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Isolation and Partial Characterization of Surface Producing Bacterial Strain Producing Amylase from Soil

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Abstract

Starch is an important constituent of the human diet and is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. The production of α-amylase is essential for conversion of starches into oligosaccharides. Amylase is one of the most important enzymes used in industry such as in textile, food, paper and pharmaceutical industry etc. Amylase has ability to hydrolyzed starch molecules into polymer composed of glucose unit. The aim of present study was to isolate amylase producing bacteria from soil samples, screening of amylase producing strains, morphological and biochemical characterizations of strains, and optimized cultural conditions of the strains. The soil samples were collected from deep soil of campus at Chandigarh with the help of aseptic spectula. The bacterial strains were isolated by serial dilution and plating method. Three bacterial strains such as RT03, RT5 and T5 having amylolytic activity was isolated. Based on intensity of clear zone one strain were selected for further study. The morphological characterization of strain RT5 showed that it is rod shaped bacilli, cream color, raised elevation, entire margin, moderate size and Gram negative in nature. Enzymatic activity of strain RT5 was estimated at various pH (6, 7 and 8) and temperatures (30 °C, 37 °C, 50 °C and 60 °C). The best amylolytic activity of RT5 was observed at pH 6 with optimum temperature 50 °C.

Keywords: Amylase, bacterial strain enzyme, morphological characters, pH

1. Introduction

Amylase is one of the most important industrial enzymes that have been used in biotechnology process particularly in starch hydrolysis (Kumar et al., 2013). Amylase is an extracellular enzyme which hydrolysed internal α 1,4- glucan links of starch into dextrin and other small polymers which is made up of glucose unit (Behal et al., 2006). Starch is a complex carbohydrate made up of amylose and amylopectin (Singh et al., 2014). The concentration of amylose and amylopectin in starch ranged from 20–25% and 75–80% respectively depending upon the plant. Greatest application of this enzyme is in formation glucose and fructose syrups, detergent, sweetner, in paper industry, textile industry, food industry, fermentation industry, digestive aid and spot remover in dry cleaning (Sani et al., 2014; Singh et al., 2011). This enzyme is mainly derived from the microorganism, higher plants and animals. Microbial production of amylase reduced the use of chemical for the hydrolysis of starch in starch processing industry (Dehkordi et al., 2012). The α-amylase is an metalloenzyme which are unable to do their function in the absence of Mn^{2+} and Ca^{2+} (Annamalai et al., 2011; Raul et al., 2014). They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes (Sivaramakrishnan et al., 2006). The amylase mainly the α amylase is present in salivary and pancreas of human. The α- amylase has a ability to act at any position of substrate thats by it act faster than that of β-amylase (Singh et al., 2011). The α-amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying, or transglycosylating enzymes. The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps (Sivaramakrishnan et al., 2006). Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques.

2. Materials and Methods

2.1. Sample collection

The soil samples were collected from 3–4 cm deep soil from campus at Chandigarh with the help of aseptic spectula. These soil samples then transferred to the plastic bag. These soil samples were stored at 4^0 C till further use.

2.2. Isolation of bacterial culture

Isolation of bacteria is performed by serial dilution followed by spread plate method. In this process 1gm of the soil sample were dissolved in the sterilized distilled water to obtain a concentration ranged from 10^{-1} to 10^{-4}. Then 0.1ml were taken out from sterilized distilled water containing soil sample which then spread through glass rod on the surface of solidify agar plate. These plates were incubate at 37 °C for 24 hrs. The bacterial colonies were streaked to obtain pure culture. Different purified isolates were maintained at 4 °C.

2.3. Screening of amylase producing bacteria

Bacteria which produced amylase were screened on the surface of starch-agar plate. These bacteria were streak on the surface of starch-agar plate and incubate at 37 ^{0}C for 24 hrs. After incubation, plate was flooded with 1% of iodine solution. Presence of zone of clearance showed positive results selected for further investigation.

2.4. Morphological characterization

The isolated strain was characterized on the basis of size, shape, color, nature of colony and pigmentation.

2.5. Biochemical characterization

Selected strain was biochemically characterized using Catalase test oxidase test, indole production, methyl red test, Voges Proskuer test, citrate utilization, Hydrogen sulfide producton, amylase production, Cmcase test, Lipase production and protease test. Fermentation test which were performed by using Lactose, maltose, dextrose, galactose, melibiose, L-arabinose, inulin, dulcitol glycerol, sorbitol, adonitol, rhamnose, melezitose, ONPG, D-arabinose, malonate Utilization, xylose, fructose, raffinose, trehlose, Sodium gluconate, sucrose, mannose, salicin, inositol, mannitol, ribose, cellobiose, esculin hydrolysis, xylitol, sorbose and citrate utilization.

2.6. Amylase production

The production medium was inoculated with overnight grown inoculum. For the preparation of inoculum a loop full of bacterial isolate was transferred in 50 ml of inoculum medium. The flask was placed on a rotary shaker incubator at a speed of 150 rpm at 37°C for 12 hrs. Amylase production was carried out by submerged fermentation. 25 ml of bacterial inoculums was added into 250 ml of the production medium (same as inoculation medium). The flask was placed on a rotary shaker incubator at a speed of 150 rpm at 37 °C for 24 hrs. After incubation, fermented broth was centrifuged at 8000 rpm for 20 min in a cooling centrifuge. Supernatant was collected which was crude enzyme and used for the estimation of amylase.

2.7. Optimization of reaction conditions of crude amylase

2.7.1. pH optimization

The pH of the enzyme is optimized using phosphate buffer of different pH. Phosphate buffer of pH 6, 7 and 8 are prepared.

2.7.2. Temperature optimization

To find out the optimum temperature at which the enzyme shows maximum activity, enzyme assay is performed at different temperature of incubation. Enzyme activity is checked by incubating at different temperature of 40 °C, 50 °C, 60 °C.

2.8. Partial purification of amylase enzyme

The ammonium sulphate precipitation followed by dialysis was used for partial purification of amylase enzyme. Ammonium sulphate was saturate up to 80% of 100 ml cell free extract. After overnight incubation, the content was centrifuged at 10000 rpm for 30 min. Supernatant was collected and saturated up to 90% with ammonium sulphate. Then the content was further centrifuged at 10000 rpm for 20 min and pellet was collected for further analysis. The enzyme mixture was transferred in a dialysis bag and immersed in phosphate buffer at 4°C for 24 hrs. buffer was continuously stirred using a magnetic stirrer throughout the process with regular change of buffer.

2.8.1. Enzyme assay for amylase enzyme

1.0 ml of culture broth was taken in test tube and 1.0 ml of substrate (starch) was added in test tube. The test tubes were covered and incubate at 35 °C for 15 minutes in water bath. Then 2.0 ml DNS reagent was added in each tube to stop the reaction and kept in boiling water bath for 10 minutes. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer.

3. Results and Discussion

Amylase, producing organisms like fungi and bacteria are generally isolated from soil and most of the work is focused on the amylase. The present study deals with isolation of amylase producing bacteria from soil. The collected soil samples producing different types of colonies on the surface of nutrient agar after serial dilution (Alariya et al., 2013). The isolated bacterial colonies were analyzed for their colony morphology and Gram's staining (Figure 1). Three bacterial strains were isolated i.e. RT03, RT5, T5. These bacterial strains were tested for amylolytic activity with iodine test. The zone clear is developed around the bacterial colony due the utilization of the starch present in the media by the amylase produced by the bacterium (Haribhau et al., 2015). The RT5 strain produced more amylase enzyme as compare to RT03 and T5. Similar method was also used by Singh, et al 2016 to isolate 5 bacterial strains out of 10, on the basis

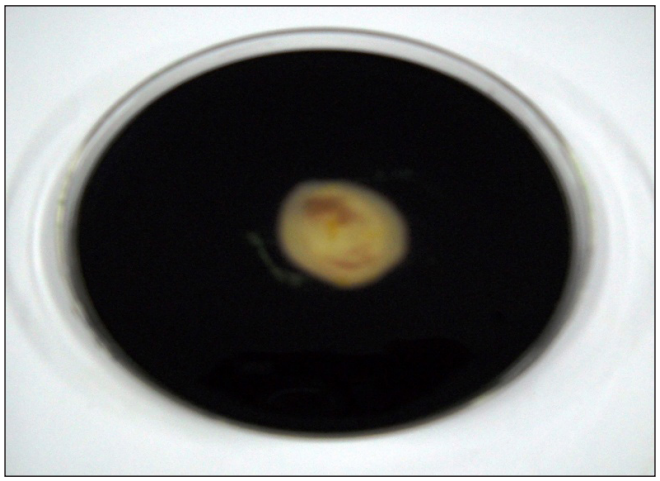

Figure 1: *Bacillus* sp. showing positive starch hydrolysis

of zone of clearance. The morphological characterization of strain RT5 showed that it is rod shaped bacilli, cream color, raised elevation, entire margin, moderate size and Gram negative in nature (Figure 2). Enzymatic activity of strain RT5 was estimated by Inoculating culture on 50ml of Luria broth containing 1% of starch in two flasks and incubates

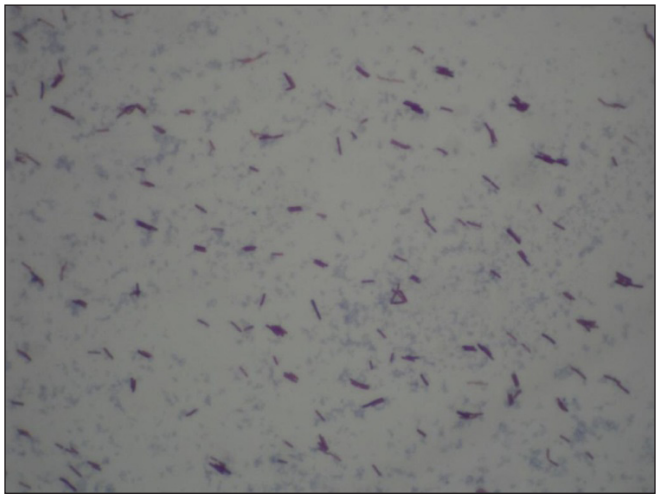

Figure 2: *Bacillus* sp. showing gram negative nature

at 37 °C, 150 rpm for 24 hrs. The activity was checked after 48hrs of incubation. Enzyme shows higher activity at 24 hrs of incubation i.e. 3.34 U mg^{-1} as compare to 48 hrs of incubation i.e.1.332 U mg^{-1}. This means that amylase activity decrease from 3.34 to 1.332 U ml^{-1} as the incubation time increase from 24 to 48 hours at 35 ±2 °C (Table 5). Similar work was done by singh et al 2016 reported decrease in amylase activity from 0.981 to 0.215 U ml^{-1} as the incubation time increase from 24 to 72 hrs at 35 ±2 °C. Hence, 24 hrs broth is taken for enzyme isolation and its characterization. Physiological characterization of RT5 showed negative results for growth at 30° C and positive result at 37 °C, 50 °C and 60 °C (Table 1). For further optimization of enzyme production was carried out for pH and Temperature. This enzyme produced

Table 1: Physiological characteristics of bacterial strain RT5

Growth Temperature	Strain RT 5
30 °C	-ve
37 °C	+ve
50 °C	+ve
60 °C	+ve

maximum activity at pH 6 i.e. 4.08 U mg^{-1} as compare to pH 7 and 8 i.e. 2.42 U mg^{-1} and 1.298 U mg^{-1} respectively (Figure 3). Mahdavi et al., 2010 and Demirkan, 2011 in *Bacillus subtilis* showed that this enzyme was optimally active at pH 6.0. The enzyme showed maximum activity at 50 °C i. e 0.80 as compare to 40 °C and 60 °C i.e. 0.74 and 0.75 respectively (Figure 4). A similar result was also found by Mahdavi et al., 2010; Singh et al., 2016 and Annamalai et al., 2011 in *Bacillus*

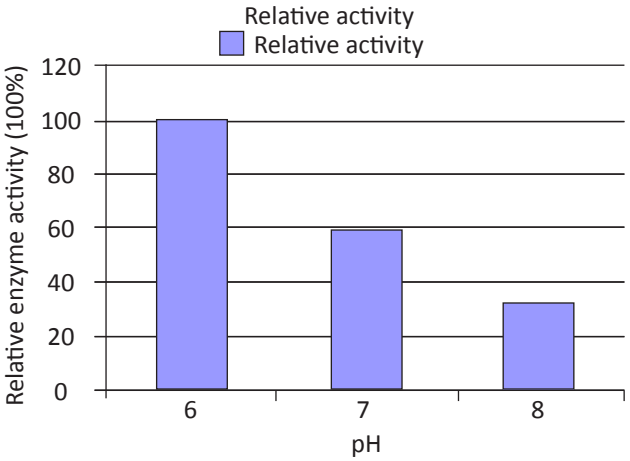

Figure 3: Relative activity of enzyme at different pH

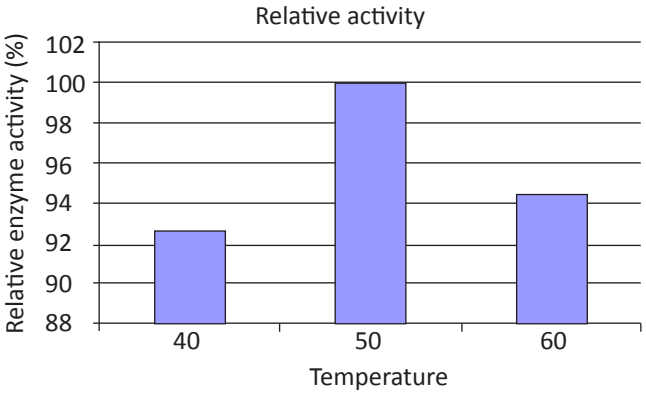

Figure 4: Relative activity of enzyme at different temperature

cereus. Various biochemical tests (Table 2, 3) had been done with RT5 which showed positive result for catalase test, oxidase test, amylase production tests but negative for indole production, Methyl red test, Voges Proskuer test, Citrate utilization, Hydrogen sulfide production, cmcase test, lipase production and protease test. Catalase test, oxidase test and starch hydrolysis were also found positive

Table 2: Biological characteristics of bacterial strain RT5

Biochemical characteristics	Characteristic (-ve / +ve)
Catalase test	+
Oxidase test	+/-
Indole production	-
Methyl red test	-
Voges Proskuer test	-
Citrate utilization	-
Hydrogen sulfide producton	-
Amylase production	+
Cmcase test	-
Lipase production	-
Protease test	-

Table 3: Fermentation tests of bacterial strain RT5

Carbohydrate	Characteristic (+ve / -ve)	Carbohydrate	Characteristic (+ve /-ve)
Lactose	-	Xylose	+
Maltose	+	Fructose	+
Dextrose	+	Raffinose	-
Galactose	-	Trehalose	+
Melibiose	-	Sucrose	+
L-Arabinose	-	Mannose	+
Inulin	-	Sodium gluconate	-
Glycerol	+	Salicin	-
Dulcitol	-	Inositol	-
Sorbitol	-	Mannitol	+
Adonitol	-	Ribose	-
Rhamnose	-	Cellobiose	-
Melezitose	-	Xylitol	-
ONPG test	-	Esculin Hydrolysis	-
D-Arabinose	-	Citrate utilization	-
Malonate Utilization	-	Sorbose	-

by Pokhrel et al., 2013; Patel et al., 2013. Fermentation test with RT5 showed that it is positive for maltose, dextrose, glycerol, xylose, fructose, trehalose, sucrose, mannose and mannitol utilization but negative for lactose, galactose, melibiose , L-arabinose, raffinose, inulin, sodium gluconate, salicin, dulcitol, inositol, adonitol, sorbitol, cellobiose, ribose, rhamnose, xylitol, melezitose, ONPG test, esculin hydrolysis, D-arabinose, citrate utilization, malonate utilization and sorbose test. Biochemical test such as indole test, sugar utilization test, methyl red test, citrate utilization Test, voges proskauer test, starch hydrolysis, catalase test, casein hydrolysis were carried out to find the enzymatic activity of isolated organism by Gowsalya et al., 2014. Microorganism always require specific environment for their growth. So, it is important to know which parameters are important for the growth of a given bacterial taxon. Therefore, one alternative is to take the bacteria back to the environment to grow them, often by moving a portion of the environment into the laboratory (Table 4 and 5).

Table 4: Standard graph of glucose using dns method

Concentration of glucose (mg ml^{-1})	Absorbance at 540 nm
0.0	0.00
0.2	0.36
0.4	0.68
0.6	1.00
0.8	1.20
1.0	1.70

Table 5: Production of amylase by strain RT after partial purification

Sl. No.	Time	Enzyme activity (u ml^{-1})
1.0	0 hrs	o.oo
2.0	24 hrs	3.34
3.0	48 hrs	1.332

4. Conclusion

From our work on bacterial strain RT5 we concluded that it is an amylase producing bacteria which is rod shaped, gram negative, having cream color, raised appearance, moderate size and showing maximum activity at pH 6 and 50ºC temperature. The bacteria were grown on medium (nutrient agar) containing 1% starch. Many of the bacterial species reported were not thermostable. But the amylase from the present study is thermosatble. So, the amylase used in present study has various industrial applications.

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