

Production of α -amylase using new strain of *Bacillus polymyxa* isolated from sweet potato

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Abstract: In this study, a new amylase producer strain was isolated from sweet potato tuber. This strain was able to grow at 37 °C and produce α -amylase in high quantity compared to other standard strain cultures. In the first part, cultivation in shake flask in standard medium was carried out to give complete information about the growth and production kinetics of this strain. The results clearly demonstrate that the isolated strain is able to production α -amylase in submerged culture with concentration up to 2050 μ kat/L after 20 h cultivation. Furthermore, medium optimization was carried out by changing the starch concentration and cell cultivation in medium of mixed carbon source (composed of starch and glucose of ratio 15:5 g/g) to enhance the production process and to increase the growth rate. The volumetric and specific α -amylase production in this optimized medium were 4550 μ kat/L and 1060 μ kat/g, respectively. Further improvement in enzyme production process was achieved by scaling up the process from shake flask to 3-L stirred tank bioreactor under non-oxygen limiting condition. The maximal volumetric and specific α -amylase productions in bioreactor batch culture were 5210 μ kat/L and 1095 μ kat/g, respectively, after only 14 h cultivation.

Keywords - α -amylase, *Bacillus polymyxus*, bioprocess optimization, batch cultivation

I. Introduction

Starch is the most abundant storage carbohydrate in plant kingdom and the second feed stock of plant material next to cellulose. Thus, it is the major energy storage material in many crops such as wheat, maize, rice, cassava and potato [1]. Starch is glucose polymer of different chain molecular weight and morphology (chain length and branching) depending on its origin. Starch needs to be hydrolyzed first to monosaccharide (glucose) or oligosaccharide (maltose) to be easily metabolized. Different classes of starch degrading enzymes are produced naturally by different types of plant, animal and microorganisms. Among different starch degrading enzymes, the most industrially important amylases are: α -amylase (EC 3.2.1.1.), β -amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3). These enzymes are require individually or in combination during industrial application. The production process of these enzymes involve mainly strains belong to bacteria (especially strains belong to *Bacilli* such as *B. amyloliquefaciens* and *B. cereus*), and fungi (mainly belong to *Aspergillus* group such as *A. oryzae* and *A. awamori* [2-5]).

α -amylase (1,4- α -D-glucan-glucanhydrolase, EC 3.2.1.1; CAS # 9000-90-2) is the most industrially important enzyme. This biocatalyst is a classical calcium containing protein and belongs to endo-amylases family and catalyzes the cleavage of α -D-(1-4) glycosidic bonds in starch and related carbohydrates with retention of the α -anomeric configuration in the products [6]. Therefore, in addition to its latest application in biorefinery and biofuel production, this enzyme finds many applications in different sectors of wellness industries including: starch liquefaction, food industries, pharmaceutical and drug industries [7-9]. Large scale industrial production of this enzyme is carried out mainly in submerged culture using different types of microorganisms. However, most of industries preferred using bacterial strains belongs to *Bacillus* strain because of the long history of this type of organisms in industry for the production of different hydrolytic enzymes [10]. The most widely used *Bacillus* strains for amylase production are *B. subtilis*; *B. amyloliquefaciens*; *B. licheniformis* [11-13]. However, most of industrial strains of high productivity grew at 30 °C. Thus, cooling is required during the cultivation process when cells cultivated in large scale submerged culture especially in sub-tropical and tropical countries. The aim of this work was to develop a cultivation strategy for amylase production with short cultivation time by applying a new α -amylase producer strain which is able to grow and produce enzyme effectively at 37 °C. Applying mesophilic microbes of high ability to grow and produce

enzyme at relatively high temperature will reduce the production cost. This based on the fact that the cooling water cost have high share in the bioreactor operation cost especially in sub-tropical and tropical countries.

II. Materials and Methods

Isolation and selection of α -amylase producer strain

Sweet potato tuber sample was obtained from local market of Tripoli, Libya. 10 gm of decayed part of the tuber was taken in 100 ml water. The sample was then diluted by using serial dilution method and plated on selective agar plate to isolate the most effective enzyme producer strain according to Samie *et al.* [14]. Agar medium was composed of (g/L): soluble starch 1.0; KH_2PO_4 1.0; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.05; $(\text{NH}_4)_2\text{SO}_4$ 2.0; Tryptone 2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 3.13; Agar 20. The medium was adjusted to pH 7.0 before sterilization. The amylase producer strain was isolated by flooding the agar plates with 1% (w/v) iodine solution. The most effective isolate was selected, subcultured and further identified.

Identification of the α -amylase producer strain

Morphological and biochemical methods were carried out to identify the enzyme producer strain. The initial morphological observation shows that the strain is rod-shaped, G+ve and endospore former bacteria. Therefore, biochemical identification was carried out using standard API biochemical test for identification of Bacilli [15] (BioMérieux Co, France). The data obtained from API tests were used for strain identification using API software. The results are fully matched with the morphological and biochemical feature of *Bacillus polymyxa*.

Strain preservation and inoculum preparation

The isolated strain was preserved in frozen in 50% glycerol solution (v/v) at -80°C . Before use, the cells were activated twice in LB agar medium of the following composition (g/L): beef extract 5, peptone 10, yeast extract 5, NaCl 5, and agar 20 (pH 7). After 24 h day cultivation, the arisen colonies were used to inoculate 250-ml Erlenmeyer flask (of 50 ml working volume) containing LB broth. The inoculated flasks were incubated on rotary shaker at 37°C and 200 rpm for 24 h.

Fermentation medium and cultivation conditions

The medium used for enzyme production had the following composition (g/L): $(\text{NH}_4)_3\text{PO}_4$, 5; Yeast extract 1, K_2HPO_4 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5; Sodium citrate 0.1; CaCl_2 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.1 and soluble starch 10 [12]. Cultivations were carried out in shake flask and in bioreactor at 37°C . In case of shake flask, 250 ml Erlenmeyer flask of working volume of 50 ml was used in this study. The inoculum was in form of 4 ml of cell suspension (of 1 OD at 600 nm) obtained from previously cultivated cells in LB medium for 24 h. The inoculated flasks were incubated on rotary shaker at 200 rpm and 37°C (Innova 44, New Brunswick Scientific, NJ, USA). Cultivations in bioreactor scale were carried out in 3-L stirred tank bioreactor (Bioflo III, New Brunswick Scientific, NJ, USA) with a working volume of 2L. Agitation was performed by two 4-bladed Rushton turbine impellers (d_i , impeller diameter = 65 mm; d_t (tank diameter) = 135 mm, $d_i/d_t = 0.48$) at 400 rpm. Aeration was performed by filtered sterile air at the rate of 1 v/v/min. pH values and Dissolved oxygen concentrations was measured during cultivation using on-line electrodes attached to the bioreactor controller (Mettler Toledo, Switzerland). Foam was suppressed by the addition of silicon antifoam reagent (Antifoam A, Sigma, USA).

Analytical methods

Samples were taken at different time intervals in form of 10 ml and 50 ml in case of bioreactor and shake flasks, respectively. After sampling, the optical density of culture was determined immediately by using spectrophotometer (Novaspec II, Pharmacia Biotech, Sweden) at OD_{600} . Samples were centrifuged at 6000 RPM and the cells were washed/centrifuged twice before drying in an oven at 110°C for 24 h. One OD_{600} unit was equivalent to 0.29 g (cell dry weight/L). The supernatant was frozen at -20°C and stored for further analysis. Determination of α -amylase activity was carried out according to the method of Bernfeld and modified by Stellmach [16]. In this method, 0.5 ml of sample, previously diluted in 0.05 M acetate buffer of pH 4.9, was incubated for 3 min at 25°C with 0.5 ml of 1% soluble starch solution. The reaction was terminated by addition of 1 ml of dinitrosalicylic acid color developing agent. The sample was then heated for 5 min in boiling water bath and cooled immediately in ice bath to stop the color developing reaction. After addition of 10 ml of distilled water, the sample optical density was determined at 540 nm. The enzyme activity is expressed in Katal unit which defined as the amount of activity that produces one mole of reducing group per second [17].

III. Results and Discussion

Isolation and Identification of the bacterial strain

Ten g of decayed part of sweet potato tubers were taken and washed with sterile saline solution and further homogenized using food mixer with 20 ml of double distilled water. The obtained homogenized mixture was diluted up to 1×10^{-8} from each sample 1 ml was taken and plated over starch selective medium as described in materials and methods part and incubated at 37 °C for rapid isolation of amylase producers. The arisen colonies were isolated and tested for their potency for amylase production using agar plate method. The highest producer strain was identified using morphological, biochemical and API identification kits, API 20E and API CHB50 (Biomerieux, France). The tests included: Gram staining, incubation temperature, anaerobic growth, Voges-Proskauer and methyl red test, catalase production, growth in NaCl solution, growth at pH 5 and 7, fermentation of carbohydrate, starch hydrolysis, utilization of citrate, formation of indole, formation of dihydroxyacetone, deamination of phenylalanine, casein hydrolysis, tyrosine degradation, getalin hydrolysis, egg yolk lecithinase, growth in the presence of lysozyme) according to the standard method of bacilli identification [18,19]. Identification of the bacterial strain was carried out by using API-web program of the manufacturer. The obtained results clearly demonstrate that this strain excellent identification with high percentage matching to *Bacillus polymyxa*.

Kinetics of cell growth and α -amylase production in standard fermentation medium

For better understanding of the kinetics of cell growth and enzyme production of the newly isolated strain, cultivations were carried out in shake flask to evaluate the enzyme production process. As shown in fig. 1, after a lag phase of about 4 hours, cells grew exponentially with rate of 0.123 g/L/h and reached maximal biomass of about 2 g/L after 20h cultivation. In parallel to cell growth, cells produced α -amylase in culture continuously mainly after lag phase of about 6 hours and accumulated in culture with almost constant rate of 140 μ kat/L/h reaching a maximal enzyme concentration of 2050 μ kat/L. On the other hand, for better understanding of the cell performance for enzyme production, the yield coefficient $Y_{P/X}$ which give the value of specific enzyme production per gram of biomass was also calculated. As shown in figure 1, the maximal value of yield coefficient of about 1200 μ kat/g was obtained after 16 h cultivation and kept more or less constant for the production time. This clearly indicate that the cell performance for enzyme production was changed during cell cultivation and cells need time to reach maximal cell performance for enzyme production which is almost close to the late exponential growth phase. However, the amount of enzyme produced in this culture was higher than other published standard bacillus strain such as *B. amyloliquefaciens* [11]. The phenomena of parallel relation between cell growth and α -amylase production profile was also observed in other strains such as in case of *B. licheniformis* [20], *B. amyloliquefaciens* [12] and *Micrococcus varians* [21].

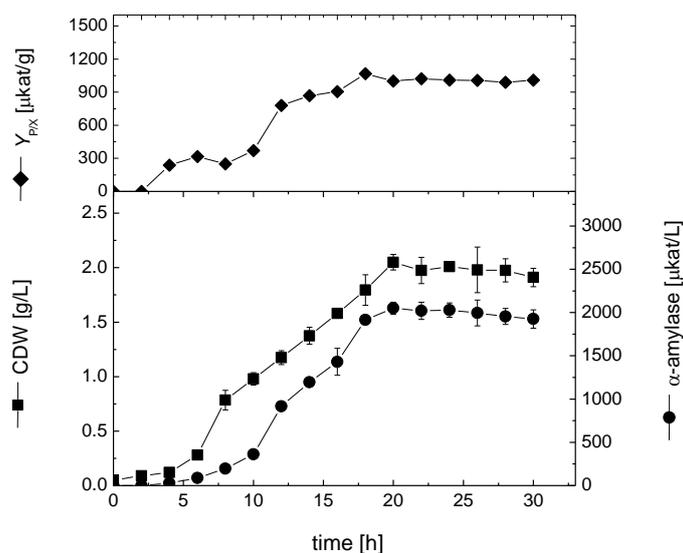


Figure 1. Time profiles of cell growth and α -amylase production during submerged fermentation of *B. polymyxa*. (Values are mean \pm standard deviation, $n=3$)

Different starch concentrations

In this experiment, further attempt was done to increase the volumetric production of α -amylase using the newly isolated strain. Thus, cultivations were carried out in shake flask in media of different starch concentrations ranged between 0-40 g/L and data taken after 20 h cultivation based on the results of the previous experiment. Fig. 2 demonstrates the results of biomass, volumetric and specific α -amylase production. As shown, the maximal biomass of 3.55 g/L was obtained in 30 g/L starch culture and further increase in starch concentration resulted in reduction in biomass. For α -amylase production, the maximal volumetric and specific yield of about 3500 μ kat/L and 1046 μ kat/g, respectively, were obtained in culture supplemented with only 20 g/L starch.

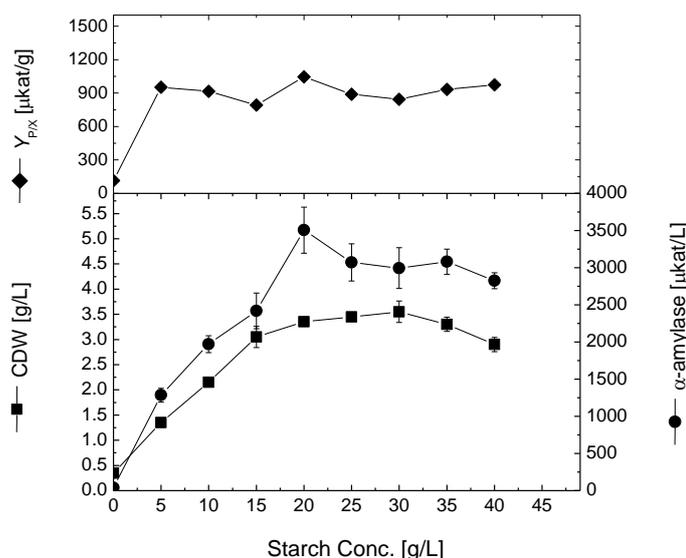


Figure 2. Effect of different starch concentration on cell growth and α -amylase production during submerged fermentation of *B. polymyxa*. Data were taken after 20 h cultivation (Values are mean \pm standard deviation, $n=3$)

On the other hand, the values of specific α -amylase production in all starch supplemented culture were ranged between 850 to 1050 μ kat/g. Thus, we can conclude that the optimal starch concentration for α -amylase production in this medium is 20 g/L.

Effect of different starch/glucose ratio

Based on the growth curve (fig. 1) of this study, after inoculation of cells into the production medium, cells entered lag-phase of about 4 hours as adaptation phase prior exponential cell growth. This phase is due to the big different between the composition of vegetative growth medium and fermentation medium. In the later medium, carbon source is mainly starch (with small fraction of carbon source in yeast extract). Therefore, cells need to produce starch degrading enzyme first to degrade starch to C-6 monomer (glucose) or disaccharide maltose for cell utilization. To reduce this problem of long lag phase, cells were cultivated in different media of range of (starch:glucose) ratio to investigate the effect of (starch:glucose) mixture when applied as carbon source on cell growth and α -amylase production. As shown in figure 3, the maximal biomass of about 7 g/L was obtained in pure glucose supplemented culture. This value is about two fold higher compared to starch supplemented culture. On the other hand, the maximal volumetric and specific α -amylase production of about 4580 μ kat/L and 1077 μ kat/g, respectively, were obtained in culture supplemented with mixed carbon source of starch:glucose in ratio of (15:5) g/g. This clearly indicates that, the production of enzyme is induced with the presence of starch in culture. This results are also further increase in glucose ratio beyond 5 g/L in culture resulted in significant reduction in both specific and volumetric enzyme production. The lowest values of both specific and volumetric enzyme production were obtained in starch free culture. This repression effect of glucose on enzyme production in our study was also reported in other authors during α -amylase production by *B. subtilis* [22-26-29].

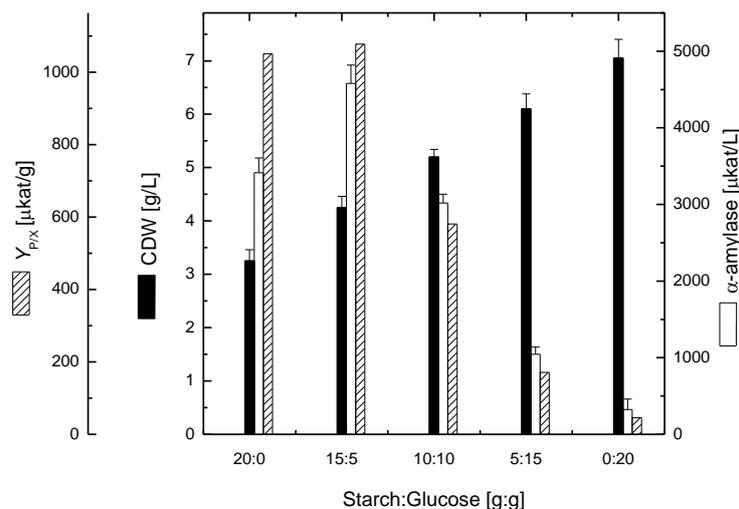


Figure 3. Effect of different starch:glucose ratio on cell growth and α -amylase production during submerged fermentation of *B. polymyxa*. Data were taken after 20 h cultivation (Values are mean \pm standard deviation, $n=3$)

Kinetics of cell growth and α -amylase production in optimized medium

For better understating of the advantages of the new medium formula (which includes starch and glucose in ratio of 15:5 g/g, in culture), cultivations were conducted in shake flask culture in batch mode. As shown in figure 4, cells grew exponential after inoculation without any observed lag phase as in medium of only starch as carbon source. In this culture, cells utilized the available sugar in culture (glucose) during the adaptation phase until starting amylase production. The growth rate of cells in this culture was 0.283 g/L/h and reached maximal biomass of 4.3 g/L after only 16 h cultivation (about 4 hours less than the corresponding batch culture using only starch as substrate). During this phase, α -amylase was produced continuously in culture by cells with production rate of 322 μ kat/L/h and reached the maximal value of 4550 μ kat/L after 16 h. This value was more than double of those obtained in initial medium which include starch and sole carbon source. On the other hand, the cell specific production was increased gradually by time and reached about 1060 μ kat/g after 16 h when cells entered the stationary phase which is also the same of termination of enzyme production phase. Further increase in the value specific production was as a result of the decrease of biomass during the decline phase and not due to a real increase in cell productivity [27].

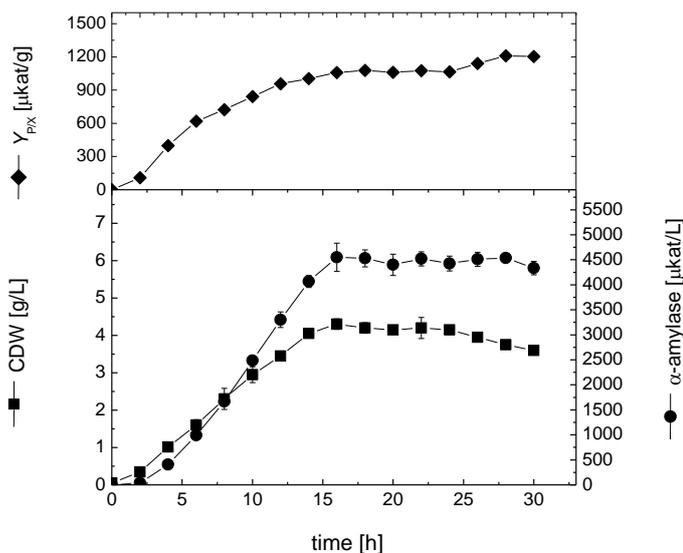


Figure 4. Time profiles of cell growth and α -amylase production during submerged fermentation of *B. polymyxa*. In starch:glucose medium in ratio of 15:5 [g/g] in shake flask culture (Values are mean \pm standard deviation, $n=3$)

Production of α -amylase in 2-L stirred tank bioreactor

For better understanding of enzyme production kinetics and scalability of this process, cultivations were carried out in 3-L stirred tank bioreactor with working volume of 2-L, inoculum size, medium composition and other cultivation parameters were the same as in shake flask cultures. As shown in figure 5, cells grew exponentially without significant lag phase with rate of 0.425 g/L/h reaching stationary phase by biomass of about 4.7 g/L after 12 h. This time was shorter by 4 hours compared to the corresponding batch culture in shake flask. During this active growth phase, the DO in culture decreased gradually as a result of high oxygen consumption in culture and reached about 28% saturation after 12 h (the time of late exponential growth phase). As cells entered the stationary phase, the DO value increased again and reached about 80% at the end of cultivation time. However, in parallel to active cell growth α -amylase was produced in culture without any significant lag phase with rate of 417 μ kat/L/h and reached the maximal volumetric production of about 5210 μ kat/L after only 14 h (compare to only 4550 μ kat/L in case of shake flask culture after 16 h cultivation).

The higher cell growth and enzyme production when the process transferred from shake flask to bioreactor in our study is due to the fact that cultivations in stirred tank bioreactor provides better aeration and agitation compared to shake flask culture and thus increase cell performance and productivity of aerobic microorganisms during biosynthesis of different primary and secondary metabolites [23-25-28].

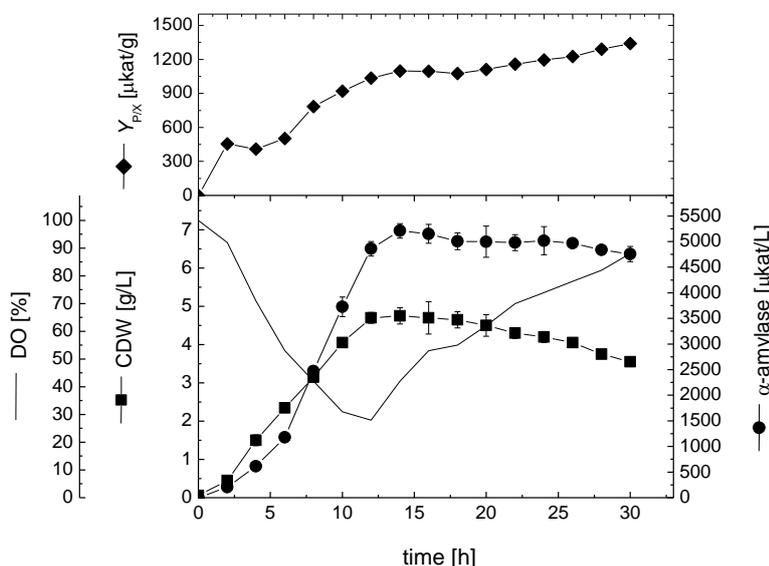


Figure 5. Time profiles of cell growth and α -amylase production during submerged fermentation of *B. polymyxa*. Using optimized medium in 3-L stirred tank bioreactor (Values are mean \pm standard deviation, $n=3$)

IV. Conclusion

This study demonstrated clearly the high performance of the new isolated strain *B. polymyxa* which was isolated from sweet potato. The production process was first improved through the increase of starch concentration in culture from 10 g/L to 20 g/L. Further improvement of this process was process by applying a mixed carbon source in form of starch:glucose in ratio (15:5), this not only resulted in an increase in enzyme production but also significant reduction of the production phase from 20 h to only 16 h. By scaling up the process from shake flask to bioreactor level, the enzyme production was further increased and the production time was further shortened to only 12 h. The obtained results in this work clearly demonstrate that the isolated strain in this study could serve as alternative biofactory for α -amylase production in industrial scale.

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