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RESEARCH ARTICLE

A Study on Optimization of Microbial Alpha-Amylase Production

[*]C.R Shalinimol[1]

[1]Department of Microbiology, Scott Christian College, Nagercoil, Kanyakumari, India.

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ABSTRACT

The present study is concerned with the production of α- amylase by the bacterial species Bacillus. The study is focused mainly on the optimization of α- amylase from sago industrial waste. Various optimization parameters like carbon sources, nitrogen sources, incubation time, pH and temperature are preferred for getting good results. Morphological and biochemical characteristics are used to identify the strain of Bacillus species. The maximum amount of amylase production is observed under various measurements. The total protein content of amylase enzyme is found out. The optimum temperature for the production of high amount of amylase is categorised. It is noted that the carbon source like maltose could improve the production of amylase. Casein acts as a good nitrogen source for the production of amylase. pH values are maintained individually in each growth. These results provide the ways in which α- amylase is produced in considerable amounts by bacterial species which are isolated from the industrial waste soil. These amylases are important hydrolase enzymes which have been widely used to convert starch molecules to simple sugars. Among amylases, α-Amylase is in maximum demand due to its wide range of applications in the industrial front. They are used in detergents, paper industry, textile industry, food industry and many other industrial applications. Nowadays consumers are very much aware of the health and environmental problems and hence enzymes are used instead of chemical catalysts. Though enzymes are obtained by several plants, animals and microbial sources, enzymes from microbes are preferred due to its availability and ease of production in large scale sectors. One of the leading environmental issues is waste management. Hence, by considering the waste samples of industries for the production of amylase, effective and intensive utilisation of waste can be measured thereby obtaining useful products in biological ways. Biochemical characterization of sago industry waste shows that it is rich in carbohydrates and contains 55-60% of starch.

Keywords: α- amylase, Optimization, Casein, Waste management, pH.

1. INTRODUCTION

The most important enzymes are the amylases which are of greater significance in the field of biotechnology. They can be obtained from several sources such as plants, animals and microorganisms. A large number of microbial amylases are available. These amylases are used instead of chemical hydrolysis of starch because amylases are bio-catalysts, which increase the rate of any reaction in starch processing industries. The amylases resulting from microorganisms have a broad spectrum of industrial applications as they are more stable when compared to plant and animal α-amylases. The crucial boon of using microbial amylase is that the multiplication in the growth of microorganisms is rapid, resulting in economical bulk production capacity of enzymes and hence enzymes of appropriate characteristics are obtained which are preferred by large scale industries. Amylases are characterised as follows:

α-Amylase: The main sources of α-amylase are bacteria, fungi and yeasts. Among these sources enzymes from fungal and bacterial sources have a greater influence over other applications in industrial sectors. The hydrolysed composition obtained from the hydrolysis of starch depends upon the effect of

*Corresponding author. Tel.: +919790539264
Email address: shalinimol_2008@yahoo.com (C.R.Shalinimol)
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temperature, the conditions of hydrolysis and the origin of enzyme. The optimum pH is 7.

β-Amylase: Primary sources of β-amylase are the seeds of higher plants and sweet potatoes. During ripening of fruits, β-amylase breaks down starch into maltose resulting in the sweetness of the ripened fruit. The ideal pH of the enzyme ranges from 4.0 to 5.5.

γ-Amylase : γ-amylase is highly stable and efficient in acidic environments and has an optimum pH of 3.

Among the various physiological parameters, pH of the growth medium plays a vital role by inducing morphological changes in the organism and enzyme secretion. Usually pH from 6 to 10 is maintained for the growth of the medium and enzyme production. The results of the present study are found to be ideal at pH of 7 which starts declining due to increase in alkalinity. Physical factors such as pH, temperature, aeration and agitation are significant for optimization process. The decrease in enzyme production is associated with the reduction in cell growth and inactivation of enzymes. Several factors that affect the outcome are culture medium, concentration of acids, temperature, presence of solvents, nitrogen and carbon sources and moisture content.

2. RELATED STUDIES

[1] The enzyme activity is regulated by a reaction mixture which contains 1 ml of casein in 0.05 M glycine-NaOH buffer. The optimum nitrogen source for the production of enzyme is casein and yeast extract. It is found that the ideal temperature for its production is 30^0 C. The result shows that the strain growth and enzyme production varies with incubation period and the ideal time is found to be 72 hours. [2] Starch converting enzymes are currently in use in various industries. Various starch converting enzymes correspond to α-amylase family. (β/α) 8 barrel structure, hydrolysis and amino acid residues are its characteristics and it includes 21 reactions. Protein crystallization and X-ray crystallography techniques produce 3D structures of this family. [3] For the production of alpha amylase, industrial residues of banana peel and corn pith are used as substrates which are rich in organic compounds. Here, Taguchi method and orthogonal array design are used to improve the quality of amylase production and to examine the factors affecting its outcome. [4] The paper deals with the production of extracellular α-amylase from Bacillus subtilis AX20. Several biochemical tests are performed to characterise the species of microbes. At 37°C of its stationery phase, utmost amylase activity of 38 U/ml is obtained where ideal pH and temperature are maintained. [5] The studies focus on improving the efficiency of alpha amylase production by the temperature shifting strategy. i.e. the temperature is shifted from higher to lower levels or vice-versa. Hence the overall production of α-amylase is significantly improved. Constant incubation period is maintained over all the temperature variants. [6] To increase the productivity of amylase, culture conditions are optimized with soluble starch as the substrate. The effects of temperature, acidity regulation, fermentation time and the culture mediums are analysed and optimum values are provided. In this research, initial pH and incubation period are set to be 7 and 48 hours respectively at 33°C. [7] This research was based on the production of alpha amylase by using the agro-industrial by-products and organic materials such as maltose, sucrose, lactose and starch. Ideal constraints are regulated by Box-Behnken experimental design which contributes for the response surface methodology. It is concluded that increase in starch leads to increase in production yield. [8] It indicates that the major source of α-amylase is microorganisms. The experiment emphasizes on the optimization of amylase production in which pasta cooking water is selected as the basal medium. pH is maintained to be 6 and the incubation period and temperature as 72 hours and 40°C accordingly. [9] Purification of the enzymes after its production is discussed in this paper. It is found that the specific activity of the enzyme amylase is said to be increased with respect to purification. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis and zymography analysis are the test analysis carried out for molecular weight determination and enzyme activity processing respectively. The study in [10] employs response surface methodology and central composite design to provide ideal solid state fermentation condition. To analyse the maximum enzyme production, the variables including moisture absorption, pH and temperature are selected. It is experimented that the amylase activity

decreases with increase in incubation period and temperature. [11] Amylase production takes place using submerged and solid state fermentation where solid state fermentation is preferred over submerged fermentation. Since the enzymes are thermo-stable, they emerge as the significant part in large scale industrial sectors. Effects of fermentation time, temperature, inoculum size, nitrogen and sugar source and moisture content are optimised for improved alpha amylase production. [12] evaluates the thermo-stable amylase activities of the isolates. It includes the selection of appropriate medium of cell growth for the synthesis of α-amylase. The ideal temperature and acidity level are said to be 65°C and 8 correspondingly. Among the several mediums in which the isolate is allowed to grow and examined, the optimum medium is chosen.

3. MATERIALS AND METHODS

Alpha amylase is referred as calcium metallo-enzyme and it is confirmed that the presence of calcium in enormous quantities may enhance gain of the substance. Strain growth depends on the presence of amino acids and other saturated amino acids. A series of fermentation process is been carried out in certain cases. Cell growth, sugar utilization, nutrient absorption, pH and temperature levels are regularly monitored. Below certain pH range, alpha amylase may not be stable and such conditions are examined and regulated.

3.1. Bacterial isolate and its culture medium

Samples of bacteria are separated from sago industrial wastes. About 25 samples are considered and among that around eight strains showed enzyme activity. Under specific cultured conditions, one strain is determined to be the best producer of alpha amylase which is the species Bacillus.

3.2. Optimization

Optimization is the process which is used to maintain a balance between the medium components and to minimize the utilization of unused products at the end of the processing. Since the article paves way for the production of enzymes using industrial residues, assessing of cost effectiveness can be achieved.

The factors affecting the enzyme production are pH, temperature, nitrogen and carbon sources, incubation period, starch and its nutrient medium. The formation of clear zones around the colonies refers to the hydrolysis process of amylase production. The enzymes maintain high stability over a particular setting of temperature and pH. Temperature is said to be the prime aspect for almost all enzyme activities. It is also essential for the enzymes to be active and stable even at peak temperature levels.

3.2.1. Effect of incubation time

Production of amylase may not take a lot of time, but considerable increase in its yield is noted after its submission to an incubation period of several minutes. Bacterial isolate is grown in a production medium of pH 7. It is carried out individually at a time interval of 12 hours each, incubated at 37^{o}C. The maximum and minimum amount of amylase production is 352.94 U/ml and 150.46 U/ml respectively. Table 1 displays the effect of incubation time.

Table 1.Incubation time v/s alpha amylase production

Incubation time (hours)	Alpha amylase (U/ml)	Protein (mg/ml)
12	150.46	10.15
24	202.88	25.44
36	221.41	40.18
48	352.94	95.64
60	290.14	85.88
72	195.33	80.81

3.2.2. Effect of pH and temperature

Bacterial isolate is cultured in different production mediums with a pH of 5, 6, 7, 8, 9 and 10 with 1N NaOH and 1N HCl before bacterial inoculation followed by amylase enzyme assay as in the previous experiment. It is incubated at 20^{o}C, 30^{o}C, 40^{o}C, 50^{o}C, 60^{o}C and 70^{o}C for 48 hours. After the incubation period, the culture broths are centrifuged to obtain the supernatant of the culture.

The amylase production is assessed after 48 hours of incubation period at 37^{o}C. The enzyme production started to increase when the pH moved from 5- 10. The maximum and minimum amount of amylase production is observed at pH 7 and pH 10 respectively. There is decrease in enzyme production when

the pH becomes alkaline. The results for the effect of pH in amylase production are given in table 2.

The maximum amylase enzyme production is obtained at 40°C. The minimum amylase production is observed at a temperature of 70°C and the tabulation is provided in table 3.

Table 2.Effect of pH

pH	specific activity (U/ml)
5	130.23
6	162.88
7	250.45
8	184.28
9	78.15
10	72.75

Table 3.Effect of temperature

Temperature (°C)	Specific activity (U/ml)
20	90.48
30	105.23
40	148.33
50	113.21
60	85.88
70	67.94

3.2.3. Effect of carbon and nitrogen sources

The carbon source of the production medium is replaced with glucose, galactose, maltose, sucrose, lactose, fructose and dextrose and the nitrogen source with peptone, yeast extract, casein, ammonium sulphate, ammonium nitrate and ammonium chloride separately at 0.5% concentration. The effects of carbon and nitrogen sources are tested after 48 hours of incubation at 37°C. 1 ml inoculum culture is inoculated in each flask and incubated at room temperature for 24 hours on a rotary shaker at 120 rpm. The effect of carbon sources is displayed in table 4.

Table 4.Effect of carbon sources

Carbon sources	Specific activity (U/ml)
Glucose	42.3
Galactose	155.45
Fructose	102.04
Maltose	210.46
Lactose	118.19
Sucrose	155.58
Dextrose	112.95

Production of enzymes is observed using the above mentioned carbon sources. Based on the results, maximum amylase production occurs when maltose is used.

Table 5 represents the effect of nitrogen sources and the maximum amylase production is observed where the minimum activity is observed in ammonium chloride.

Table 5.Effect of nitrogen sources

Nitrogen sources	Specific activity (U/ml)
Peptone	75.48
Yeast extract	85.22
Casein	155.54
Ammonium sulphate	65.38
Ammonium nitrate	55.46
Ammonium chloride	48.23

3.2.4. Effect of inoculum size

For the production of amylase, inoculum size plays a significant role. It is analysed that by increasing the overall size of the inoculum, its productivity is improved. An accurate inoculum size is not usually followed. It varies from 2-3%.

Alpha amylases are universally distributed throughout animals, plants and microbes and can be produced by different species of microorganisms. α- amylase derived from the genus Bacillus is commercially applied to many fields. The Bacillus species include Bacillus licheniformis, Bacillus stearothermophilus and Bacillus amyloliquefaciens. α- amylases are employed in clinical, medicinal and analytical chemistry besides their uses in food fermentation, brewing, textile, paper, detergent, distilling and pharmaceutical industries.

4. RESULT ANALYSIS

25 isolates of bacterial samples are derived from the natural sago industrial wastes, where eight strains are found to be α- amylase producers. Several tests based on the morphology and biochemical reactions are performed and strain AB-19 is recognised as the best zone producer which is called Bacillus sp. The resultant pH and temperature after the growth of Bacillus species are 6 and 70°C for 24 hours accordingly. It is aerobic with 40°C and 48 hours as optimum temperature and duration for maximum enzyme production respectively. Its growth starts decreasing due

to the declining phase and low amylase synthesis of the cells from 48 hours.

Depending upon the nutrients present in the medium and culture conditions of the organisms, the incubation time gets varied. It is observed that the growth and amylase production is improved by the addition of 10 mg calcium and 1% of peptone to the liquid medium.

The most favourable conditions for the growth and synthesis of amylase by microbes are calcium, yeast extract and peptone.

It is analysed that at stationary phase, maximum amylase activity is reported and the protein content was 95.64 mg/ml where the culture is grown at 40^{o}C. The fermentation of enzymes depends on the size of the inoculum and the ideal inoculum size would be 1000µl which gives the highest yield of enzyme production.

5. CONCLUSION

The breaking down of biopolymer molecules into smaller polymers to simple glucose units is done by alpha amylase. Though it is being produced by animals, plants and animals, microbes are considered to be the best producers because it is widely present in liquid and solid states and among bacteria of different types, the Bacillus species produces industrial amylases in an enormous amount. It is aimed to construct an ideal medium for the growth of microorganisms. Broth medium is chosen as the basal medium because it is rich in nutrition. The productivity is determined after a period of five days initially at 37^{o}C in 25 ml medium with shaking at 150 rpm and the initial pH set at 7. The prime components of medium include 0.5 ml of 1% of starch, 0.1M phosphate buffer and 0.5 ml of enzyme solution which are incubated for 30 minutes at 37^{0}C. 1 ml of 3, 5-dinitrosalicylic acid is added to cease the reaction. It is followed by heating the medium by placing it over the water bath for five minutes and allowed to be cooled under room temperature. 8% of deionized water is added to the mixture. The substitutes for nitrogen sources are fructose, galactose, maltose and sucrose where carbon sources are replaced by peptone, yeast extract, casein, ammonium sulphate, ammonium nitrate and ammonium chloride. This review brings forth the optimization of amylase using liquid state fermentation. This method is highly favourable for bacterial growth. This substrate is selected due to its starch abundance.

The results assist in better understanding of the interaction between the substrate and amino acids present in the medium. It is identified that by proper recycling methods, several strains of bacteria would be separated from such industrial wastes and thereby enhances the productivity of bio-catalytic enzymes. This work illustrates the optimum requirements for the production of alpha enzymes and future scopes based on filtering techniques to overcome the industrial wastes and it is necessary to find adequate principles to maximize the quality of enzymes by minimising the cost functions. The other aspect to be improved is the efficiency in nutrient medium preparation which is the sole platform of enzyme activity. Certain other techniques in the purification of the resulting amylase have to be formulated. Temperature shifting strategies can be promoted in improving the efficiency of alpha amylase production. Therefore agro-industrial wastes are economically utilized owing to the advances in microbiology.

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