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RESEARCH ARTICLE

Identification and Evaluation of Bacillus Species Bacteria from Sago Industrial Waste

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ABSTRACT

Amylase is one of the significant enzymes which are of much use in the real world. These enzymes convert complex starch molecules into glucose or simple sugar units. Amylases are widely used in a number of industrial processes such as food, fermentation, textile, paper, detergent and pharmaceutical industries. Enzymes from fungal and bacterial sources play a key role in industrial sectors. Amylases are classified into three types: α- amylase, β- amylase and γ- amylase. α-Amylase is obtained from plants, animals and microorganisms. The α-amylase production is crucial for the conversion of starch into simple sugar. Starch is an important ingredient of human food and is a major storage product of many economically important crops. In this article, the production of α-amylases has generally been carried out using sago industrial waste, where a few samples are taken and subjected to bacterial growth under separate specified substrates. About 25 waste samples are collected and the availability of bacteria is noted. It is found that, among these 25 samples, around eight strains showed the growth of bacteria producing alpha amylase and based on several studies, it is identified that the best source of this particular amylase production is by the bacterial species, Bacillus. Different approaches are exhibited based on temperature, pH, incubation period, nutrient medium and other constraints. It is concluded that alpha amylase enzyme is been produced from bacillus species bacteria. Enzymes are sometimes referred as bio-catalysts which are highly specific and catalyse reactions faster than chemical catalysts. α-Amylase is been extensively used as the result of microbial action under controlled conditions. These has been in increasing demand due to its vital role of starch hydrolysis and applications.

Keywords: Enzymes, Hydrolysis, Industrial waste, Bacillus species, Bio-catalyst.

1. INTRODUCTION

All enzymes are proteins which catalyse the biochemical reactions taking place in living cells. A large number of industrial applications use enzymes in environment and food technology. Enzymes accelerate biochemical reactions by its extreme catalytic power. Enzymes depend on various environmental factors. Many enzymes are produced in industries which are utilised for several purposes. Enzymes produced in industries are lipase, protease, amylase, cellulose, etc. Many plants, animals and human beings are capable to produce different types of enzymes and the most commonly used microorganisms for the production of enzymes are bacteria and fungi. One of the most important enzymes used in microbiology is amylase. These amylases are of three types α-amylase, β- amylase and γ- amylase where β-amylase cannot be developed by animal kingdom. α and γ- amylases are produced in animal system. Although amylase is derived from several sources such as animals and plants, the enzymes from microbial sources generally meet industrial demands. In most cases, amylases from plants and microbial sources have been employed as food additives. Barley and fungal amylase are been used in brewing industry and for the preparation of oriental foods. Alpha amylase utilizes starch as carbon and energy sources which are the key enzymes in the metabolism of a spacious diversity of living organisms. Starch is a

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carbohydrate source consisting of two molecules namely, amylase and amylopectin. Amylase functions by hydrolysing the straight chain bond between the individual glucose molecules that make up the starch chain. The single straight chain and the branched starch chain are called amylose and amylopectin respectively. These starches are polar molecules and have different ends. Enzymes from fungal and bacterial sources have influenced the industrial sector applications by various means. α- amylase breaks down long chain carbohydrates to maltose and maltotriose by limiting dextrin from amylopectin. The present study is aimed at the in- vitro production of α- amylase enzyme from an isolated bacterium obtained from sago industrial waste. Strains are separated from different natural substrates. Based on evaluation, one of the strains is considered to be Bacillus species. By optimization of culture conditions, production of alpha-amylase is high. Alpha-amylase production is stimulated by peptone and yeast extract which is independent of the presence of starch in the culture medium. The enzyme is thermo-stable and retains amylolytic activity after incubation at a specified temperature. High performance is maintained over a broad pH range and the enzyme remained active after alkaline incubation. α-Amylase is popularly used due to its activities that can be carried out in hydrolysis in the conversion of glucose and fructose syrup from starch.

2. LITERATURE REVIEW

[1] The article presents the production of amylase from Bacillus isolate. Amylase is yielded from such bacteria in smaller amounts even without the existence of starch, but its production is high when sufficient starch is provided. Several types of sugars are available such as galactose, fructose, sucrose, lipase, glucose and maltose of which fructose is suitable for maximum amylase production. [2] Based on the research, it is found that bacteria species Bacillus produces considerable amount of amylase. The production of amylase depends mainly on nutrient medium, pH, temperature and the incubation time. Enzyme activity is higher with respect to rise in temperature, but extreme heat affects its yield. [3] The study deals with the production of α-amylase where its outcome is stimulated by galactose, urea and peptone. It is focussed on mixing two or more substrates for better results rather than using a single substrate. Ideal temperature, inoculum and incubation period are maintained. The process involved the production of alpha amylase by submerged fermentation method. [4] It focuses on the generation of amylase from waste supplements of mussel processing. The increase in enzyme production is examined by injecting the strains to different culture mediums. It is concluded that the enzyme production depends on nutrient contents, nitrogen and carbon sources where glucose also plays a vital role in the processing. To enhance the processing, five nitrogen sources are formulated in this process. [5] The paper submits the process of solid state fermentation to the collected strains of bacteria to produce thermo-stable α-amylase. The process is triggered by amylo-glucosidase and any availability of metal ion does not show any remarkable changes. Substrate, pH, moisture content, mineral absorption, nitrogen and carbon sources, incubation period and temperature are the factors determining the production of amylase. [6] Scientific analysis is been carried out in the production of α-amylase by subjecting Bacillus strains to complex and synthetic culture mediums. According to the analysis, it is stated that the rate of growth alters in both culture condition i.e. complex medium is favoured for fast growth rate because of the existence of saturated fatty acid whereas synthetic medium for slow growth rate. Therefore further results are analysed by providing additional fatty acids in the growth medium. [7] It includes the yield of enzymes using the laboratory strains of bacteria, Bacillus species. The stains are placed in separate medium and the effects are viewed. The total production seems to be varied in each case where the nutrient medium, temperature level, acidity factor, incubation time, inoculum size and presence of amino acids are considered. [8] Amylases are enzymes which converts starch products into simple sugars. α-Amylases are the one which are widely used in industries where the usage is extended from various food processing and textile factories to medical fields. Among various sources of amylase production, their productions from microbes are favoured in wide-reaching sectors. [9] At considerable lower temperatures, alpha amylase productivity is high where the composition of starch is low. When low concentrated starch is replaced with

either starch of high concentration or calcium, high temperature level is required for its production. In both cases, the developed amylase is highly thermo-stable. Experiments are conducted by allowing the strains to emerge in various starch medium such as potato, rice, corn, oats, etc and the results are examined accordingly. [10] This paper evaluates the isolation of starches from plant, animal and microbial sources which are further processed in industries. It is found that amylolytic enzymes are largely available which are produced by several organisms living in moderate and extreme temperature conditions. This includes fermentation systems where the measure of fermentation varies with the concentration of enzymes.

3. ENZYME DESCRIPTION

The most important natural glucose polymers in plants are starch. Starches usually initiate the generation of various types of vital compounds. Commercial enzymes are produced by microorganisms. Since the sago waste is rich in nutrient content, it provides a source for many microbes and for this study, bacteria is chosen which produces considerable amount of amylase. Studies reveal that maltose is known to be a better inducer when compared to starch content. Starch is highly available in seeds, tubers, leaves, bulbs and fruits of plants. It includes amylose and amylopectin in which its proportion varies with respect to the medium. These starches bear variety of products which are applied in various industries. These bio- enzymes are favoured instead of chemical enzymes resulting in better results. Generally samples are compiled from industrial wastes and defiled products from trees. This article indicates considering 25 samples from sago industry. By inoculating the samples in agar nutrient medium, these can be isolated.

3.1. Measurement of enzymes

The quantity of enzymes can be stated as moles. Quantity measurement of active enzyme represents the enzyme activity. It depends on the amount of substrate. Another enzyme constraint is its specific activity which relates to the assessment of enzyme purity in the blend. It is defined as the moles of product in a specified time per milligram of proteins present in its composition. The measurement of enzyme relates with the usage of substrate or the resultant products.

3.2. Factors limiting the measurement of enzymes

Enzymes may be sensitive to heat, temperature, pH, carbon and nitrogen sources and salt concentration. Certain enzymes cannot withstand extreme temperature and concentration of salts. The rate of reaction increases with increase in temperature up-to certain limits. It gets affected by very high rise in temperature due to the conversion of protein elements. All enzymes have ideal pH and temperature levels. Activity of enzymes can be ceased due to obvious pH range.

Samples are allowed to grow in agar nutrient medium. It may contain starch with several other forms of nutrients required for bacterial growth. The production of enzyme is indicated by the formation of halo around the colony. Enzyme production takes place by allowing the isolate to develop in broth medium which contains plenty of nutrients, employed for bacterial growth. It is usually composed of peptones, required salts and amino acids. This article deals with using of agar nutrient medium for microbial growth.

4. METHODS

4.1. Identification of amylolytic bacteria

Sago waste samples are collected from industrial field of sago industries. It is a mixture of soil and industrial wastes. One gram of this mixture is diluted with distilled water under serial dilution technique. One millilitre of appropriate dilutions (10-5- 10-7) of sago waste samples are placed on nutrient agar plates, which is the nutrient medium used for the cultivation of microbes, supporting growth of a wide range of microorganisms.

Figure 1.Bacillus species colonies over agar plate

Nutrient agar is popular because it can grow a variety of types of bacteria and fungi

which contain many nutrients needed for the bacterial growth as shown in figure 1. It is applied for the isolation of amylolytic bacteria. The plates were incubated at 37^{o}C for 24 hours. The bacterial colonies are purified and preserved for further procedures.

4.2. Isolation of amylolytic bacteria

A total of 25 isolates on the nutrient agar medium is yielded. Each strain which is the genetic variant or subtype of amylolytic bacteria has been designated with unique accession number, prefixed with two letters AB (Amylolytic Bacteria) which is followed by two numerical digits. All the 25 isolates are then screened for amylase production by subjecting it to starch hydrolysis test to enable whether the strains can consume the nutrients present in the starch medium. Alpha amylase enzyme provides considerable consumption of such starch producing maximum production. It is examined to identify the presence of starch even after its consumption at regular time intervals. The production of amylase enzyme by isolated amylolytic bacteria is shown in figure 2.

Figure 2.Production of amylase enzyme by isolated bacteria

4.3. Evaluation of amylolytic bacteria

Isolates are named from AB-1 to AB-25 and the isolate AB-19 shows the maximum inhibition zone. Hence the strain AB-19 is selected and subjected to further morphological and biochemical tests. When the industrial sago wastes are diluted, it is placed in nutrient medium where bacteria are cultivated resulting in isolation of bacterial cultures. Isolated bacterial cultures are evaluated by starch hydrolysis test on starch agar plates. It is then incubated at 37^{o} C for 24 hours which is followed by immersing in iodine solution for 30 seconds on the starch agar plates. By this chemical reaction, amylase is produced.

4.3.1. Production of amylase enzyme

The pure culture of bacteria is introduced into starch nutrient broth and incubated for 24 hours. After 24 hours of inoculation period, these vegetative cells are used as an inoculum source. The bacterial strain is then subjected to liquid state fermentation process. Amylase enzyme is produced using basal medium and starch as substrates. Initial pH of the medium was adjusted to 7. The organism was developed at 37^{o} C for 5 days in 25 ml medium at 150 rpm. Samples are taken at the time interval of 12 hours and up-to five readings are determined. Samples are centrifuged at 10,000 rpm for about 10 minutes. The supernatant of the culture after centrifugation is used to determine the α- amylase activity.

4.3.2 Enzyme analysis

Pure enzyme is taken in a loop and was streaked on the surface of starch agar plate with single line. In-order to prepare crude enzyme preparation, 10 ml of bacterial culture broth was centrifuged at 5000 rpm for 20 minutes and the resulting Cell free supernatant is recovered by centrifugation which is used for amylase activity.

The reaction mixture containing 0.5 ml of 1% starch in 0.1 M phosphate buffer (pH 7.0) and 0.5 ml of enzyme solution is incubated for 30 min at 37^{o}C. The reaction includes addition of 1 ml of 3,5- di-nitro salicylic acid. It is heated in a boiling water bath for 5 minutes and cooled at room temperature followed by the addition of 8 ml of deionized water. Absorbance of each solution containing the brown reduction product is measured at 540 nm in a UV- visible spectrophotometer which is then converted to maltose. One unit of α- amylase activity is defined as the amount of enzyme that releases 1µ mol of reducing sugar as maltose per minute under assay conditions.

One unit per ml per minute is given by enzyme activity / molecular weight of maltose multiplied by the time of incubation.

4.3.3. Protein determination

For the determination of protein concentration in crude sample, one ml of the sample is taken and 0.2 ml of alkaline copper reagent is added to it where it is incubated for 10 minutes at room temperature and 500 µl of diluted Folin's reagent (1:1 with distilled

water) is added. Tubes are incubated at room temperature for 30 minutes and the sample is analysed.

The waste soil sample from the sago industry yielded 25 isolates on the nutrient agar medium which are then screened for amylase production by allowing it to starch hydrolysis test.

4.3.4. Morphological and biochemical characteristics of the strain AB- 19

Morphological and biochemical characteristics are depicted in table 1. The strain AB-19 is creamy white in appearance. Its motility and the gram reaction are positive which shows aerobic growth. Optimum pH and temperature are said to be 7 and 30-35^{o}C correspondingly. Its nitrate reduction, citrate utilization and catalase reaction are known to be positive where tyrosine utilization and triple sugar ion values are negative. The starch and gelatine hydrolysis are referred to be positive and negative values respectively. Based on these results, the strain AB-19 was identified as Bacillus species.

Table 1.Morphological and biochemical characteristics of the strain AB-19

Parameters	Characteristics
Colony colour	Creamy white
Morphology	Rods
Gram reaction	+
Motility	+
Aerobic growth	+
Anaerobic growth	-
Optimum pH	7.0
Optimum temperature	30-35o C
Oxidase	-
MR-VP test	-
Catalase	+
Nitrate reduction	+
Indole production	-
Citrate utilization	+
Tyrosine utilization	-
Triple sugar iron	-
Starch hydrolysis	+
Carbohydrate fermentation	+
Gelatin hydrolysis	-
Cellulose	+
Lactose	+

4.3.5. Initiation of amylase production

Isolates require starch and sugar as the main components. It is needed to be incubated for several hours based upon the medium. Cells are harvested by configuration techniques where the resulting supernatant is isolated which determines the total mass of the enzyme produced. Strains show higher enzyme activity when subjected to growth in agar medium. The activity rate is enhanced after incubating the medium.

5. RESULT ANALYSIS AND CONCLUSION

It is categorised that all the good alpha amylase producers correspond to the species Bacillus. The production of amylase depends mainly on pH and temperature. Maximum amylase productivity lies with the presence of glycerol, proline and vitamin B supplements. In certain cases, the resultant enzyme is refined by ammonium sulphate precipitation, gel filtration and hydrophobic activities. Thermo-stability of the amylase enzymes enhances with respect to temperature levels and starch concentrations. Cost limitation occurs due to the fact of employing the supernatant of enzyme source, where the supernatant after centrifugation is subjected for the further processing. Most of the substrates provide higher rate of hydrolysis as the result of increase in temperature. The study deals with the scope of further increase in the presence of bacteria in the waste soil of industries. Hence this method of producing amylase from such bacteria paves way for the recycling of industrial waste too.

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