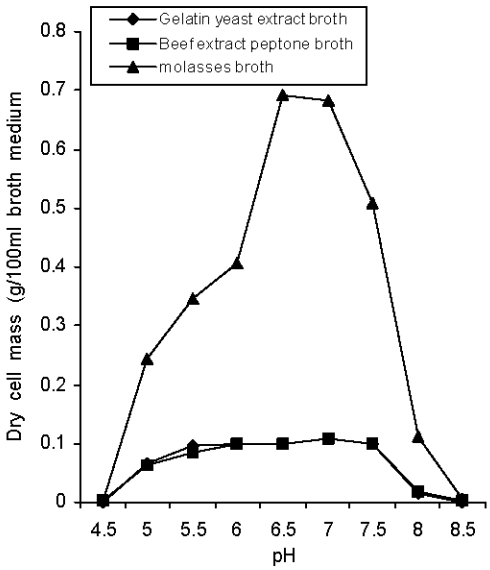

Fig. 4: Response to pH represented in DCM

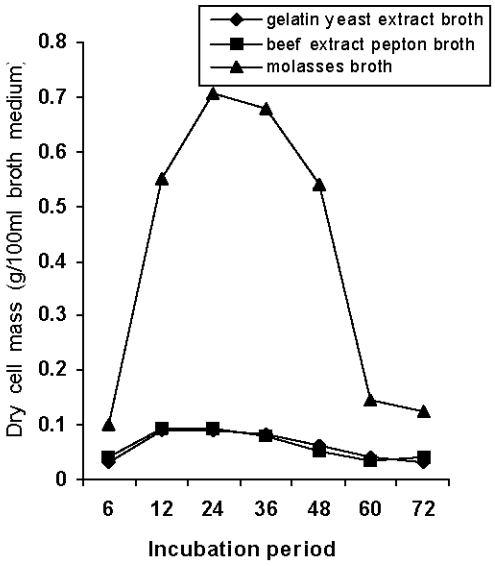

Fig. 5: Dry cell mass at different incubation periods

correlated with the results obtained by [28]. Molasses could be used as an integrated medium for the growth of microorganisms as well as the production of valuable products with economic importance because it is rich in minerals, nutrients and even vitamins and other compounds [22, 29]. Optimum conditions of molasses concentration, temperature, pH, incubation period, different substrate concentrations (gelatin) and different inorganic nitrogen sources for *Bacillus subtilis* KO strain were determined via determination of DCM.

Determination of Optimum Growth Conditions: Data of Figure 2. Showed the growth response of *Bacillus subtilis* KO strain to different molasses concentrations. Maximum growth was 0.780 g/100 ml broth medium at molasses concentration of 10 %, w/v.

Bacillus sp. JMA 5 was isolated from molasses contaminated soil and was used to produce polyhydroxybutyrate (PHB) in presence of 25 % (w/v) molasses [30]. Molasses was also used as the sole carbon source in a concentration of 3 % (w/v) in presence of corn steep liquor (w/v) as nitrogen source for PHB production [31].

From Figure 3, it was found that *Bacillus subtilis* KO strain exhibited its maximum growth 0.750 g/100 ml broth medium at 45°C, where there was no growth above 50°C. Optimum temperature for maximum growth for *Bacillus subtilis* 115 and *Bacillus subtilis* was 40°C [32].

Figure 4. Showed that the maximum growth of *Bacillus subtilis* KO strain was from 0.693 & 0.682 g/ml broth medium in a pH range between 6.5 and 7, respectively. This result was in correlation with the finding of other workers for different *Bacillus subtilis* strains e.g. *Bacillus subtilis* BS59 and *Bacillus subtilis* 38 [33, 34, 35].

Figure 5. showed that the best growth of *Bacillus subtilis* KO strain was 0.706 g/100 ml broth medium after 24 h. incubation period. This result was correlated with the data recorded for *Bacillus subtilis* CBTK 106 exhibited its maximum growth after 24 h incubation period [36], 48 h for *Bacillus subtilis* BS1 and *Bacillus subtilis* IKBS-10 [37, 38].

Table 1: Dry cell mass at different gelatin concentrations

	Dry cell mass (g/100ml)		
Gelatin concentration	Gelatin yeast extract broth medium	Beef extract peptone broth medium	Molasses broth medium
Control	0.019	0.060	0.542
0.05%	0.068	0.065	0.550
0.1%	0.088	0.074	0.549
0.5%	0.089	0.075	0.552
1%	0.098	0.092	0.562
2%	0.146	0.098	0.606
3%	0.159	0.136	0.608
4%	0.137	0.124	0.531
5%	0.124	0.069	0.512

Table 2: Dry cell mass at different inorganic nitrogen sources

	Dry cell mass (g/100ml broth medium)		
Inorganic nitrogen sources (0.1%, w/v)	Gelatin yeast extract broth medium	Beef extract peptone broth medium	Molasses broth medium
Control	0.001	0.0236	0.715
Amm.phosphate	0.098	0.099	0.741
Amm. acetate	0.096	0.098	0.512
Amm. oxalate	0.078	0.079	0.402
Amm.chloride	0.069	0.078	0.382
Sodium nitrate	0.053	0.065	0.372
Amm. sulphate	0.042	0.054	0.356

Data presented in Table 1 showed that the maximum growth of *Bacillus subtilis* KO strain 0.608 g/100 ml of molasses broth medium at gelatin concentrations between 2 and 3 %, w/v. This result was in complete accordance with the finding of [24] who found that maximum protease productivity by *Bacillus anthracis* S-44 and *Bacillus sp.* K 30 was exhibited with gelatin concentration between 1.5 and 2 %, (w/v). It was interesting to find that *Bacillus subtilis* KO strain could give also a significant growth on beef extract peptone broth medium using beef extract as a substrate beside the maximum growth on molasses broth medium using molasses as a substrate. This may be because beef extract or molasses contain a substrate in its structure.

Data of Table 2 showed that the maximum growth of *Bacillus subtilis* KO strain was 0.741 g/100 ml broth medium when molasses broth medium was supplemented with 0.1% (w/v) ammonium phosphate as inorganic nitrogen sources. It has slight effect on growth when compared with control (0.715 g/100 ml broth medium). Results of Table 2 exhibited the fact that ammonium acetate and ammonium oxalate has a slight inhibitory, enhancement or even no effect of the DCM of *Bacillus subtilis* KO strain. Our findings confirm the fact that strain *Bacillus subtilis* KO succeeded to grwth strongly in the absence of any of the inorganic nitrogen source supplied to the molasses broth medium, considering molasses as the sole carbon and nitrogen source. This findings were in complete accordance with the result obtained by [39-41].

From the results of the factors investigated in the present study an ideal medium and ideal condition for cultivation and growth of *Bacillus subtilis* KO strain were determined and used to further studies.

From the data obtained in the present study, it could be recognized that this study may be the unique to use molasses not only to isolate the bacterial strain *Bacillus subtilis* KO strain, but also to grow and maintain it. Moreover, it used a medium contain only molasses to produce considerable amount of valuable products with economic importance by *Bacillus subtilis* KO strain [22].

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