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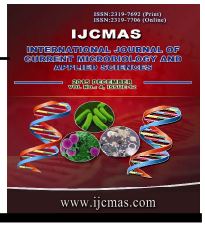

Original Research Article

Solid Substrate Fermentation using Agro Industrial Waste: New Approach for Amylase Production by *Bacillus licheniformis*

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Keywords

Amylase, Optimization, Agro industrial, Solid substrate fermentation

A B S T R A C T

In the present study amylase production was optimized using Solid Substrate fermentation after screening of four types of agro industrial waste material as substrate i.e wheat straw, paddy straw, sugarcane waste, and maize straw. The results obtained in the present study indicated *Bacillus licheniformis* as a potential strain for a-amylase production using solid-state fermentation with paddy as substrate. Various physiochemical parameters were also optimized like fermentation period, pH and inoculum concentration. Maximum enzyme production was found to be 37ºC and pH7. Effect of inorganic salt (NaCl, $FeCl_2$, $MgSO_4$, KH_2PO_4 and $CaCl_2$) and various surfactants (Tween20, Tween80, PEG,

Introduction

Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market (Rajagopalan and Krishnan, 2008). There has been an increasing trend towards the utilization of the solid state fermentation (SSF) to produce several enzymes from micro-organisms (Sodhi, 2005).

The selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation. Need for improved enzyme production technology for amylase has attracted attention due to its increasing demand in the food, brewing and textile industry. It has also become necessary to find cheap alternative

substrates for amylase production. The agro-industries produce large quantities of residues that pose serious problems of disposal, in spite of them being sources of biomass and nutrients. These substrates can be used for production of valuable compounds such as enzymes and various secondary metabolites (Soccol and Vandenberge, 2003). In the view of advantages of amylase, the present study was aimed at optimizing the fermentation parameters for the enhanced production amylase enzyme through solid substrate fermentation.

Materials and Methods

Microorganism

The bacterial strain *Bacillus licheniformis* MTCC 1483 was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. The bacterial strain was revived in Brain heart infusion broth. The bacterial strain was sub-cultured periodically after 4 days and stored at 4°C until further use.

Agro-Waste as Substrates for Amylase Production

Wheat straw, sugarcane bagasse, maize straw and paddy straw were chosen as a substrate for the production of α- amylase by SSF. They were procured from local market of Mohali (Landran). Substrates were washed with distilled water for 2-3 times and then treated with 1% NaOH for 30 min. After that substrates were autoclaved and dried in oven at 80^0C for two days. Dried substrates were ground in the grinder to make small particles.

Fermentation Medium

Solid-state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks containing 5 g of each substrate i.e paddy straw, wheat straw, sugarcane and maize straw. The substrate was moistened with 10 ml of distilled water (KH_2PO_4 0.1g/L, NaCl 0.25g/L, $MgSO_4$ 0.01g/L, $CaCl_2$ 0.01g/L) autoclaved at 121°C for 15 min and cooled. The flasks were inoculated with 1% (v/w) bacterial inoculum and incubated at 37°C.

Enzyme Assay

Amylase was assayed using supernatant containing crude enzyme by Dinitrosalicylic method (Miller, 1959) and optical density was taken at 540nm, on UV-spectrophotometer. For this method 1 ml enzyme extraction was taken and 1% starch and 1 ml phosphate buffer. Mixture was incubated at 50^0C for 30 min. After incubation added 2 ml DNS reagent and again incubated at boiling temperature for 5 min cooled at room temperature. After that add 1 ml potassium sodium tartrate and made up the final volume up to 10ml with distilled water and took the absorbance at 540nm.Blank was prepared by without adding starch. The amylase production was determined in IU/ml/min by applying the standard formula.

Enzyme activity = micromoles of glucose released per ml per minute (IU/ml/min)

Effect of Carbon and Nitrogen Source

Different carbon sources (1%w/v) such as lactose, glucose, starch and fructose were incorporated into the basal medium by replacing the carbon source in the

production medium to analyze its role on the production of both the enzymes. Similarly, the enzyme production was optimized by different nitrogen sources (1%w/v) such as yeast extract, peptone, ammonium chloride, ammonium nitrate and ammonium sulphate incorporated in the basal medium.

Optimization of Culture Conditions

The various operating variables for fermentation were optimized which included pH, temperature, time of incubation and inoculum concentration. Optimal temperature and pH were obtained by varying the temperature range from 30°C to 50°C and pH 4.0 to 9.0. In addition, production of enzymes was monitored from 12 to 72hr of incubation.

Effect of Inorganic Salt and Surfactants

The effect of metal ions on the production of α-amylase was studied using the following metal ions i.e NaCl, $FeCl_2$, $MgSO_4$, KH_2PO_4 and $CaCl_2$. The effect of various surfactants on α-amylase production was evaluated by adding Tween20, Tween80, PEG and SDS.

Partial Purification of Enzyme

The crude enzyme produced was subjected to ammonium sulphate precipitation to remove the unwanted protein components (40% saturation for α-amylase).

Molecular Weight Determination

Molecular weight was determined using SDS- polyacrylamide gel electrophoresis (Kariya et al, 2003). The separation was done in denaturing conditions on 12.5% polyacrylamide gel. Samples in 10 µl quantities were loaded into electrophoretic wells and electrophoresis done at room temperature using a constant current of 150 mA per gel for 2 hours. The standard marker was used for reference purpose and Coomasie brilliant blue staining was done for band visualization.

Statistical Analysis

All the readings are taken in triplicate and expressed as Mean±S.E.M.

Results and Discussion

Selection of Agro-Industrial Waste for Amylase Production

Selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation. Different solid substrates were found to effect the production of enzymes. Four substrates i.e wheat straw, paddy straw, sugarcane waste and maize straw were used in the present study. The highest enzyme activity (0.104 IU/ml/min) was observed from the extract obtained using paddy straw (Table. 1). So paddy was chosen as best substrate for amylase production and further parameters were optimized using paddy straw as substrate in the medium.

Optimization of Carbon and Nitrogen for Amylase Production

Various carbon sources (Table. 2) and nitrogen sources (Table. 3) were tested for their ability to increases amylase production. It was found that starch (carbon source) and yeast extract (nitrogen source) were able to

increase amylase activity to 0.359 IU/ml/min and 0.675 IU/ml/min respectively in the medium.

Optimization of Physico-Chemical Parameters

Evaluation of various physico-chemical parameters are required in order to determine the optimum conditions for amylase and protease production by *Bacillus sp*. (Mukesh et al., 2012). In the present study various operating variables for fermentation were optimized which included pH, temperature, time of incubation and inoculum concentration. Optimal temperature and pH for amylase production were found to be 35°C and pH 7 (Table. 4 and 5). These results corroborate with findings of the study conducted by Mather et al., 2011 and Mukesh et al., 2012. In addition, production of enzymes was monitored from 12 to 72hr of incubation. It was found that maximum enzyme production occurred after 24hr during the stationary phase of growth. There was a decline in production of enzyme as indicated by reduced enzyme activity after 48hr (Table. 6). However, maximum amylase enzyme production can also occurs at 96hr (Ashwaniet et al., 2011). Results of varying inoculum concentration on amylase production are given in Table. 7, which indicate that inoculum concentration higher than 1% resulted in decreased enzyme activity per ml. This could be attributed to the fact that bacteria might have utilized medium faster and has undergone decline phase due to nutrient depletion.

Effect of Inorganic Salts and Surfactants on Amylase Production

Results of effect of various inorganic salts and surfactants on amylase activity are given in figure 1. It was found that enzyme produced by *Bacillus licheniformis* MTCC 1483 is stable in the presence of most of the inorganic salts and surfactants at 1% (w/v) concentration. Amylase activity was found to be maximum with Tween-80. There are reports that Tween 80 significantly increase amylase production at lower concentration (Bhardwaj et al., 2012). However, PEG and $FeCl_2$ proved to be most inhibitory.

Crude enzyme was partially purified using ammonium sulphate precipitation (40% saturation). After that the protein was denatures using SDS and run through PAGE (Polyacrylamide gel electrophoresis). It was found that molecular weight of enzyme was 27.5 kDa (approx.).

In the present study, culture conditions and media components were optimized for better production of the amylase enzyme from *Bacillus licheniformis* MTCC 1483 using solid state fermentation. The optimized production medium was found to be cost effective, convenient and easier to scale up. Also the enzyme was found to be effective against inhibitory surfactants and inorganic salts.

Table.1 Production of -Amylase by SSF using Different Agro-Waste as Substrates

Sr. No.	Substrate	Amylase activity (IU/ml/min)
1	Sugarcane bagasse	0.062±0.002
2	Wheat straw	0.060±0.001
3	Paddy straw	0.104±0.002
4	Maize straw	0.077±0.001

Table.2 Effect of Different Carbon Sources (1% w/v) on Production of Amylase by SSF

Sr. No.	Carbon source	Amylase activity (IU/ml/min)
1	Starch	0.359±0.001
2	Starch + Glucose	0.248±0.002
3	Starch+ Lactose	0.337±0.001
4	Starch + Fructose	0.277±0.003

Table.3 Effect of Different Nitrogen Sources (1% w/v) on Production of Amylase by SSF

Sr. No.	Nitrogen source	Amylase activity (IU/ml/min)
1	Ammonium chloride	0.389±0.002
2	Ammonium sulphate	0.378±0.003
3	Ammonium nitrate	0.488±0.001
4	Peptone	0.410±0.003
5	Yeast extract	0.675±0.001

Table.4 Effect of Temperature on Production of Amylase by SSF

Temperature in ° C	Enzyme production(IU/ml/min)
30	0.013±0.001
35	0.017±0.004
40	0.011±0.003
50	0.009±0.002

Table.5 Effect of pH on Production of Amylase by SSF

pH	Enzyme production(IU/ml)
4	0.790±0.002
5	0.770±0.001
6	0.890±0.002
7	0.950±0.004
8	0.820±0.002
9	0.170±0.003

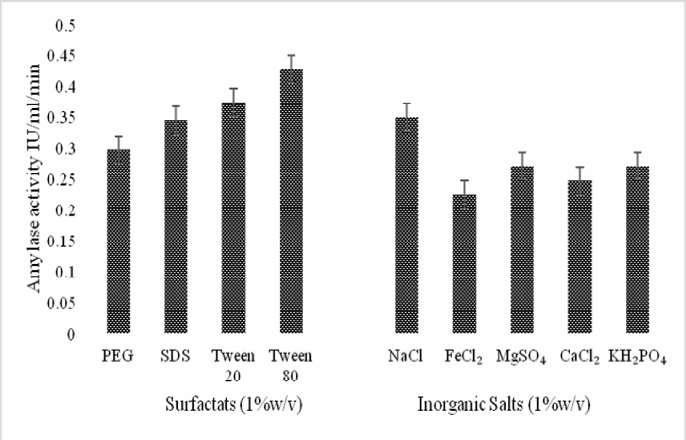
Table.6 Effect of Incubation Time on Production of Amylase by SSF

Incubation time (hr)	Enzyme production(IU/ml/min)
12	0.006±0.001
24	0.130±0.002
48	0.034±0.001
72	0.009±0.003

Table.7 Effect of Inoculum Concentration on Production of Amylase by SSF after 24hr

Inoculum ($2x10^6$ cells /ml)	Enzyme production(IU/ml/min)
1%	0.170±0.002
2%	0.160±0.001
3%	0.130±0.001
4%	0.119±0.002
5%	0.090±0.003

Figure.1 Effect of Various Inorganic Salts and Surfactants on Amylase Activity



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