Microbial Production and Maximization of α-Amylase by Submerged Fermentation

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Abstract: ***Amylases are enzymes, which break down starch into simple sugars. They are one of the most important industrial enzymes. For large scale production microorganisms are widely used. The aim of the study was, α - amylase production and maximization, through submerged fermentation time courses. Submerged fermentation time courses with Bacillus amyloliquifaciens, under standard condition and varied parameter conditions were carried out in shake flasks. Throughout the work α- amylase assay, Lowry method and reducing sugar assay were done. The fermentation after change in parameter showed that the nitrogen source and controlled pH- 7 is essential, for the production of α-amylase enzymes.***

Keywords: Bacillus amyloliquifaciens, submerged fermentation, α- amylase assay, nitrogen source.

1. Introduction

The enzymatic hydrolysis of starch is one of the most important enzymatic reactions that is carried out at an industrial scale [1] and the amylase family enzymes, is of great significance due to its wide area of potential application [2]. Though it can be derived from several sources such as plants, animals, and microorganisms, the microbial sources generally meet the industrial demand [2], [3].

The amylases, which have been identified in microbes, include alpha amylase, beta amylase and glucoamylase among these three amylases the studies on alpha amylases are well documented [1],[4],[5],[6],[7],[8].

Even though several microorganisms produce the amylases, it remains still as a challenging task to obtain the strains, which are capable of producing commercially acceptable yields and this selection of suitable strain is the most significant factor. Various steps are required for the commercial production of amylases. It is because the environmental factors required for the optimum growth of microorganisms which are employed in production may differ from those required for the production of enzymes. Some of these factors include nutrient supplementation, pH of the medium, osmotic pressure, degree of aeration, temperature and purity of the media [2].

There are two main methods for amylase production, submerged fermentation and solid-state fermentation [2]. A semi-quantitative estimation was done to compare amylase production on solid medium, which involves growing the organism on media containing starch and testing starch hydrolysis by flooding with iodine [9]. For calculating the α-amylase activity, α- amylase enzyme assay was used [10]. Hence α- amylase is an inducible enzyme and the presence of reducing sugars in the media can affect the amylase enzyme production, the reducing sugar was determined using the DNS method [11]. This method is one of the widely used methods for determining reducing sugar and is well documented by many of the research workers [4],[6], [12]. For the estimation of biomass an indirect method was used (Lowry method) [13].

Here submerged fermentations were carried out in conical flasks. *Bacillus amyloliquifaciens*, a potent α- amylase producer [14], which was selected as the best strain, has been used to study the enzyme secretion. The present investigation involves the microbial production and optimization of α-amylase by using different carbon and nitrogen source.

2. Materials and Methods

2.1 Maintenance and determination of amylolytic microorganisms

The amylolytic micro organisms were grown in starch agar plates. The zone of clearance by the amylase producing colonies on starch agar plates due to the hydrolysis of starch [9] was compared after flooding the plate with iodine. The provided strains *Bacillus amyloliquifaciens and Bacillus subtilis* form University Of Western Sydney, was maintained. Sub culturing was done once in three weeks.

2.2 Inoculum medium and enzyme production medium: A single isolated colony was inoculated in to a 50 ml medium containing basal medium, yeast extract and soluble starch was used as the inoculum. The enzyme production medium was prepared in 250ml conical flasks with 50ml medium, which contained basal medium ($(NH_4)_2SO_4$- 5g/l, K_2HPO_4- 9g/l, KH_2PO_4- 3g/l, Sodium Citrate- 1g/l, $CaCl_2$ $2H_2O$- 0.1g/l, $FeCl_3$ $6H_2O$- 0.14g/l, $FeSO_4$ $7H_2O$- 0.01g/l, $MgSO_4$ $7H_2O$- 0.5g/l, $MnSO_4$ H_2O- 0.01g/l, $ZnSO_4$ $7H_2O$- 0.001g/l).

2.3 Fermentation time courses

2.3(a) Shake flask fermentation under standard condition: The first time course experiment was done under standard conditions for *Bacillus amyloliquifaciens* (Carbon source- 1% Soluble starch,1%yeast extract, temperature – 37°C, pH – 7, DOT – Aerobic, Inoculum size – 5% v/v). Conical flasks containing 50ml the production mediums were autoclaved at 121°C for 15minutes and were cooled at room temperature. It was then inoculated with inoculum and was placed in orbital shaking incubator. The time courses were carried out for a period of four days and the samples were collected for assays.

2.3(b) Shake flask fermentations under optimized conditions: For optimization three different sets of fermentation time courses were done in shake flasks. The first one was done with 1% Wheat starch as carbon source, 1% yeast extract as nitrogen source at pH-7. The second time course were done with 1% Soluble starch, 1% peptone at pH-7 and the third time course with 1% Soluble starch, 1% meat extract at pH-7. The fermentation time courses were run for 4 days and each day samples were collected and frozen for the assays.

2.4 Monitoringfermentations: The samples collected from each day of the fermentation time courses were centrifuged for about 5 minutes at the maximum rpm and the supernatant obtained was used for Amylase assay [10] and reducing sugar assay [11] was used. The pellet obtained, after washing it with saline and again re-suspending it in saline and 0.3 M NaOH, was kept in boiling water bath for 20 minutes and was centrifuged and then the supernatant was used for the biomass assay [13].

3. Results

3.1 The semi quantitative estimation on starch agar plates

After spot inoculation on starch agar plates followed by incubation and flooding with iodine, given strains *Bacillus amyloliquifaciens* and *Bacillus subtilis* were observed for the zone of clearance and *Bacillus amyloliquifaciens* was selected as the best amylase producer. *Bacillus amyloliquifaciens* was used for the further fermentation time courses. (Table-1)

Organism	Zone of clearance in cm
Bacillus amyloliquifaciens	0.8 cm
Bacillus subtilis	0.4 cm

Table 1

3.2 Submerged fermentations (Standard)

For the control fermentation experiment, the liquid medium containing 1% Soluble starch (carbon source), 1%yeast extract (nitrogen source) at pH-7, temperature – 37°C, DOT – Aerobic, the growth pattern, α- amylase activity and reducing sugar concentration were observed for four days and recorded (Figure.1). The enzyme production was not recorded in day 0, and the maximum α-amylase activity was found in day 1, 2.5×10^3 U/ml, following gradual depletion in the following day due to the depletion of nutrition.

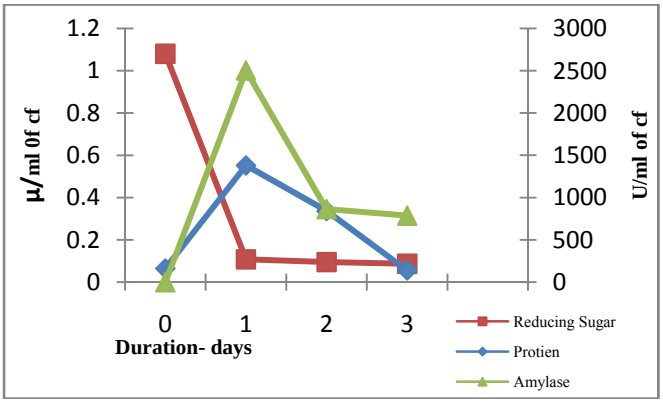

Figure 1

Time course of *Bacillus amyloliquifaciens* on 1% soluble starch, 1% yeast extract

Fermentation Conditions: Temperature - 37°C, pH – 7, DOT - Aerobic

3.3 Submerged fermentations (Using different carbon and nitrogen sources)

Three submerged fermentations were carried out differing carbon and nitrogen sources; Figure-2 shows the result of submerged fermentation using 1% wheat starch and 1% yeast extract as the carbon and nitrogen source. Here maximum α-amylase activity was found in day2, 7.6×10^3U/ml. Figure-3 shows the result of fermentation using 1% soluble starch as carbon source and 1% peptone as nitrogen source, in this time course the maximum amylase activity was recorded on day2, 1.3×10^4U/ml. Figure-4 shows the result of fermentation using 1% Soluble starch and 1% meat extract as carbon and nitrogen source and the best amylase activity was found on day 1, 6.08×10^3U/ml.

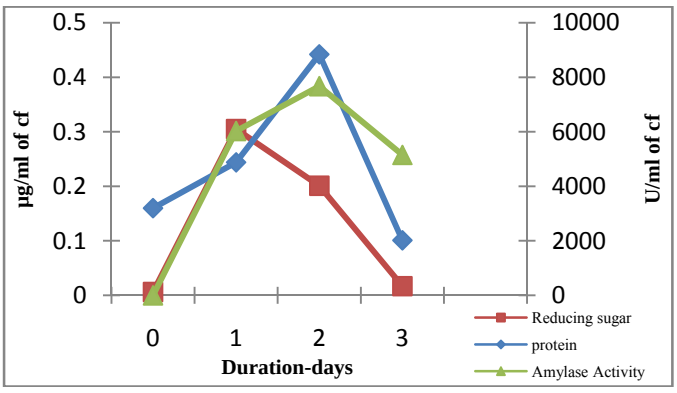

Figure 2

Time Course of Unknown isolate on 1% Wheat starch, Yeast extract1%

Fermentation Conditions: Temperature - 37°C, pH - 7, DOT – Aerobic

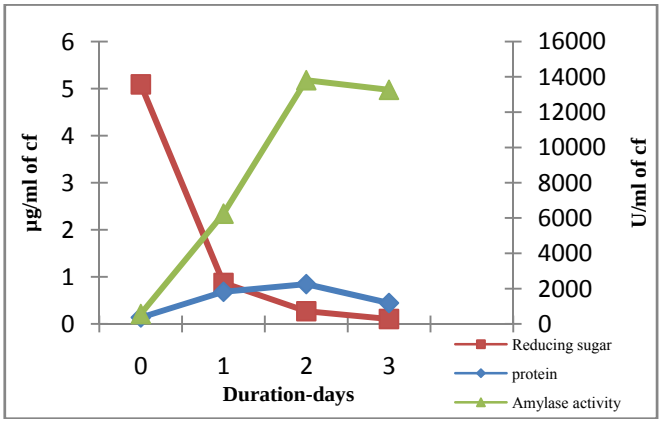

Figure 3

Time Course of *Bacillus amyloliquifaciens* on 1% Soluble Starch, 1% peptone

Fermentation Conditions: Temperature - 37°C, pH - 7, DOT – Aerobic

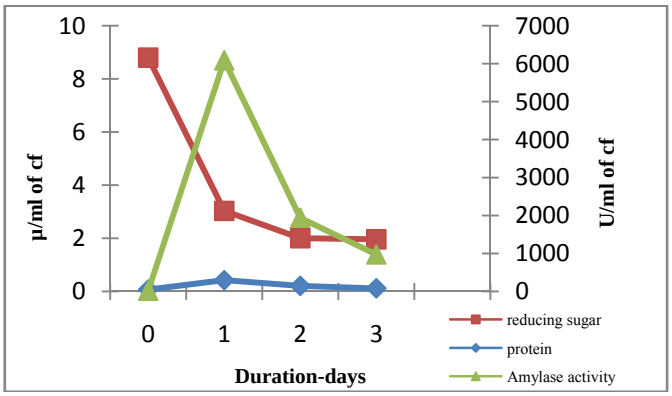

Figure 4

Time Course of *Bacillus amyloliquifaciens* on 1% soluble starch, meat extract-1%

Fermentation Conditions: Temperature - 37°C, pH - 7, DOT – Aerobic

4. Discussion

Primarily a semi quantitative estimation was done successfully on a solid medium to detect the amylase production. This was a very rapid and simple method which was effective in determining the amylase producers [9].

The submerged fermentation results shows that, Carbon sources greatly influence α-amylase production and the most commonly used substrate is starch. According to a previous report it shows that cells grown in a medium containing a carbon source other than sugars (citrate or glutamate) produce more enzyme than the cells grown in a medium with starch [15]. And this report is proven in the present study. The standard fermentation and other two fermentations, which used soluble starch, had the enzyme production faster than the fermentation which used wheat starch. And among the different nitrogen sources tried peptone was found to be best than yeast extracted and meat extract. It provides the approach to the proven study, that the adequate amount of nitrogen source in the microbial production of enzymes increase the production of enzymes [3]. By adding 1% peptone in the production medium, as the nitrogen source, the micro organisms had a long stationary phase along with constant increase in amylase production.

For the production of stable α- amylase different parameters like carbon source, temperature, inoculum age, inoculum size, pH, and modification of existing enzymes through protein engineering [3] are being studied and many works are in progress due to the application and necessity of enzyme.

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References

1) Basks T, Janssen AEM and Boom RM. The effect of carbohydrates on α- amylase activity measurements. Enzyme and microbial technology, (39), pp.114-119, 2006.
2) Pandey A, Nigam P, Soccol CR, Soccol VT, Singh S and Mohan R. Advances in Microbial amylases. Biotechnology applied biochemistry, (31), pp. 135-152, 2000.
3) Gupta R, Giras P, Mohapatra H, Goswami VK and Chuhan B. Microbial α- amylases: a biotechnological prespective. Process biochemistry, (38), pp. 1599-1616, 2003.
4) Mitidieri S, Martinelli AHS, Schrank A and Vainstein MH. Enzymatic detergent formulation containing amylase from *Aspergillus niger*: A comparative study with commercial detergent formulations. Bioresource technology, (96), pp. 1217-1224, 2006.
5) Santamaria RI, Rio GD, Saab G, Rodriguez ME, Soberon X and Munguia A. Alcholysis reactions from starch with α- amylases. FEBS LETT, (452), pp. 346-350, 1999.
6) Haq I, Ashraf H, Javed I and Quadeer MA. Production of α- amylases by *Bacillus lichiniformis* using an economical medium. Bioresource technology, (87), pp. 57-61, 2003.
7) Murayama T, Tanabe T, Ikeda H and Ueno A. Direct assay of α- amylase using flurophore-modified cyclodextrins. Bioorganic and medicinal chemistry, (14), pp. 3691-6, 2006.
8) Biely P, Misloviocova D, Markovic O and Kalac V. A new chromogenic substrate for assay and detection of α-amylase. Analytical biochemistry, (172), pp. 176-179, 1988.
9) Akpan I, Bankole MO and Adesemowo AM. A rapid plate culture method for screening of amylase producing microorganisms. Biotechnology Techniques, (13), pp. 411-413, 1999.
10) Wilson JJ, and Ingledew WM. Isolation and characterization of Schwanniomyces alluvius Amylolytic enzymes. Applied and environmental microbiology, (44), pp. 301-107, 1982.

11) Miller GL, Blum R, Gennon WE and Burton AL. Measurement of carboxymethylcellulase activity. Analytical biochemistry, (2), pp. 127-132, 1960.
12) Staden JF and Mulauduzi LV. Flow injection spectrophotometric assay of α- amylase activity. Analytica Chemica Acta, (421), pp. 19-25,2000.
13) Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Journal of Biology and chemistry, (193), pp. 265-275, 1951.
14) Zhang Q, Tsukagoshi N, Miyashiro S and Udaka S. Increased production of α- amylase by *Bacillus amyloliquifaciens* in presence of glycine. Applied and environmental microbiology, (46), pp. 293-295,1983.
15) Eliana de Oliveria Santos and Meire Lelis Leal Martins. Effect of the medium composition on formation of Amylase by *Bacillus* sp, (46), pp. 129-134, 2003.

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