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ENHANCED PRODUCTION OF AMYLASE FROM GARDEN SOIL ISOLATE V_2

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ABSTRACT

The present work comprises the amylase enzyme production by isolated amylase producing microorganism. Out of 14 bacterial strains, were isolated and screened for amylase production and bacterial isolate V_2 showed maximum amylase activity (52.88 U/ml/min) and it was found that the maximum yield of alpha amylase activity was observed at 18 hrs of incubation period. Maximum amylase production was observed at pH 7.0 (69.04 U/ml/min) and temperature 30°C (71.52 U/ml/min). The maximum amylase production was found in production media containing fructose as carbon source with amylase production; (89.44 U/ml/min) and yeast extract as nitrogen source with amylase production; (90.2 U/ml/min). Thus in present studies bacterial isolate V_2 found to be promising amylase producing bacterial isolate.

KEY WORDS

Amylase, Microorganism, starch, temperature.

INTRODUCTION

Amylases are extracellular enzymes that catalyze the hydrolysis of starch, liberating linear oligosaccharides, branched oligosaccharides and glucose. Amylases are universally distributed in the plant, animal and microbial kingdom [1]. The substrate of amylase is starch which is a carbohydrate source consisting of two molecules amylose and amylopectine. A single straight chain of starch is called an amylose and a branched starch chain of starch is called an amylopectin [2].

There are three stages in conversion of starch; (1) Gelation (2) Liquefaction (3) Saccharification. In gelation starch granules dissolved to produce viscous suspension. In liquefaction partial hydrolysis of starch leads to loss of velocity. In saccharification hydrolysis produce glucose and maltose [3]. Amylases are important biocatalyst due to their nature to consume wide spectrum of substrates, high stability towards extreme temperature, pH, etc. [4]. The amylases from microorganisms have a broad spectrum of industrial applications as they are more stable when compared with plant and animal amylases [5].

Amylases have wide spread application in industries like food, brewing, textile, detergent and pharmaceutical. Amylase also applied in detergents production, to improve cleaning effect and used for starch de-sizing in textile industry [6, 5, 7, 8, 9]. The industrial enzyme production value of world market is about US $2.7billion where detergents (37%), textiles (12%), starch (1%), baking (8%) and animal feed (6%) are the main industries [10, 11]. Thus in order to produce amylase enzyme, the present study is focused on screening of amylase producing organism for various soil sample and optimization of various process parameters for enhanced production of amylase.

MATERIALS AND METHODS

In present study from various biotopes of vapi soil sample was collected and serially diluted and plated on Starch agar plate and further allowed to incubate at 30°C for 24 hrs.

Enrichment medium; Starch medium (gm/liter); Beef extract - 3.0, Peptone - 5.0, Starch - 20.0, Agar - 15.0, pH - 7.0(+0.2)

Isolated colonies from starch agar plate were picked and streaked in on starch agar plates with starch as the only carbon source and allowed to incubate at 30 C for 24 – 48 hrs. After incubation period, individual plates were flooded with Gram's iodine (Gram's iodine- 250 mg iodine crystals added to 2.5gm potassium iodide solution, and 125ml of water, stored at room temperature) to produce a deep blue colored

starch-iodine complex and the zone of degradation of blue color complex forms, which is the basis of the detection and screening of an amylase producing strain. The colonies showing zone of clearance in starch agar plates were selected as amylase producing strain and maintained on nutrient agar slants.

Storage and maintenance of pure culture

The amylase producing strains were streaked on starch agar slants. The slants were incubated at 30°C for 48 hrs to obtain pure culture of the bacteria. The pure culture stored in refrigerator at 4°C and sub cultured periodically.

Amylases production and assay

Extracellular amylases was produced in submerged fermentation, this production was carried out in 250 ml Erlenmeyer flasks containing 100 ml of liquid medium for enzyme production [12].

Inoculumn preparation

An isolated colony, from the preserved culture plate was transferred in to 50 ml Erlenmeyer flask containing nutrient broth. The flasks were incubated at 30°C for 24 hrs at 150 rpm. The freshly grown 24 hrs old culture with 1.0 O.D. at 600 nm is used as Inoculumn to inoculate in production medium.

Production medium

The production medium containing (gram/liter); Beef extract - 3.0, Peptone - 5.0, Starch - 20.0, Agar - 15.0, pH - 7.0(+0.2) was sterilized at 121°C, 15 lbs for 15 minutes.

Inoculation of production medium

The sterilized production medium was inoculated with 1% (v/v) of 24 hrs old culture. The inoculated flask is allowed to incubate at 30°C for 48 hrs at 150 rpm. The sample is taken at an interval of 0, 6, 12, 18, 24, 30, 36 and 48 hrs of incubation, centrifuged at 5000 rpm and supernatant is used for enzyme assay. The pellet was washed with equal volume of distilled water and two times with normal saline and then mixed in equal volume of normal saline. The growth was measured by taking the O.D. at 600 nm.

Enzyme assay for amylase enzyme

A suitable volume of isolated culture broth incubated for 48 hrs was centrifuged at 5000 rpm for 20 min and supernatant was recovered. Amylase was determined by spectrophotometric method. 1.0 ml of crude enzyme and 1ml of 1% soluble starch in phosphate buffer (pH 7) was added in test tube. The test tubes were covered and incubate at 30°C for 10 minutes**.** Then 1.0 ml DNS reagent was added in each tube to stop the reaction and kept in boiling water bath for 10 minutes. After boiling cool the tubes at room temperature, final volume was made to 10 ml with distilled water. The absorbance was read at 540 nm by spectrophotometer. One unit of amylase was defined as, "The amount of enzyme that catalyses the release of one µ mol of reducing sugar (equivalent to glucose) per minute per ml of culture broth from starch under standard assay conditions".

Optimization of culture condition

Effect of incubation period

The effect of incubation period was determined by incubating production medium with 1% Inoculumn for 48 hrs at 30°C. The sample was withdrawn at regular time interval from fermentation flask and centrifuge at 5,000 rpm for 20 min and the supernatant was analysed for amylase assay.

Effect of production medium

In present study various production medium were evaluated for their effect on amylase production. The effect of production medium on amylase production was done by incubating the 100 ml of inoculated production medium with 1 % inoculums. The flasks were incubated at 30°C for 18 hrs. After incubation period, 5ml sample was withdrawn from fermentation flask and centrifuge at 5,000 rpm for 20min. The supernatant was analysed for amylase assay. The medium used in this study are as follows:

Medium I (gm / liters): Beef extract - 3, Peptone - 5, Starch - 20, pH - 7.0(+0.2).

Medium II (gm / liters): KH_2PO_4 – 1, Na_2HPO_4 – 2.5, NaCl - 1, $(NH4)_2SO$ - 2, $MgSO_4.7H_2O$ – 0.05, $CaCl_2$ – 0.05, Tryptone – 2, Soluble starch - 10, pH 6.5.

Medium III (gm / liters): Starch -10, Yeast extract - 2, Peptone - 5, $MgSO_4$ - 1, NaCl - 1, $CaCl_2$ - 0.2

Medium IV (gm / liters): Peptone - 6, $MgSO_4$ - 0.5, KCl - 5, Starch – 1.

Medium V (gm / liters): Glucose - 2, Yeast extract – 0.3, Peptone – 0.5, NaCl – 1.5, $Na_2HPO_4.2H_2O$ - 1.1, $NaH_2PO_4.2H_2O$ – 0.61, KCl – 0.3, $MgSO_4.7H_2O$ – 0.01.

Medium VI (gm / liters): Soluble starch - 5, Ammonium nitrate – 5, $CaCl_2$ – 1, NaCl – 1, $MgSO_4.7H_2O$ – 1, K2HPO4 – 1, KH_2PO_4 – 1, pH – 7.

Medium VII (gm / liters): Soluble starch – 4, $(NH_4)_2SO_4$ – 5, Peptone- 6, $FeCl_3$ - 0.01, $MgCl_2{\cdot}6H_2O$ - 0.01, $CaCl_2{\cdot}2H_2O$ - 0.01, KH_2PO_4 – 4, K_2HPO_4 - 7.5, pH - 7.0.

Medium VIII (gm / liters): K_2HPO_4 - 2.5, KH_2PO_4 - 3.75, $MgSO_4$ - 0.125, NaCl - 3.75, $(NH_4)_2SO_4$ - 2.5, $CaCl_2.2H_2O$ - 0.05, $FeSO_4.7H_2O$ - 0.05, Yeast extract - 1.25, Starch - 2.5.

Effect of pH on amylase production

The pH of production medium was adjusted as 4, 5, 6, 7, 8 and 9 using 1 N HCl or 1N NaOH. The effect of pH

on amylase production was done by inoculating the above production medium with 1 % inoculums and the flasks were incubated at 30°C for 18 hrs. After incubation period, 5 ml of the sample was withdrawn from fermentation flask and centrifuge at 5,000 rpm for 20 min. The supernatant was analysed for amylase assay.

Effect of temperatures on amylase production

The effect of temperature on amylase production was carried out by incubating the 100 ml of inoculated production medium with 1 % inoculums. The inoculated medium was incubated in the temperature range of 15° – 60° C for 18 hr. After incubation period, 5 ml sample was withdrawn from fermentation flask and centrifuge at 5,000 rpm for 20 min. The supernatant was analysed for amylase assay.

Effect of carbon sources on amylase production

In order to determine the effect of carbon sources on amylase production various carbon sources such as sucrose, glucose, fructose, maltose & lactose were evaluated for their effect on amylase production. The fermentation flasks were inoculated with 1% Inoculumn and incubated at 30°C for 18 hrs. After incubation period, 5 ml sample was withdrawn from fermentation flask and centrifuge at 5,000 rpm for 20 min. The supernatant was analysed for amylase assay and the effect of carbon source concentration on amylase production was done by taking the carbon source range of 0-2 % w/v.

Effect of nitrogen source on amylase production

In order to determine the effect of nitrogen sources on amylase production various organic and inorganic nitrogen sources such as Peptone, Urea, Yeast extract, Beef extract, Ammonium sulphate, Ammonium chloride and Sodium nitrate were evaluated for their effect on amylase production. The fermentation flasks were inoculated with 1% Inoculumn and incubated at 30°C for 18 hrs. After incubation period, 5ml sample was withdrawn from fermentation flask and centrifuge at 5,000 rpm for 20 min. The supernatant was analysed for amylase assay and the effect of nitrogen source concentration on amylase production was done by taking the nitrogen source range of 0-2 % w/v.

Results and Discussion

Screening & Isolation of amylase producing bacteria from soil sample

A total of 14 bacterial isolates were obtained, showing clear zone around the bacterial colonies in starch agar plates when flooded with iodine solution. This 14 isolates were selected as amylase producing organism and purified by sub-culturing on starch agar plates. Further, all the isolates were analyzed for amylase production in starch medium and the amylase activity was determined after 24 hr of fermentation period under submerged culture condition. The results showed isolate no. V_2 showed maximum amylase activity of (52.88 U/ml/min) after 24 hrs of incubation at 30°C under shaking condition. Isolate no. F_3 showed amylase activity of 47.28 U/ml/min and isolate no. F_1 also showed comparable amylase activity of 37.52 U/ml/min after 24 hrs incubation at 30°C. However, isolate no. U_2 showed negligible amount of amylase activity 0.56 U/ml/min after till 72 hr of incubation at 30°C. Thus isolate V_2 was selected as reference culture for further study as it produces maximum amylase enzyme amongst various isolates. **Optimization of culture conditions**

The production of amylase by submerged fermentation (SmF) has been thoroughly investigated and is affected by various factors such as effect of incubation period, effect of pH, effect of temperature, effect of carbon sources, effect of nitrogen sources and effect of time course study.

Effect of incubation period on amylase production

Incubation period plays an important role in substrate utilization and its protein enrichment for enzyme production. Bacterial amylase from selected isolate V_2 was produced by using submerged fermentation at different time intervals ranging from 0, 6, 12, 18, 24, 30, 36, 42 and 48 hrs incubations. As the incubation time increase amylase activity also increase and it was found that the maximum yield of amylase activity was observed at 18 hrs of incubation (66.8 U/ml/min) however, further incubation results in decreased amylase production (Fig. 1). Time course study for the production of amylase governed by characteristics of the culture has been studied by [13].

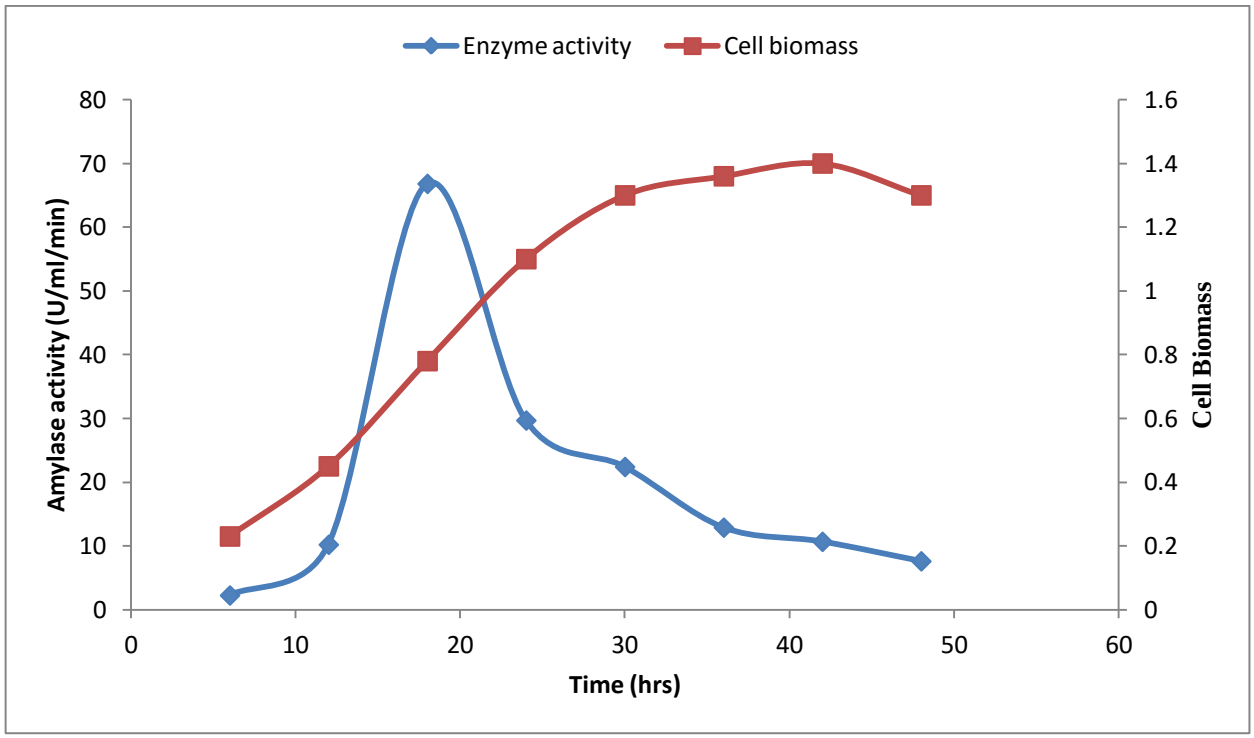

Figure 1: Effect of different incubation period on amylase production.

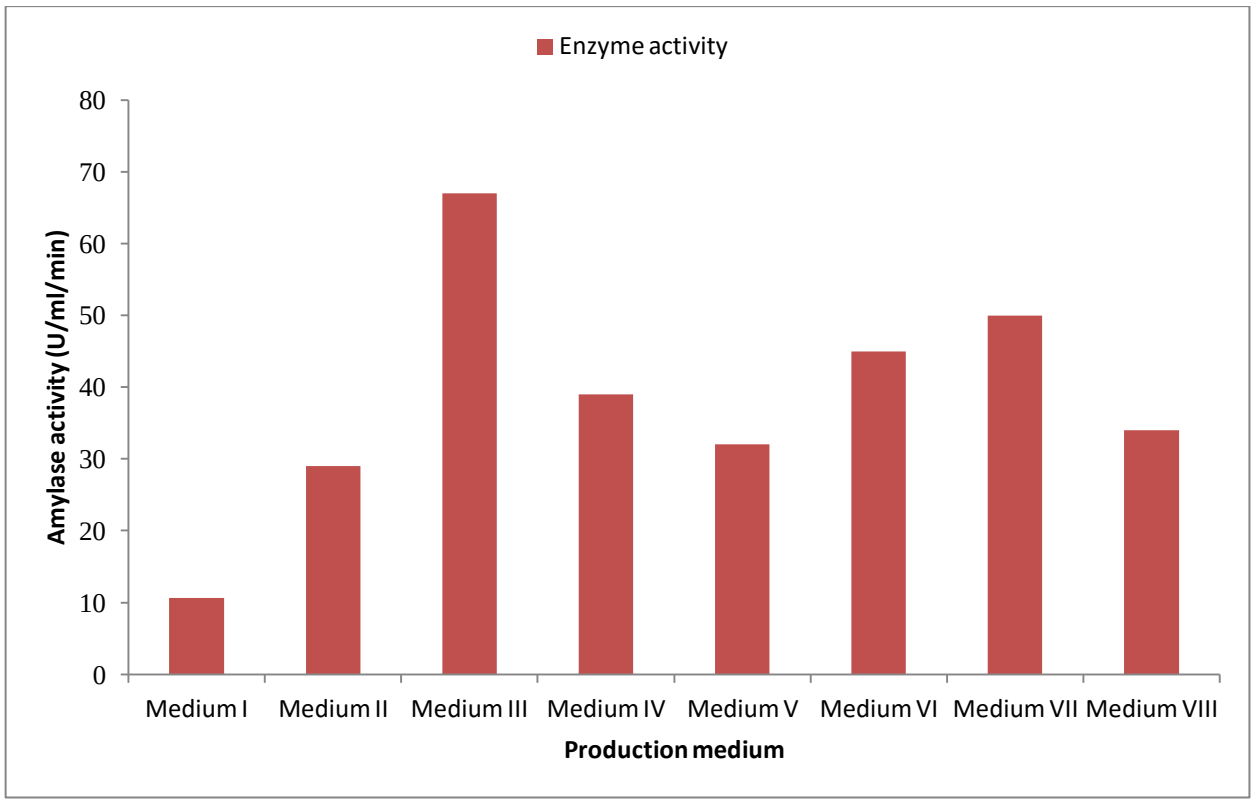

Figure 2: Effect of different production medium on amylase production.

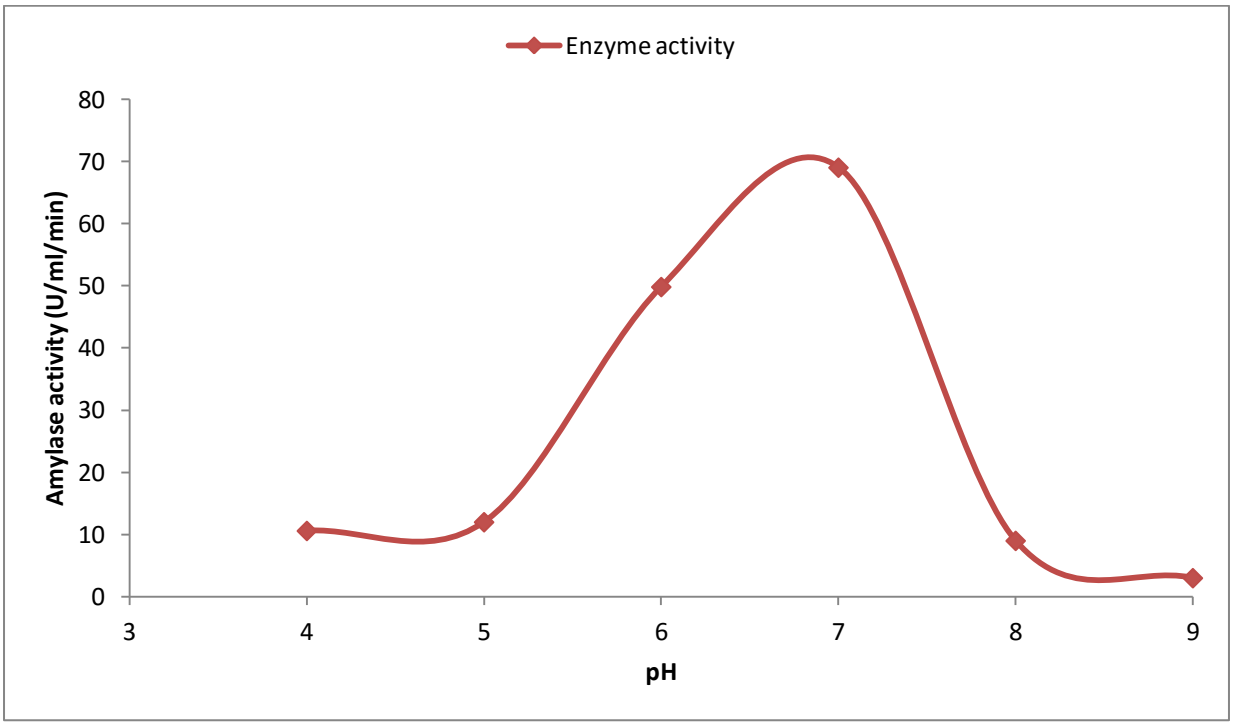

Figure 3: Effect of different pH on amylase production.

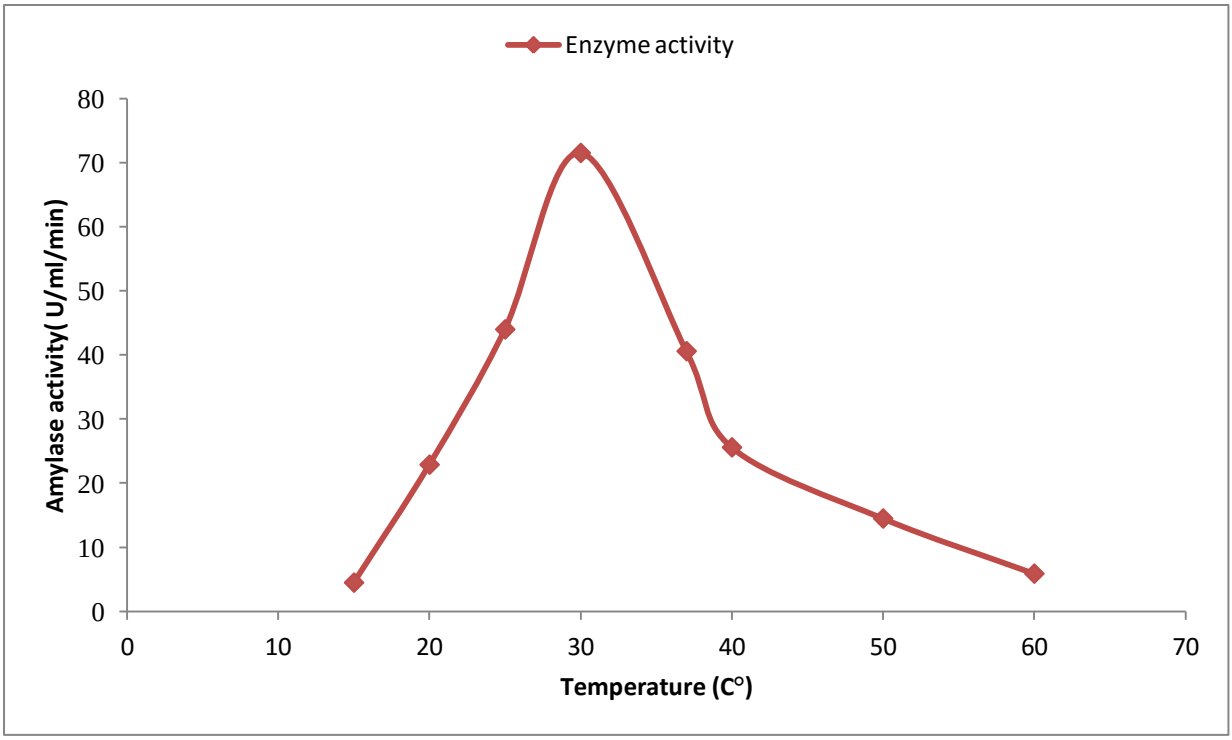

Figure 4: Effect of different Temperature on amylase production.

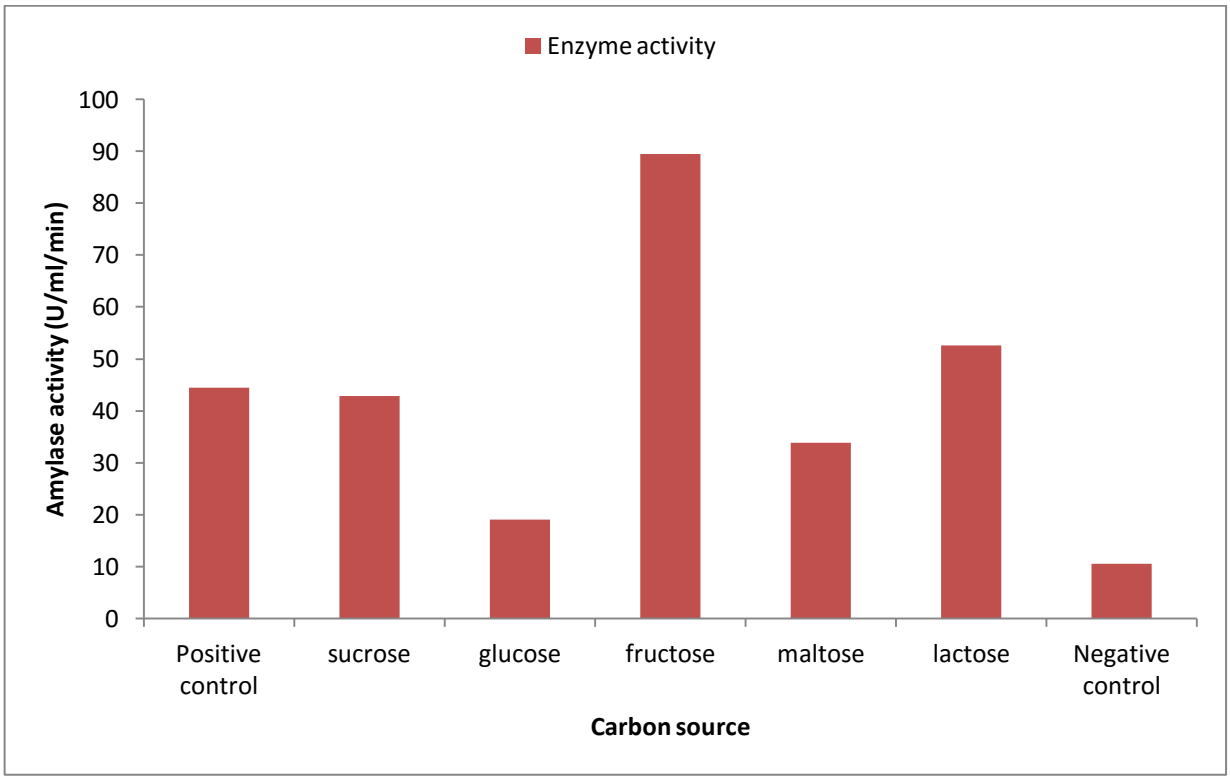

Figure 5: Effect of different Carbon sources on amylase production.

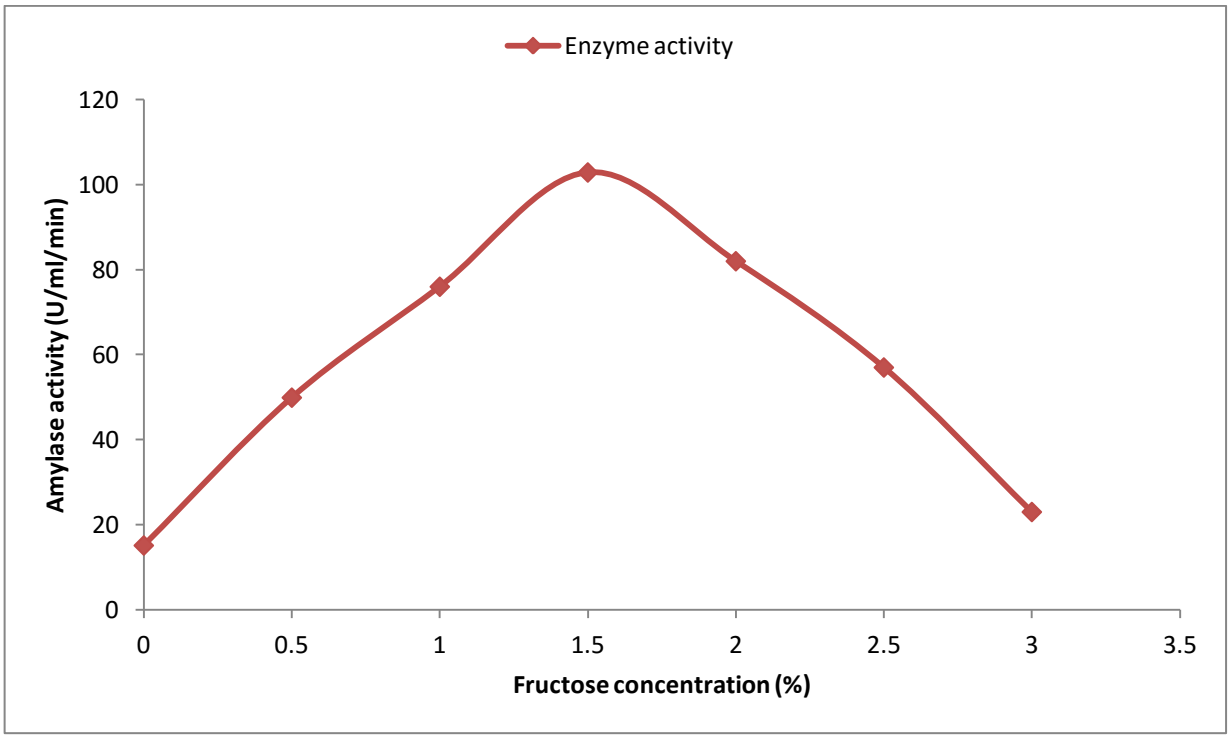

Figure 6: Effect of different fructose concentration on amylase production.

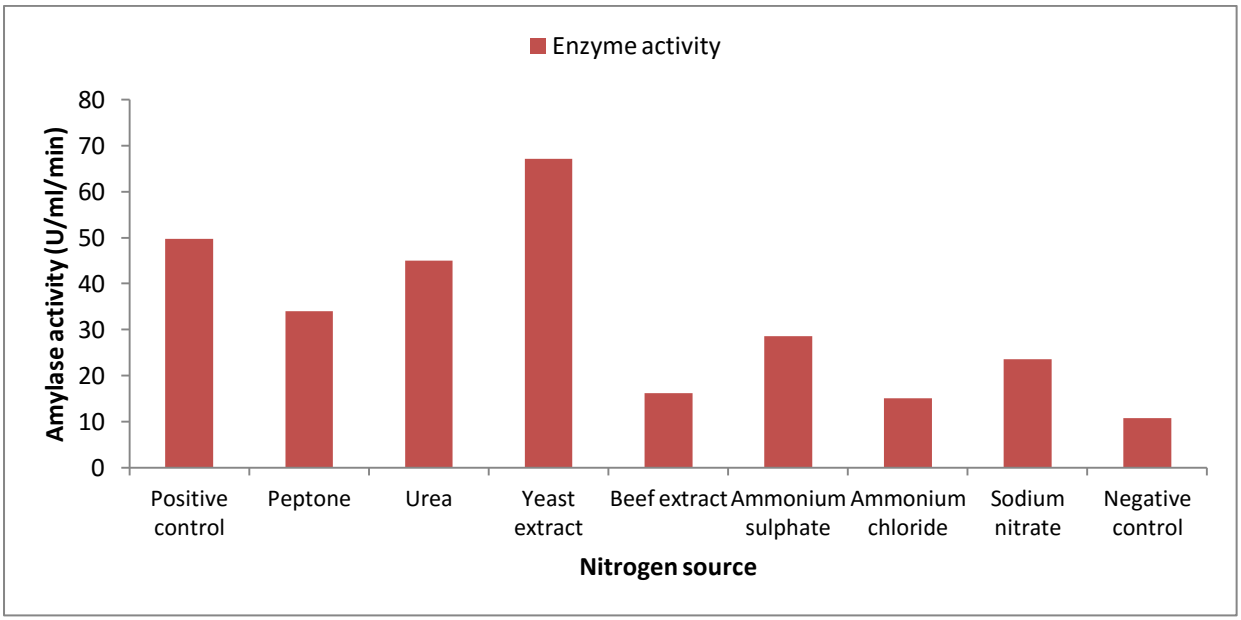

Figure 7: Effect of different nitrogen sources on amylase production.

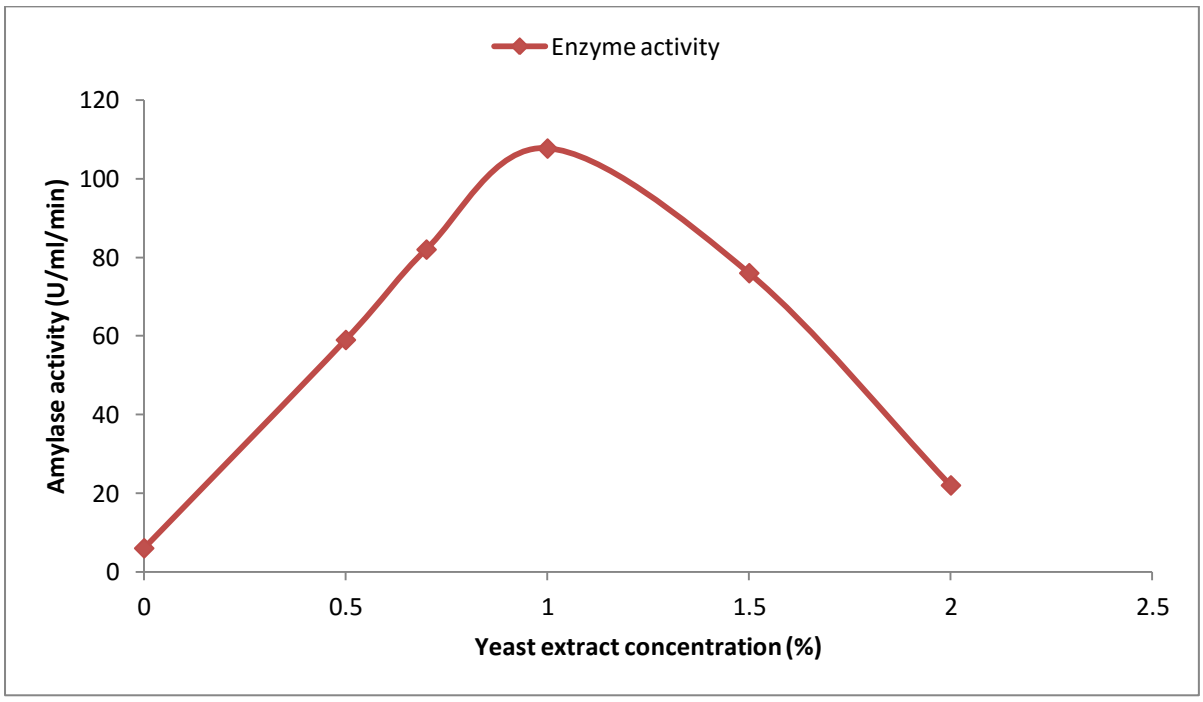

Figure 8: Effect of different nitrogen concentration on amylase production.

Effect of production medium on amylase production

Various production medium were evaluated for their effect on amylase production. The medium used in this study are as follows: Medium I, Medium II, Medium III, Medium IV, Medium V, Medium VI, Medium VII & Medium VIII. Maximum production was found in Medium III with amylase activity; (67 U/ml/min), whereas minimum production was found in Medium I with Amylase activity; (10.64 U/ml/min) (Fig. 2). Results showed that the highest amylase activity for amylase production obtained from Medium III, which may provide appropriate quantity

of ions which are essential for bacterial growth and enzyme production [14].

Effect of different pH on amylase production

It was found that pH can significantly affect the growth of microorganisms because change in pH can influence fermentation by change in growth pattern of the culture as a result it will influence the metabolic activities. Thus, to check the effect of different pH on enzyme production, the experimental design with production medium adjusted to different pH inoculated with the culture and was incubated at 37°C. In our study the amylase production was found to be maximum at 7.0 pH (69.04 U/ml/min). Although pH 4 and 9 also supported amylase production. Terui [15] who reported 6.8 as optimum pH for the production of -amylase by *B. subtilis*. Arikan [16] reported production of amylase by *Bacillus* sp. A3-15 at pH 8.5 and in between pH 7-11.0 by *Bacillus* sp. isolate ANT-6.

Effect of temperature on amylase production

Temperature is an effective parameter for production of amylase enzyme by bacteria. To study the effect of temperature on amylase activity, the experimental flask containing starch medium were incubated in a temperature range (15 ^{o}C - 60 ^{o}C) respectively. Maximum amylase was recorded at 30°C and above 30°C the amylase of isolate V_2 decreased sharply up to 60°C. Results are supported by earlier studies carried out for maximum production of amylase at temperature 30^{o}C by Sajitha et al [17], Elizabeth et al [18], Mishra et al [19]. Ashwini et al [20] also reported that *Bacillus sp*. was not capable of producing the enzyme at temperature below 25^{o}C on other hand, a progressive decline in enzyme production was observed at 45^{o}C and no enzyme production was observed at 50^{o}C as the temperature increased or decreased.

Effect of carbon source on amylase production

To study the influence of carbon source on the amylase activity the medium was supplemented with different sugars like Glucose, Sucrose, Maltose, Fructose and lactose at 1.0% concentration. The isolated bacteria showed efficient growth in all the substrates and amylase production but maximum amylase production was found in production media containing fructose as carbon source with amylase production; (89.44U/ml/min), whereas minimum amylase production was found in media containing glucose as a carbon source with amylase activity; 19.04 U/ml/min as shown in the (Fig. 5). Results are supported by earlier studies carried out for production of amylase with fructose as carbon source for *Bacillus sp.* by Ashwini et al [20], Govardhan et al [21].

The effect of fructose concentration on amylase production was done by taking the carbon source range of 0-3 % w/v. The optimum concentration obtained was 1.5 % fructose in production medium. The above findings are very close to the findings of Annamalai et al [22] where they found maximum growth of *B. cereus* and α-amylase activity at 1 % of substrate concentration.

Effect of nitrogen source on amylase production

To study the influence of organic and inorganic nitrogen source on the amylase activity the medium was supplemented with different nitrogen source like Peptone, Urea, Yeast extract, Beef extract, ammonium sulphate, ammonium chloride and sodium nitrate. Maximum growth and enzyme production (67.2 U/ml/min) was achieved when yeast extract used as the nitrogen source. Ammonium chloride (15.0 U/ ml/min) and beef extract (16.24 U/ml/min) showed minimum amylase production.

The effect of yeast extract concentration on amylase production was done by taking the nitrogen source range of 0-2 % w/v. The optimum concentration obtained was 1% yeast extract in production medium. Yeast extracts showed maximum activity which has been supported by some results Thippeswamy et al [23], Nguyen et al [24]. Yeast extract has been reported best nitrogen source for amylase production by *Bacillus* sp. IMD 435 by Lynn et al [25].

CONCLUSIONS

In the view of amylase importance present study is undertaken with submerged fermentation for the production of amylase. Amongst various isolates V_2 shows maximum amylase activity; (52.88 U/ml/min) in submerged fermentation. The optimum time of maximum amylase production was found 18 hrs. After 18 hrs the growth of isolate V_2 decreased sharply up to 48 hrs. The amylase maximum production was found in Medium III with amylase activity; (67 U/ml/min). The optimum pH and temperature for amylase production was found to be pH 7.0 and temperature 30°C respectively. The best 'carbon' and 'nitrogen' source for amylase production was fructose and yeast extract respectively. The optimum concentration obtained was 1.5 % fructose in production medium. The optimum concentration obtained was 1% yeast extract in production medium. Thus isolate V_2 found to be promising culture for amylase production, which will be further evaluated for amylase production on high scale.

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