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Original Article

Optimization of Nitrogen Source(s) for the Growth and Amylase Production from *Bacillus licheniformis* JAR-26 under Submerged Fermentation

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Abstract

The growth and enzyme production in microorganisms need optimum physical (temperature, pH, and aeration) and chemical (carbon, nitrogen and mineral ions) environment which have critical role in determining growth behaviour and biochemical production in cultured microorganisms. The present study aimed to investigate effect of different concentrations of organic nitrogen sources i.e. peptone, tryptone, soytone, beef extract, malt extract and yeast extract (0.5, 1.0, 1.5, 2.0, 2.5% w/V) and inorganic nitrogen sources i.e. $NaNO_3$, KNO_3, $(NH_4)_2SO_4$, NH_4Cl and $(NH_4)_2HPO_4$ (0.1, 0.25, 0.5, 1.0, 1.5% w/V) on growth and amylase (EC 3.2.1.1, 1, 4-á-D-glucanglucanohydrolase) enzyme production from *Bacillus licheniformis* JAR-26. Among the tested nitrogen sources, organic sources proved superior over inorganic sources and malt extract proved best for amylase production (maximum at 1.5%, 4.427 U/ml of medium) with growth/OD of 1.766 and bacteria could utilize 98.4% of the available sugar in the medium. Yeast extract was second suitable organic source for enzyme production (maximum enzyme production at 2%, 4.314 U/ml) and Growth/OD of 1.650, and bacteria could utilize 97.1% of total sugar in the medium. Soytone proved poorest organic nitrogen source for amylase production and growth of bacteria with maximum enzyme yield of 3.08 Uml^{-1} at 2.0% and growth/OD of 0.95 at 2%. Among the five inorganic nitrogen sources, none was found suitable for amylase production by *B. licheniformis* JAR-26.

Keywords: Amylase; Submerged Fermentation; *Bacillus licheniformis* JAR-26; Nitrogen Source.

Introduction

Amylases are one of the most utilized industrial enzymes for hydrolyzing starch molecules to diverse products like dextrins and progressively smaller polymers composed of glucose and/or maltose and glucose units. A significant increase in amylase production and utilization occurred in the early 1960s when *Bacillus subtilis* α-amylase and *Aspergillus niger* glucoamylase were used for the production of dextrose from starch as alternative to acid hydrolysis. Amylases hold maximum (about 30%) market share of enzyme sales with major industrial applications in starch processing, brewing and sugar production, food and paper production, textile, and detergent manufacturing [1]. Amylases are calcium metalloenzymes, divided into three groups according to their amylolytic specificity i.e. α-amylase; which cleaves the bond in interior of the substrate (endoamylase); β-amylase, which acts on the reducing end of the substrate (exoamylase); and amyloglucosidase, which liberate glucose units from the non-reducing end of substrate molecules [2]. Microbial amylases have completely replaced chemical hydrolysis in starch processing industry [3]. The production of microbial amylases from bacteria depends on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements (particularly carbon and nitrogen), incubation period, pH, temperature and metal ions [4]. Applications of bacterial amylases at industrial level have stimulated the interest to explore several microbes as bioresources for their amylolytic activity. The nature and concentration of nitrogen source in

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the culture medium affects bacterial growth and amylase production and acts as pH stabilizer. Many investigators have recorded that organic nitrogen sources support maximum amylase production in several bacteria [5]. Almost all *Bacillus* species synthesize α-amylase, thus this genus holds promise to dominate the enzyme production industries. *Bacillus licheniformis* JAR-26, isolated from spoiled tomatoes, is an acidophilic and thermostable extracellular α-amylase producing acidophilic bacteria reported in a previous study [6].

In the present study, an attempt has been made to optimize growth and amylase production from *Bacillus licheniformis* JAR-26 using different concentrations of various organic (peptone, tryptone, soytone, casamino acid, beef extract, malt extract and yeast extract) and inorganic (KNO_3, $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, and $(NH_4)_2HPO_4$) nitrogen sources under submerged fermentation.

Materials and Methods

Microorganism

Starch hydrolyzing *Bacillus licheniformis* JAR-26 was isolated from spoiled tomatoes and collected in sterilized stoppered glass vials.

Media and Chemicals

Starch,Yeast extract, Peptone, $MgSO_4.7H_2O$, KH_2PO_4, NaCl, $CaCl_2$, Agar, Distilled H_2O, Phosphate buffer, Iodine solution, 3, 5 dinitrosalicylic acid (DNS), $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, KNO_3, $(NH_4)_2HPO_4$, Malt extract, Beef extract, Tryptone, Soytone, Sephadex G-100, DEAE-Cellulose (DE-52), CM-Cellulose, Acrylamide, Bis-acrylamide, N,N,N'n'-tetramethylethane-1,2-diamine (TEMED), Sodium dodecyl sulphate (SDS) Ammonium persulphate.

Isolation of Microorganism

The thermostable, acidophilic starch hydrolyzing bacteria (*B. licheniformis* JAR 26) was screened for extracellular acidophilic amylase production by using starch medium containing (g/L): Starch (Merck, Germany), 10.0; yeast extract, 5.0; peptone, 2.0; $MgSO_4.7H_2O$,0.5; KH_2PO4, 0.5; NaCl, 1.5; $CaCl_2$, 0.1; Agar, 20.0. Initial pH was adjusted to 5.5. One gram of each sample was suspended in 9.0 ml of sterile water and 0.1 ml of suitably diluted suspension was spread on the agar plates. The plates were incubated at 45, 50, 55 and 60°C for 24 to 48 h. The isolated colonies were flooded with iodine solution and colonies bearing good colorless halos around them were picked and maintained on starch agar slants at 4 °C and further assessed for enzyme production in liquid medium. The characterization and identification of the isolate was made following Bergey's Manual of Systemic Bacteriology. The method of identification used was as given by Collee *et al*. [7].

Amylase Production

The basal fermentation medium for enzyme production contained(g/L): Starch, 10.0; Maltose, 20.0; yeast extract, 5.0; peptone, 5.0; KH_2PO4, 0.12; $CaC1_2.2H_20$, 0.12; $MgSO_4.7H_20$, 0.12; $MnSO_4.4H_20$, 0.02. Initial pH of the medium was adjusted at 5.5 and 50 ml of medium in 250 ml of Erlenmeyer flasks were inoculated with a cell suspension of optical density (OD) 0.5 (prepared from 24 h old culture). All the flasks were incubated for four days on a rotary shaker (Remi) at 170 rpm at 45°C. Samples were drawn after a time interval of 12 h, centrifuged at 8000 Xg for 10 minutes and cell free culture supernatant was used as enzyme source.

Assay of Enzyme

Culture filtrate (Supernatant) was used for assessing enzymatic activity by the method of Srivastava and Baruah [4]. One ml of 1% (w/V) starch (Merck, Germany) solution was taken in test tube and 0.2 ml of 0.2 M phosphate buffer (pH 5.5) and 0.2 ml of deionized water was added to it. The mixture was equilibrated at 70°C for 10 minutes in a water bath. 0.1 ml of supernatant was added and then reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid (DNS). The mixture was heated and the color intensity was measured at 540 nm [8] using a spectrophotometer (Systronics Spectrophotometer 169). One unit of amylase activity was defined as the amount of amylase that liberates 1.0 mg of glucose per minute under assay conditions. In all the above experiments the enzyme activity was calculated as the average of 3 independent sets of experiments (the s.d. in all cases was found negligible).

Effect of Nitrogen Source

The nitrogen content [yeast extract (10 g/L) + peptone (2 g/L)] of basal fermentation medium was replaced with different concentrations of organic nitrogen sources i.e. Yeast extract (YE), Peptone, Malt extract (ME), Beef extract, Tryptone, Soytone (0.5, 1.0, 1.5, 2.0, 2.5% w/V) and inorganic nitrogen sources i.e. $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, KNO_3, $(NH_4)_2HPO_4$

(0.1, 0.25, 0.5, 1.0, 1.5% w/V) and its effect was recorded on bacterial growth, amylase production and sugar utilization by test bacteria.

Results

The data on growth, amylase production and sugar utilization by *B. licheniformis* JAR-26 at different concentrations of organic and inorganic nitrogen supplements are shown in Fig. 1 to 6.

Among organic nitrogen sources, malt extract proved best for amylase production (maximum at 1.5%, 4.427 U/ml of medium) where growth/OD was 1.766 and bacteria could utilize 98.4% of the available sugar in the medium. Yeast extract was second suitable organic source for enzyme production (maximum enzyme production at 2%, 4.314 U/ml) with Growth/OD of 1.650, and bacteria could utilize 97.1% of total sugar in the medium. Beef extract, peptone and tryptone were moderately suitable nitrogen sources for amylase synthesis with maximum amylase yield of 4.025, 3.372 and 3.683 Uml^{-1}, respectively, at 2.0% Beef extract, 1.5% peptone and 2.0% tryptone. Soytone proved poorest organic nitrogen source for amylase production and growth of bacteria with maximum enzyme yield of 3.08 Uml^{-1} at 2.0% and growth/OD of 0.95 at 2%. Comparison of various treatment combinations revealed maximum biomass production of *B. licheniformis* JAR-26 on 2% Beef extract whereas maximum amylase production on 1.5% of malt extract under submerged fermentation.

Among the five inorganic nitrogen sources i.e. $NaNO_3$, KNO_3, $(NH_4)_2SO_4$ NH_4Cl and $(NH_4)_2HPO_4$ (0.1, 0.25, 0.5, 1.0 and 1.5% w/V), none was found suitable for amylase production by *B. licheniformis*

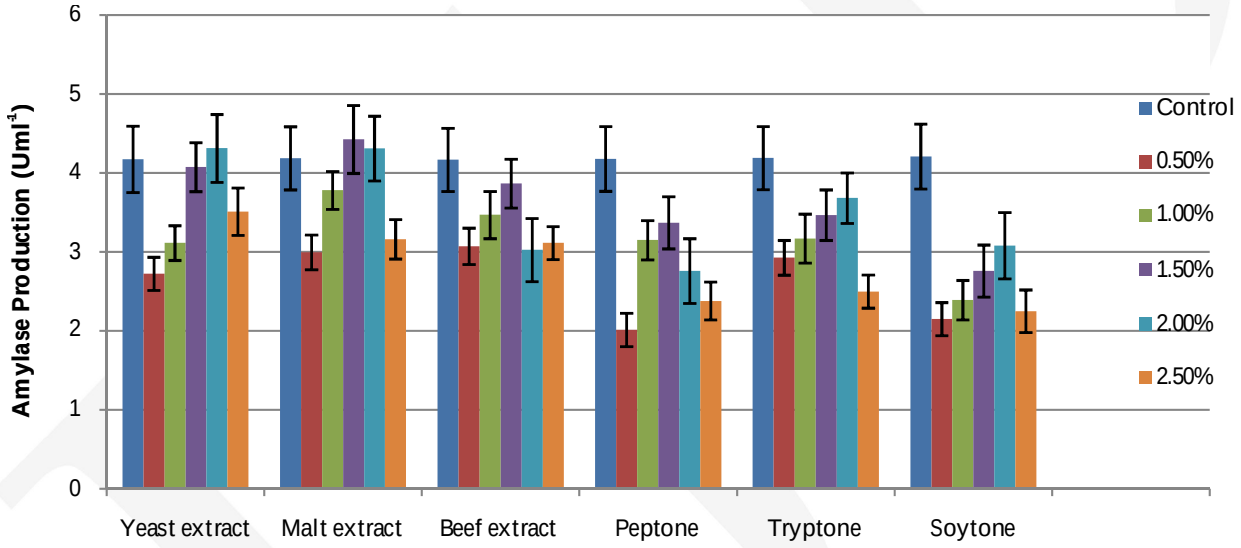

Fig. 1: Effect of different organic nitrogen source(s) on amylase production by *B. licheniformis* JAR-26

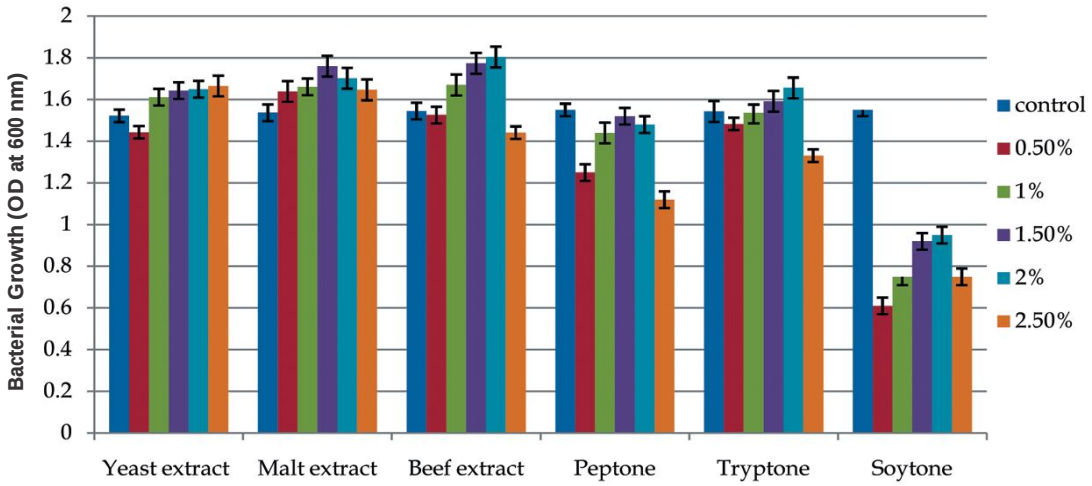

Fig. 2: Effect of different organic nitrogen source(s) on growth of *B. licheniformis* JAR-26

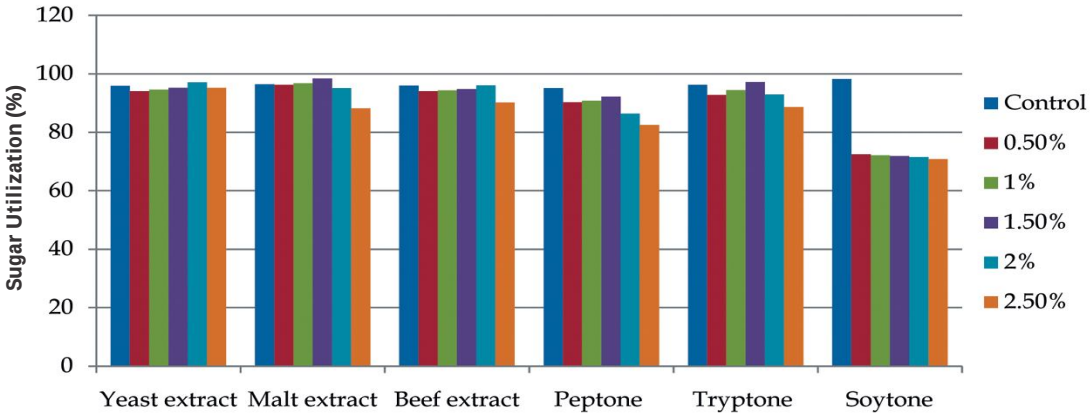

Fig. 3: Effect of different organic nitrogen source(s) on sugar utilization by *B. licheniformis* JAR-26

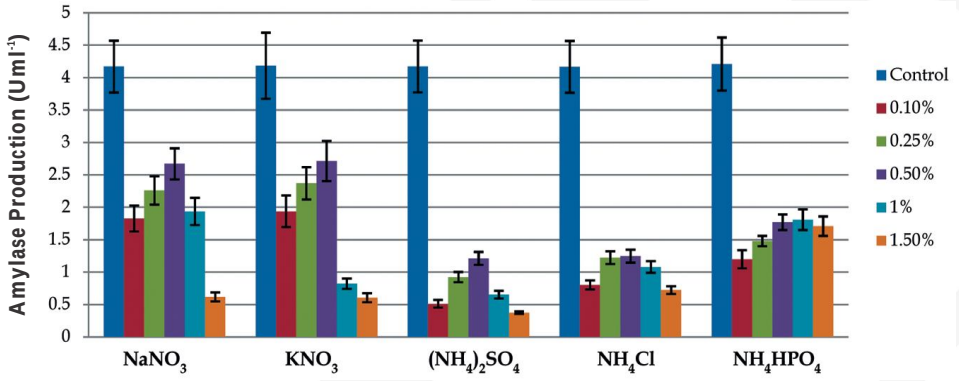

Fig. 4: Effect of different inorganic nitrogen source(s) on amylase production by *B. licheniformis* JAR-26

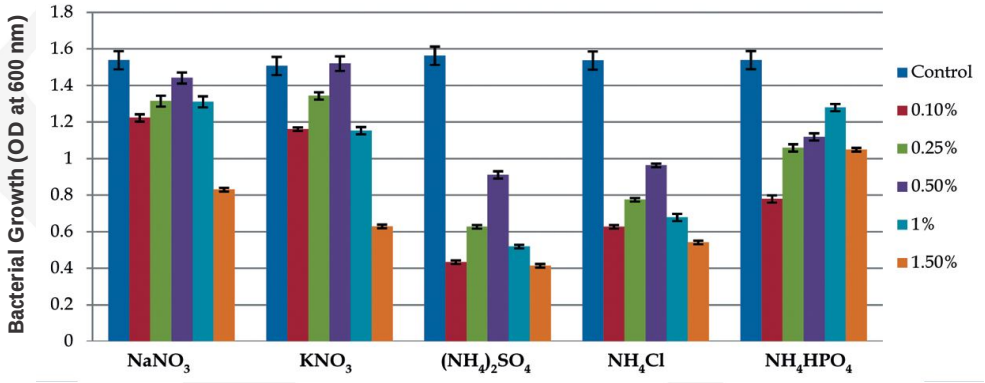

Fig. 5: Effect of different inorganic nitrogen source(s) on growth of *B. licheniformis* JAR-26

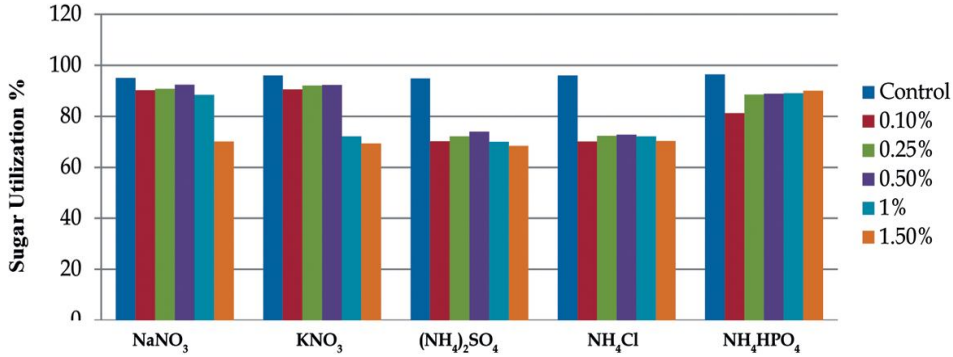

Fig. 6: Effect of different inorganic nitrogen source(s) on sugar utilization by *B. licheniformis* JAR-26

JAR-26. Both $NaNO_3$ and KNO_3 supported good growth of *B. licheniformis* JAR-26 when used as sole nitrogen source in the medium and the growth / OD obtained (1.442 and 1.521 OD at 600 nm, respectively) were more or less similar than those observed in case of control medium (1.539 and 1.521 OD at 600 nm). Among tested inorganic nitrogen sources KNO_3 shows maximum amylase production of 2.714 Uml^{-1} at 0.5% concentration. Inorganic nitrogen sources proved inferior in comparison to organic nitrogen sources with respect to amylase production as well as biomass production from *B. licheniformis* JAR-26.

Discussion

After Carbon, Nitrogen is another major nutrient that is required by the microorganisms in comparatively larger amounts. Nitrogen forms the essential part of proteins, enzymes, nucleotides and cofactors that play vital role in metabolism. The nature and relative concentration of nitrogen source in the medium affected growth and amylase production from *B. licheniformis* JAR- 26. Figures 1 to 6 indicate that the growth of *B. Licheniformis* and amylase production greatly depends upon the nitrogen source in the medium. Negi and Banerjee [9] have opined that not all nitrogen sources would act as enhancers for the production of amylases and the response differs from species to species. Among various organic nitrogen sources (peptone, tryptone, soytone, beef extract, malt extract and yeast extract) tested, malt extract was found to be the best source for amylase production (4.427 Uml^{-1}) as well as growth (1.766 OD at 600 nm) of organism in medium containing 1.5% malt extract. Very few previous studies included malt extract among their organic nitrogen sources tested for optimization of bacterial amylase production. However, similar results have been reported by Demirkan [10] on *Bacillus subtilis* wild type and mutant form (U2-6) and malt extract and tryptone were found best for amylase production from wild type and mutant form, respectively. Yeast extract was proved to be second most amylase producing nitrogen source with highest enzyme yield of 4.314 Uml^{-1} recorded at 2.0% in the present study. Similar results have been reported by Narang and Satyanarayan [11] in case of *Bacillus thermoolevorans* where Yeast extract individually and in combination with other nitrogen sources favored growth as well as amylase production.Yeast extract has also been reported better nitrogen substrate for amylase enzyme production in many previous studies of *Bacillus* species [12, 13]. In this study, beef extract, peptone and tryptone were moderately suitable nitrogen sources for amylase synthesis with maximum amylase yield of 4.025, 3.372 and 3.683 Uml^{-1} at 2.0% Beef extract, 1.5% peptone and 2.0% tryptone, respectively.

In contrast to present findings, various previous studies have reported peptone as a good organic nitrogen source for bacterial amylase enzyme production [14, 15, 16]. In case of *B. subtilis* KC-3, Vijaylakshmi *et al.* [15] proved peptone (24.64 Uml^{-1}) to be the most suitable organic nitrogen substrate followed by tryptone (21.66 Uml^{-1}). Soytone proved poorest organic nitrogen source for amylase production with maximum enzyme yield of 3.08 Uml^{-1} at 2.0%.

Among the five inorganic nitrogen sources tested, none was found suitable for amylase production by *B. licheniformis* JAR-26. Among inorganic nitrogen sources tested, KNO_3 showed maximum amylase production (2.714 Uml^{-1}) at 0.5% concentration and a slight increase in growth rate (1.521 OD at 600 nm) than control (1.508 OD at 600 nm). Avdiiuk and Varbanets [17] reported sodium nitrate (0.2%) as best inorganic nitrogen source for a-amylase production by *Bacillus subtilis* 147. Similarly, Zar *et al.* [18] reported slightly superior production of a-amylase using *Bacillus amyloliquefaciens* IIB-14 using NH_4NO_3.

Conclusion

The present findings reveal that organic nitrogen sources are better far amylase production by *B. licheniformis* than the inorganic sources. Malt extract was most suitable for amylase production at the 1.5% concentration. Yeast extract ranked second suitable source for amylase production with 4.314 Um^{-1} at 2.0%. Beef extract, peptone and tryptone were found to be the moderate and Peptone proved poorest organic nitrogen source for amylase production. Among the five inorganic nitrogen sources none was found suitable for amylase production and enzyme yields in all cases were poor than that of malt extract and yeast extract. However, comparatively good biomass growth was observed at 0.5% KNO_3 in comparison to control (OD-1.521).

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