American-Eurasian J. Agric. & Environ. Sci., 12 (11): 1507-1513, 2012
ISSN 1818-6769

DOI: 10.5829/idosi.aejaes.2012.12.11.1845

Optimization of Culture Conditions for the Production of Amylase from *Bacillus licheniformis* on Submerged Fermentation

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Abstract: The present study is concerned with the optimization of cultural conditions for the production of α-amylase from *Bacillus licheniformis*. The various parameters have conducted for maximum enzyme production. For *Bacillus licheniformis*, incubation period of 48 h, pH of 7.0, 30°C incubation temperature were found to be optimum for the production of α-amylase. Work was carried out for the optimization of "amylase production by *Bacillus licheniformis* grown on different carbon sources but maximum amount of amylase was obtained on lactose added medium. Likewise, the amylase production was also optimized by using different organic and inorganic nitrogen sources, but the optimum production of amylase were found in the presence of yeast extract and ammonium sulphate, respectively. The result on surfactants induced protease production by *Bacillus licheniformis* revealed that Tween 80 had the higher influence on enzyme production.

Key words: Protease · *Bacillus licheniformis* · Optimization · 16S rRNA gene sequence and submerged fermentation

INTRODUCTION

Enzymes are biological catalysts; they are highly specialized catalytic proteins with extraordinary catalytic power and also have remarkable specificity. They are essential for all forms of life by catalyzing the various chemical reactions in the cells. Even though enzymes catalyze only one kind of chemical reaction, there are many enzymes in a typical cell. Enzymes are soluble and colloidal substances characterized by great activity and specificity. They have their core roles in survival, growth and metabolism in living systems.

Amylases are a group of hydrolases that can specifically cleave the O-glycosidic bonds in starch. Two important groups of amylases are glucoamylase and α-amylase. Glucoamylase (exo- 1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.3) hydrolyzes single glucose units from the nonreducing ends of amylose and amylopectin in a stepwise manner [1]. Whereas α- amylases (endo-1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.1) are extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units inside the linear amylose chain [2]. Amylases can be derived from a variety of living organisms, ranging from microorganisms to plants and humans. Microorganisms are the most important sources for the production of amylases. The *Aspergillus* species produce the extracellular amylase enzyme having significant industrial importance. Microbial amylases are used in food industry, textile and paper industries [3,4]. The extracellular thermostable amylases enzyme ranks first in terms of industrial point of view due to various industrial application [5], particularly in starch processing industry. Industrially important enzymes have traditionally obtained from submerged fermentation (SMF) because of the ease of handling and greater control of environmental factors such as temperature and pH.

The use of submerged culture is advantageous because of the easy of sterilization and process control is easier to engineer in these systems. Depending upon on the strain and culture conditions, the enzyme can be cultivable or inducible showing different production patterns. The purpose of this work was to study the production of amylase by *Bacillus lichniformis* in submerged fermentation and optimized culture conditions for the production of amylase.

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MATERIALS AND METHODS

Gut Microbial Analysis: The gut was dissected out from the experimental fish under aseptic condition and then the length and weight of the gut was measured. Then the gut was ground well in phosphate saline buffer (PB5) and used as the stock. This stock solution was serially diluted and then spreaded on the agar plate. The method is used to isolate pure culture and also for estimating the total viable colonies (TVC).

CFU/ml = Number of bacterial population per 1 ml sample

Microorganism: *Bacillus licheniformis* used in the present study was isolated from the gut of estuarine fish *Etroplus suratensis* collected from Rajakkamangalam, Kanyakumari District, south west coast of India. Screening was carried on starch agar plate [6]. It was maintained on potato dextrose agar slants at 4°C.

Amylase Assay: Amylase was assayed by incubating 0.5% of soluble starch solution (Prepared in 0.1 M phosphate buffer) at 55°C for 15 min [7]. The reaction was terminated by adding 1 ml of dinitrosalicylic acid reagent followed by incubating in a boiling water bath for 10 min and the final volume was made up to 12 ml with distilled water and optical density was taken at 540 nm [8]. One unit of amylase activity was defined as the amount of enzyme that releases one micromole of reducing sugar as glucose per minute under standard assay conditions and expressed as units per gram dry substrate.

Optimization of Media Components

Effect of Incubations on Amylase Production: The effect of incubation time on amylase production was quantified by incubation interval at different hours of incubations such as 24, 48, 72, 96 and 120h.

Effect of Carbon Sources on Amylase Production: To assess the consequence of different carbon sources on amylase production was determined by using various carbon sources such as Glucose, Sucrose, lactose, Maltose, Arabinose, Sorbitol, Mannitol, Starch and Raffinose were supplemented individually. The enzyme production was monitored after 48h of incubation shaking at 120rpm.

Effect of Nitrogen Sources: The result of organic nitrogen sources was studied by using various nitrogen sources such as soya meal, beef extract, peptone, urea, yeast extract. The respective nitrogen sources were added as a sole source of nitrogen (0.5%, w/v). The enzyme production was monitored after 48 h of incubation.

Effect of Inorganic Nitrogen Sources on Amylase Production: The inorganic nitrogen sources included ammonium nitrate, ammonium chloride, sodium nitrate and potassium nitrate. The respective nitrogen sources were added as a sole source of nitrogen (0.5%, w/v). The enzyme production was observed after 48h of incubation.

Effect of Salinity on Amylase Production: Effect of salinity on amylase yield was evaluated by measuring optical density at 620 nm using casein medium (M6) supplemented with a gradient of salt (0-8%, w/v) at 35°C for 48h incubation.

Effect of Temperature on Amylase Production: The effect of temperature on amylase production was studied by varying the temperature (10-80°C). The enzyme production was measured after incubation for 48 h shaking at 120 rpm.

Effect of pH: In order to investigate the influence of pH on amylase production, the isolate was grown in a medium at varying pH (3-10). After incubation for 48 h at 35°C under shaking conditions at 120 rpm, the amylase production was quantified.

Effect of Surfactants on Protease Production: Surfactant induced protease production was assessed by using 7 different surfactants such as Tween 20, Tween 40, Tween 60, Tween 80, Triton X 100, poly ethylene glycol (PEG) and sodium dodecyl sulphate (SDS). The selected surfactants were incorporated individually into the optimized medium at 0.2% concentration and the medium without surfactant was treated as control.

RESULTS

Isolation of Amylolytic Bacterial Strains: A total of 4 out of 16 bacterial isolates /strains possessed amylolytic activity. Among the four bacterial isolates, only one bacterium was found to be higher amylase production.

Identification of Amylase Positive Colony: Based on the morphological, physiological and biochemical characteristics the suspected colony was identified as *Bacillus licheniformis* by the following standard keys of

Table 1: Morphological and Biochemical characteristics

Characteristics	Observation
MorphologyGram's staining	Gram positive rods in singles, pairs and short chains; 1.5 x 0.5µm.
Spores staining	Ellipsoidal and cylindrical, central sub terminal, swelling the sporangium.
Motility	Motile.
Colony property	On nutrient agar, colonies are 1-2 mm circular, smooth round, waxy, opaque, smooth, shiny, flat, slight cream to white, entire to erose margin and mucoid producers no pigment.
Oxygen requirement	Aerobic
Biochemical characters	Observation
Indole production test	-
Methyl red test	-
Voges Proskauer test	+
Catalase	+
Oxidase	+
Gelatin Liquefaction	+

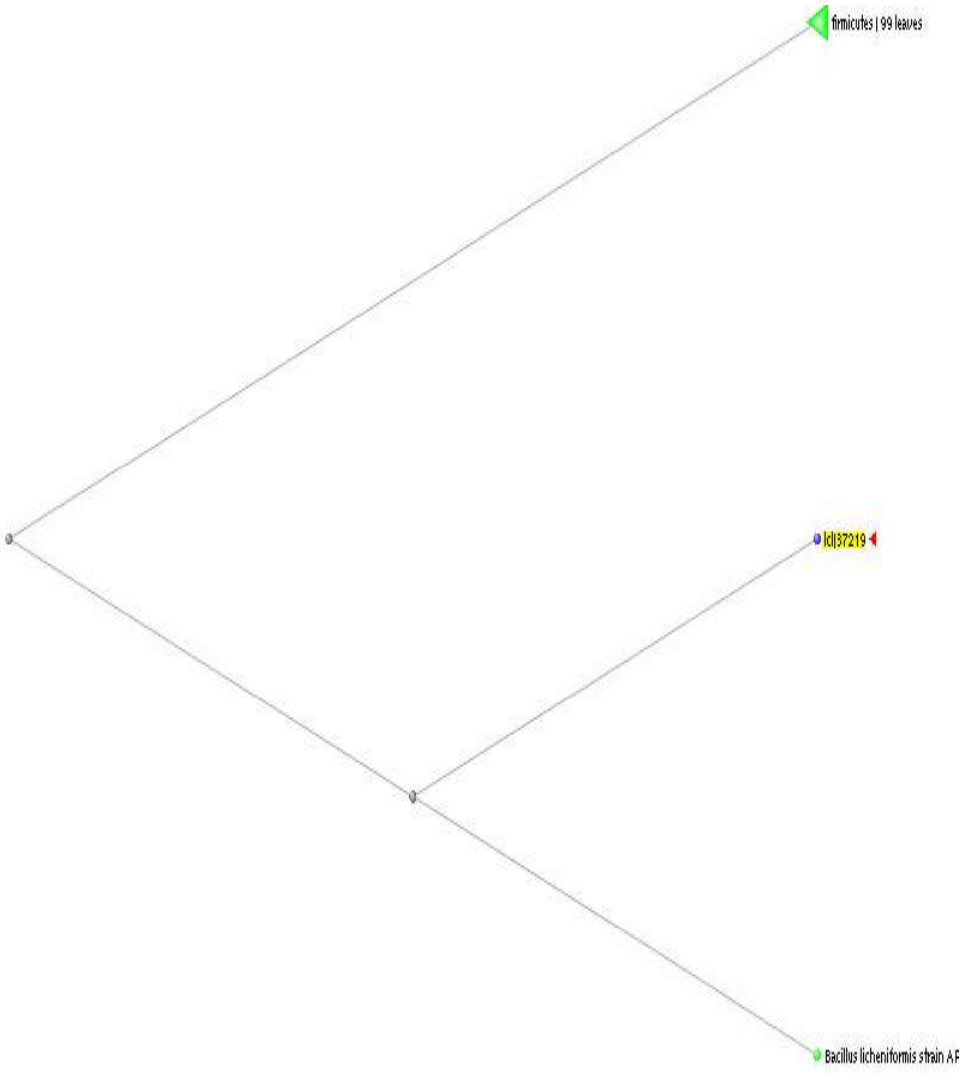

Fig. 1: Force view of *Bacillus licheniformis*

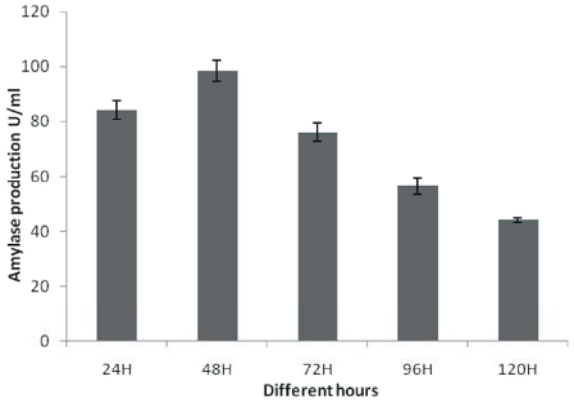

Fig. 2: Production profile of amylase in medium incubated with different hours at 35°C

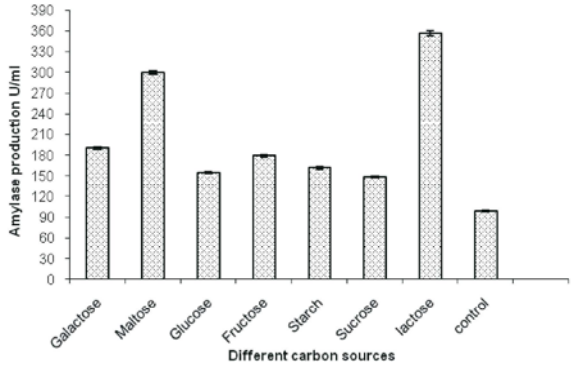

Fig. 3: Effect of various carbon sources on amylase production by *B. licheniformis* in basal medium supplemented with 0.5% carbon sources at 35°C for 48 h incubation

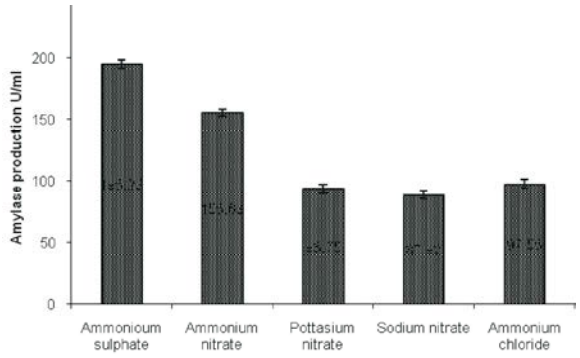

Fig. 4: Effect of various inorganic nitrogen sources on amylase production by *B. licheniformis* in basal medium supplemented with 0.5% nitrogen sources

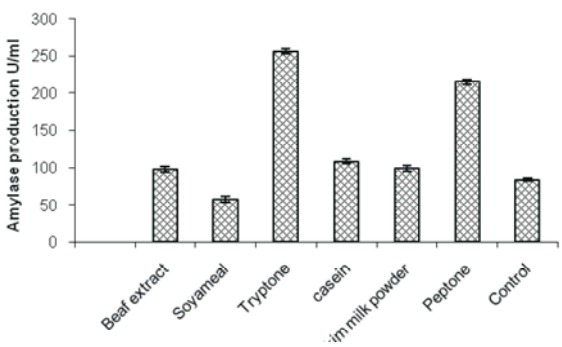

Fig. 5: Effect of various organic nitrogen sources on amylase production by *B. licheniformis* in basal medium supplemented with 0.5% nitrogen sources

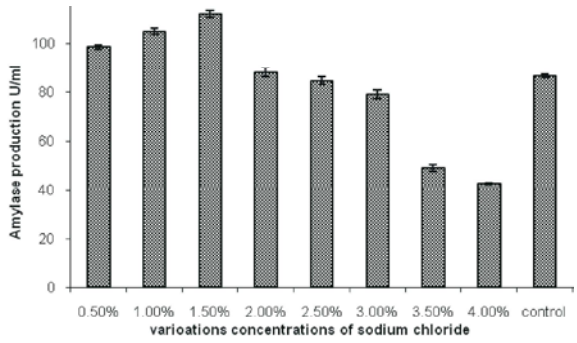

Fig. 6: Effect of sodium chloride on amylase in medium supplemented with various concentrations of sodium chloride

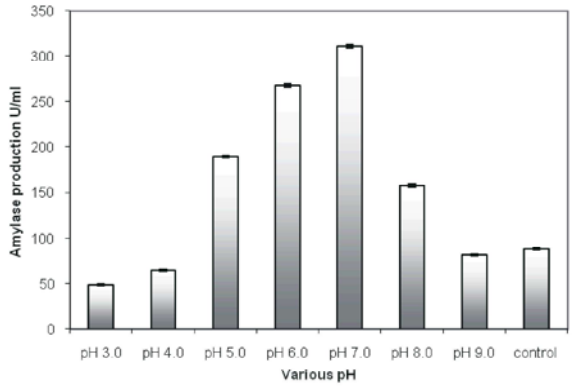

Fig. 7: Production report of different pH on amylase production. The cells were cultivated in medium with various pH by *B. licheniformis*

Bergey's Manual of Determinative Bacteriology (Table 1) and the isolated bacterial strain was screened for amylase producing ability on starch agar. The zone formation around the bacterial colony indicated the amylase positive strain. Hence the strain was identified as an amylase producer and it was taken for further experimental studies (Plate-1). Phylogenetic studies revealed that the 16S rRNA gene sequence of *Bacillus*

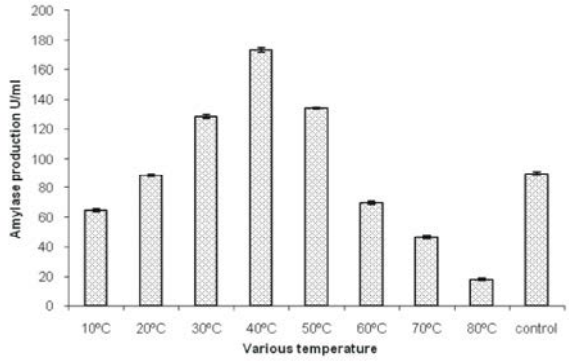

Fig. 8: The effect of various temperatures on amylase production. The cells were incubated in various temperatures at 48 of incubation by *B.licheniformis*

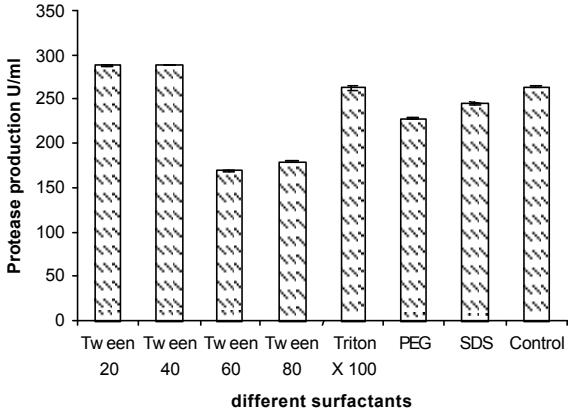

Fig. 9: Production profile of amylase in medium supplemented with various kinds of surfactants by *B. licheniformis* at 35°C for 48 h incubation

liceniformis 97% similarity with the nearest match in the Gene bank (Fig. 1).

Effect of Incubation Time on Protease Production: Figure 2 shows incubation time on the amylase production indicated that the 48 hours of incubation was suitable for *Bacillus licheniformis*. A gradual reduction of protease yield with increased in incubation time.

Effect of Carbon Sources on Amylase Production: The effect of carbon sources on amylase production by *Bacillus liceniformis* after 48 h of incubation period indicated that, it was maximum in lactose supplemented medium (Fig. 3).

Effect of Inorganic Nitrogen Sources: Among the tested nitrogen sources, the maximum amount of amylase production was registered in ammonium sulphate added medium after 48 h of incubation. But the minimum amount of amylase production was observed in ammonium chloride supplemented medium (Fig. 4).

Effect of Inorganic Nitrogen Sources on Amylase Production: Among the tested nitrogen sources, the maximum amount of amylase production was registered in tryptone added medium after 48h of incubation. But the minimum amount of amylase production was observed in peptone supplemented medium (Fig. 5).

Effect of Various Concentration of NaCL: The effect of NaCl on amylase production by *Bacillus liceniformis* inferred that, it was high in 1.5% concentration. The lowest amount of enzyme production was recorded in 4% NaCl supplemented medium (Fig. 6).

Effect of Ph and Temperature: The effect of pH on amylase production revealed that it was maximum at pH 7.0 and minimum at pH 3.0 (Fig. 7). Similarly in the experimentation on the effect of temperature (Fig. 8) the maximum amylase production was recorded in 40°C and minimum was registered at 80°C.

Effect of Surfactants: Result on surfactants induced protease production after 48 h of incubation indicated that, Tween 80 added medium produced maximum enzyme. The minimum amount of enzyme production was recorded in Tween 40 added medium (Fig. 9)

DISCUSSION

Amylases are widely distributed and are one of the most studied enzymes. These enzymes have wide scale application ranging from food to effluent treatment. Amylases are a class of enzymes (hydrolases) that are capable of digesting the glycosidic linkages found in starch or glycogen. Under aqueous conditions, amylases act on glycosidic bonds present in starch to liberate glucose, maltose, maltotriose etc [9,10]. Starch degrading enzymes like amylase have received a great deal of attention because of their perceived technological significance and economic benefits. This enzyme is also useful for the commercial production of glucose. Nowadays, the renewed interest in the exploration of extracellular amylase production in bacteria and fungi is due to various industrial applications. Few attempts have been made to elucidate the control mechanism involved in

the formation and secretion of the extracellular enzymes. The production of alpha amylase by moulds has been greatly reported [11]. In present work *Bacillus licheniformis* was found as an effective enzyme producer through submerged fermentation process. In the present study the highest amount of amylase production was observed in lactose supplemented medium. This observation was supported by earlier studies of Anto *et al*. [1] who reported that the production of amylase was stimulated by the presence of glucose, lactose and starch by *Bacillus* sp.

Among tested nitrogen sources, organic nitrogen source supported maximum amylase yield when compared to inorganic nitrogen source. In this study the highest level of amylase yield was registered in tryptone added medium. This is correlated with earlier studies of Nanag and Satyanarayana [12], who reported that starch and tryptone have important source for maximizing the amylase production. The amylase synthesis by microorganisms has been correlated to the presence (or) absence of different nitrogen sources and various amino acids in the growth medium. The differences in nutritional requirements of various α-amylase producing organisms could be attributed to the differences in their genetics [13].

In this study, the maximum amylase production was recorded in ammonium nitrate supplemented medium within the tested inorganic nitrogen sources. Michelina and Castillo [14] have reported that the supplementation of inorganic nitrogen salts greatly increased the enzyme yields in *Aspergillus foetidus*. The inhibitory effect of some of the salts may be related to the pH changes associated with their use in the medium. The enzyme is very sensitive to pH. Therefore the selection of optimum pH is very essential for the optimum production of α-amylase. In this study the highest yield of amylase was recorded in pH at 7.0 and temperature at 40°C. Narayana and Vijaylakshmi [15] also reported the optimum temperature and pH as 30°C and 6.5, respectively, for the production of α-amylase from fungal species. The present study various surfactants were used for maximizing the amylase production. Among the tested surfactants, the maximum yield was recorded in Tween 80 supplemented medium over the control. In consistence with this present study, microbes such as *Bacillus* sp [16] and *Thermomyces* sp [17] were reported that the higher yield was registered in surfactant added medium. Swain *et al.* [18] were also reported that the increased enzyme yield might be due to cell membrane permeability. The addition of carbon source in the form of either monosaccharide or polysaccharides may influence production of amylase enzyme [19].

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