The Chittagong Univ. J. B. Sci.,Vol. 4(1 &2):73-81 , 2009.

OPTIMIZATION OF CONDITIONS FOR EXTRACELLULAR AMYLASE PRODUCTION FROM *LISTERIA DENITRIFICANS*.

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ABSTRACT

The foodborne *Listeria denitrificans* was isolated from degraded rice and studied as an amylase producer. This microorganism showed maximum amylase production at temperature 27 ºC and medium pH 7.0, and on 3rd day during incubation period. Maximum amylase production by the isolate was found in medium containing 3.0 % starch as carbon source and 0.50 % yeast extract as nitrogen source.

Key words: *Listeria denitrificans*, Amylase activity, Factors influence.

INTRODUCTION

Use of microorganisms in enzyme production is much more economical and environmentally friendly than conventional chemical synthetic methods. They use less energy and utilize renewable resources, and production leftovers are highly biodegradable. They can grow in a wide range of environmental conditions (Trevan *et al*.2003). Amylases are among the most important enzymes and are of great significance in present day biotechnology. Although they can be derived from several sources such as plants, animals and microorganisms, the enzymes from microbial sources generally meets industrial demands. Microbial amylases could be potentially useful in the pharmaceutical and fine chemical industries if enzymes with suitable properties could be prepared.

With the advent of new frontiers in biotechnology, the spectrum of amylase application has widened in many other fields, such as clinical, medicinal and analytical chemistries. Wide spread application of this enzyme in starch saccharification, textiles, food, brewing and distilling industries very common.
Selection of a suitable strain is the most significant factor in the amylase production process. The environmental factors required for the optimum growth of the microorganism being employed for production may differ from those required for the production of enzymes (Pandey *et al*.2000). Parameters that

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influence microbial amylase production include pH (Mahmoud 1993, Bormiss *et al*.1981), temperature (Bormiss *et al.*1981, Hizukuri *et al.*1983, Marzan *et al.* 2001), incubation period (Bormiss *et al*.1981, Marzan *et al.* 2001, Rahman *et al*.1993) and presence of different carbon and nitrogen sources(Mahmoud1993, Guo *et al*.1988, Lachmund and Ruttkowski 1990, Okolo *et al.*1995). This paper reports on the screening and identification of a bacterial isolate obtained from degraded rice and also on the determination of its optimum culture conditions involved in maximum production of amylase.

MATERIALS AND METHODS

Microorganism

The isolate *Listeria denitrificans* (NB_2) was isolated from degraded rice by enrichment technique and purified by repeated pour plate and streak plate methods using nutrient agar (NA) medium.

Screening of the isolates for amylolytic activity

For primary selection of the isolates, hydrolysis of starch agar plate method was followed. After primary selection, secondary screening of the isolates was made by measuring amylase activity in liquid medium by quantitative method. For *in vitro* production of amylase by the isolates, three different broth media, namely- Starch yeast extract medium (Adams 1997), Defined salt medium (Almeida *et al.* 1997) and Standard production medium (Kwan *et al.* 1993) were used. After secondary Screening, on the basis of their better enzyme activity in broth medium the bacterial isolate (NB_2) was finally selected for detailed study.

Measurement of enzyme activity

One ml of culture filtrate was added to 5 ml of 1% soluble starch prepared in 0.2 M citrate phosphate buffer pH 5.8 and 1 ml of 0.2 M citrate phosphate buffer was taken in a test tube and incubated at 40°C for 1 hour (Rahman *et al*.1993). The amount of reducing sugars released during incubation was measured by Nelson's modification of Somogyi method (Nelson 1944). Enzyme activity was expressed by amount of Glucose released/ml extracted enzyme /unit time (U/mL).

Biomass yield

Bacterial biomass was determined by measuring the absorbance at 600 nm (Henriette *et al*. 1993).

Identification

The bacterial isolate NB_2 was identified on the basis of its morphological, cultural and physiological characteristics. These characteristics were compared with standard description of Bergey's Manual of Determinative Bacteriology (Buchanon and Gibbons, 1974).

Optimization of culture conditions for maximum production of amylase

Effect of incubation period

The effect of incubation periods on the maximum production of amylase by selected isolate was studied. For this, 50 ml of selected medium was taken in each 100 ml conical flask and autoclaved. After cooling the flasks were inoculated and incubated for 24, 48, 72, 96 and 120 hours. The culture was centrifuged at 8000 rpm for 15 min at 4^0C.

Effect of medium pH

To observe the effect of medium pH on enzyme production, the medium was prepared at different pH (4.5, 6.0, 6.5, 7.0 and 8.5), inoculated and incubated at 37ºC. The effect of medium pH on biomass characteristics, biomass yield and amylase activity was recorded.

Effect of temperature

After inoculation the culture medium was incubated at different temperature values such as 10^0C, 27 ± 2^0C, 37 ± 2^0C, and 45 ± 2^0C for maximum enzyme production. The effect of temperature on biomass characteristic, biomass yield and amylase production was recorded.

Effect of carbon and nitrogen sources

Four carbon (starch, rice powder, potato, wheat powder) and five nitrogen sources (malt, yeast extract, ammonium di-hydrogen phosphate, ammonium sulphate and potassium nitrate) were added separately to the medium (Standard Production Medium) and the effect of this carbon and nitrogen sources on the production of amylase, extracellular protein and biomass yield was recorded. To ascertain optimum proportion of suitable carbon and nitrogen sources, the study was carried out with 0.5 to 4% carbon and 0.025 to 2% nitrogen sources keeping other experimental conditions at optimum level.

RESULTS AND DISCUSSION

Primarily, 35 microbial isolates (fungal + bacterial) were isolated from different samples including rice, wheat and bread by enrichment and pour plating

technique. These isolates were purified, preserved and tested for their amylolytic ability.

Screening of the isolates

On the basis of their better enzymatic activity in three different broth media, the bacterial isolate NB_2 was finally selected for detailed study.

Identification of the selected isolates

On the basis of their morphological, cultural and biochemical characteristics, the bacterial isolate was found to belong to the genus *Listeria*. They were provisionally identified as *Listeria denitrificans* Prevot.

Optimization of culture conditions for maximum production of amylase

Effect of incubation period on amylase production

The bacterial isolate NB_2 exhibited different rate of enzyme activity at different incubation period and maximum at 3^{rd} day (Fig:1). The highest biomass yield was recorded after 5 days of incubation. The pH of the supernatant was found to range from 5.8 to 6.0. Production of maximum amylase within 48-72 hours of incubation period by bacterial isolate was reported by Sidhu *et al.* (1997).

Effect of incubation temperature on amylase production

The bacterial isolate NB_2 exhibited maximum enzyme production at 27ºC (Fig:2) and maximum biomass yield at 37ºC. The color of the supernatant was golden yellow and the change of pH of the supernatant was found to range from 4.76 to 5.27. Haroun *et al.* (1993) reported maximum amylase production by *Streptomyces* strain at 37ºC.

Effect of medium pH on the production of amylase

The bacterial isolate NB_2 exhibited maximum enzyme production at medium pH – 7.0 (Fig:3) and maximum biomass yield at medium pH 6.0. The color of the supernatant was golden yellow and the change of pH of the supernatant was recorded to range from 5.2 to 6.11. The isolate exhibited turbid growth with sedimentation at different medium pH. The optimum pH of the bacterial isolate NB_2 was recorded to be 7.0 which is closely related to the observations of Hisaka *et al*. (1993) in *Flavobacterium odoratum*.

Effect of carbon and nitrogen sources on the production of amylase

The pH values of the culture supernatant of the bacterial isolate NB_2 were found to vary from 5.76 to 7.15 and the colour was also found variable (transparent, golden yellow, deep brown to red oxide) with different carbon and

nitrogen sources. Highest biomass yield was recorded with starch and malt when used as carbon and nitrogen sources, respectively. The bacterial isolate exhibited highest amylase production in starch and yeast extract containing medium (Fig:4) and also showed that the optimum concentration of starch and yeast extract for maximum enzyme production were 3.0 % and 0.50 %, respectively (Fig:5). Maximum amylase production by *Aspergillus fumigatus* was reported when 0.25% $(NH_4)_2HPO_4$ and 4% starch were added in the growth medium (Cherry *et al*. 2004).

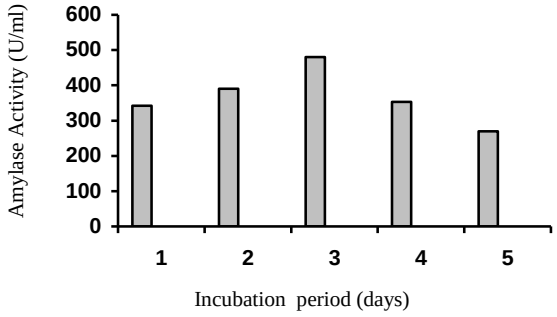

FIGURE 1. EFFECT OF INCUBATION PERIOD ON THE PRODUCTION OF AMYLASE BY THE ISOLATE *L. DENITRIFICANS*.

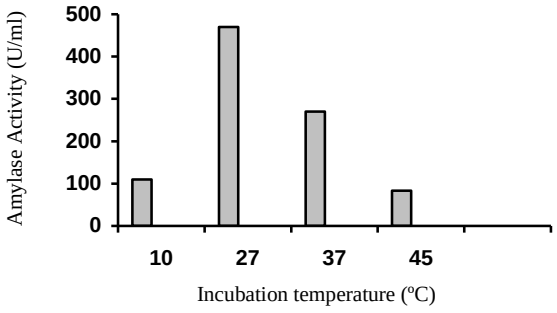

FIGURE 2. EFFECT OF INCUBATION TEMPERATURE ON THE PRODUCTION OF AMYLASE BY THE ISOLATE *L. DENITRIFICANS*.

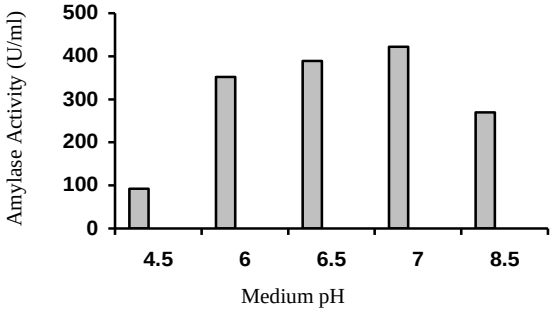

FIGURE 3. EFFECT OF MEDIUM PH ON THE PRODUCTION OF AMYLASE BY THE ISOLATE *L. DENITRIFICANS*.

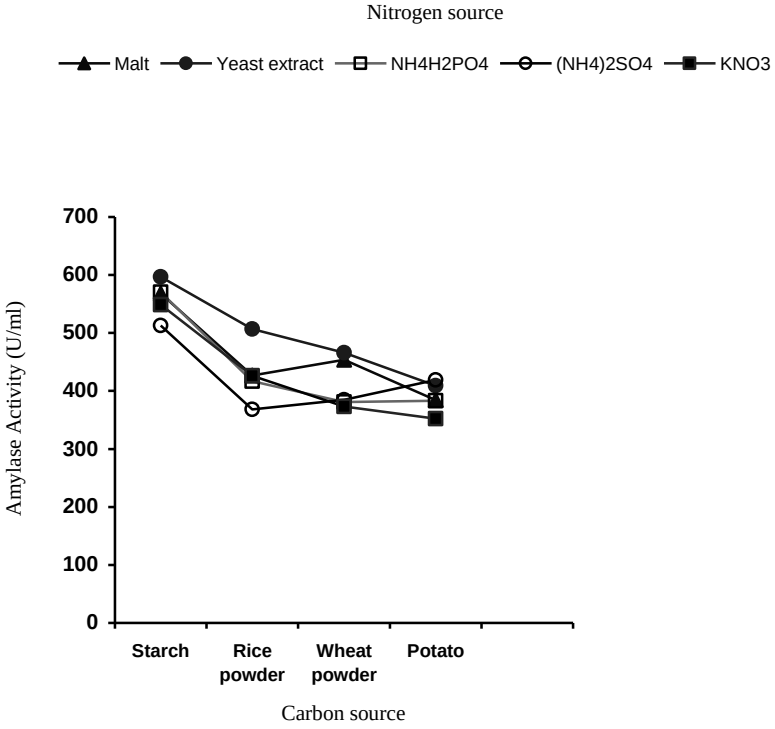

FIGURE 4. EFFECT OF CARBON AND NITROGEN SOURCES ON THE PRODUCTION OF AMYLASE BY THE ISOLATE *L. DENITRIFICANS*.

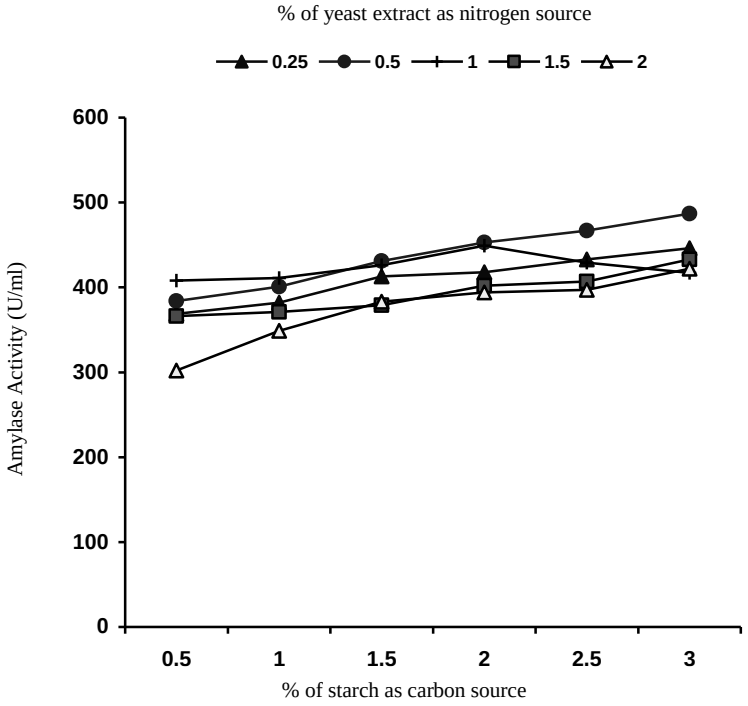

FIGURE 5. EFFECT OF CONCENTRATION OF STARCH & YEAST EXTRACT ON THE PRODUCTION OF AMYLASE BY THE ISOLATE *L. DENITRIFICANS*.

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Manuscript received on 18.6.09; Accepted on 26.6. 08

The Chittagong University Journal of Biological Sciences, Vol.4 (1 & 2): Page No : 73-81