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Production of Amylase from Agro-Industrial Wastes by Using Thermophilic Actinomycetes

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The aim of this research is to study the production of amylase by microbial action over the industrial and agricultural wastes. Actinomycetes are facultative thermophilic group of gram-positive bacteria which produce the antibiotics and a range of hydrolytic enzymes including amylases, proteases and lipases. This study describes the preliminary screening of six strains of actinomycetes isolated from indigenous sources (AH-I to AH-VI) for the production of amylases. Qualitative and quantitative tests suggested that strain AH-II was the best as a source of extra cellular amylases. Effect of metal ions on the growth and amylase production and the optimization of conditions like pH, temperature, agitation speed *etc.* (*e.g.*, 7.5, 60 ºC and 150 rpm, respectively) for the maximum amylase production were also assessed.

Key Words: Thermophilic actinomycetes, Gram-positive bacteria, Amylases, Agro-industrial wastes.

INTRODUCTION

Agro-industrial wastes are produced on large scale on yearly basis. Theses include sugarcane baggass, wheat straw, wheat bran, corn meal, cotton seed meal, soybean meal, mango peel, sunflower meal, *etc*. These wastes act as a source of C and N hence used as substrates[1] and mostly consist of ligno-cellulosic wastes containing mainly cellulose (40-50 %), hemicellulose (30-40 %) and lignin (8-10 %). Therefore, these have attracted significant interest to produce industrial important commodities like enzymes[2]. The starchy wastes used as substrates were from arrowroot, arum, maize, potato, pulse, rice, rice husk, tamarind SSF processes for production of food enzymes[3].

Submerged fermentation has been a major source for the production of the enzymes. Enzymes are remarkable and highly specialized proteins, possessing catalytic activity far greater than that of synthetic catalysts. All the starch degrading enzymes have been named as amylases. Amylases identified as α, β, γ amylases have been purified and characterized from various sources. These types of amylases catalyze the hydrolysis of starch and other polysaccharides into glucose, disaccharides and trisaccharides by transferring glucosyl residue to water[4].

Amylases are derived from human saliva, human hog and rat pancreas as well as microorganisms such as *Bacillus subtilis, Bacillus Coagulans, Aspergillus oryzae, Aspergillus candida, Pseudomonas saccharophila* and *Streptomyces* (actinomycetes)[5]. In the past, requirements for enzymatic preparations had been met by classical sources such as animal and higher plant tissues. But now the increasing requirement, for the enzymes has stimulated the search for enzyme from microbial world[6]. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer[7,8].

The actinomycetes are a large group of gram positive bacteria which inhibit a wide range of environments[9,10]. They secrete many extra cellular enzymes which contribute to the breakdown of complex organic materials such as proteins nucleic acids, polysaccharides and lignocelluloses found in soil and make the carbon available for the growth of other organisms[11,12].

Amylases are especially important for use in food, drink and textile industries for the hydrolysis, liquefaction or decomposition of starch containing materials. This enzyme is used in the production of adhesive, in human and veterinary therapeutic agents as auxillary in fundamental research[13,14].

Microorganisms have been divided into three main groups: psychrophiles (3 to 20 ºC); mesophiles (13 to 45 ºC) and thermophiles (42 to 100 ºC) or above according to their ranges for growth temperature. In this industrial age, thermophilic organisms have tremendous potential because of their commercial importance. A number of definitions or classification systems have been proposed for thermophilic organisms[15] but these

have remained somewhat controversial. Actinomycetes contain a large number of thermophilic species[16]. The optimum temperature for growth of most of the species ranges between 40 to 50 ºC and maximum 60 to 70 ºC. More recently Edwards[17] has reviewed the thermophiles and tailored the definition of thermophiles as those organisms which are capable of growth at temperatures above 55 ºC.

Thermophilic actinomycetes have also been described as potential candidates for biodegradation processes and transformation of chemicals into intermediate by-products or mineralized forms as well as in supperession of plant diseases by production of antibiotics, lytic enzymes, direct parasitism and competition for nutrition[18]. Goldberg and Edwards[19] have reported the isolation of a thermostable α-amylase from a thermophilic actinomycetes. The present study was designed to know the amylolytic activities of actinomycetes. Different physical factors that are pH, temperature, starch concentration *etc*. were also studied under which enzymatic activity was maximum.

EXPERIMENTAL

Six strains of actinomycetes (AH-I to AH-VI) were taken from Microbiology Research Laboratory, Department of Biological Sciences, Quaid-i-Azam University, Islamabad, as a source of microbial amylase.

Microscopy: Structures of the selected strains were examined under oil immersion lens through Gram's staining method.

Gram staining: Using sterile techniques, a smear of each strain on the slide was prepared, dried and heat fixed, flooded the smears with crystal violet and allowed to stand for 1 min then washed with distilled water. Smear was next flooded with Gram's iodine and allowed to stand for 1 min and was washed with distilled water. Decolourized with 95 % ethyl alcohol, washed with tap water. Then counterstained with safranin for 60 s and washed with distilled water. Slides were dried and examined under oil immersion.

Acid fast staining: Using sterile techniques, bacterial smear was prepared and allowed to be air dried. The smear was heat fixed and flooded with carbol fuchsin on a warm hot plate allowing the preparation to steam for 5 min. Stain was not allowed to evaporate but replenished as needed. It was also prevented from boiling by adjusting the hot plate to a proper temperature. Slides were cooled prior to washing. Smear was decolourized by acid alcohol and counter stained with methylene blue for 2 min. Finally the slides were washed with distilled water, dried and examined under oil immersion.

Selection of the suitable medium: Growth of actinomycetes was carried out in four different media in shake flask experiments. The media was autoclaved at 15 Ibs psi and 121 ºC for 15 min. Compositions of media are given below:

Medium (M-I): Starch (10 g), glycerol (2.5 g), sodium chloride (1.0 g), distilled water (1000 mL), pH of the medium (7.0).

Medium (M-2): Starch (10 g), peptone (1.0 g), K_2HPO_4 (0.3 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), distilled water (1000 mL), pH (7.0).

Medium (M-3): Starch (15 g), arginine monohydrocholoride (1.0 g), glycerol (10.5 g), sodium chloride (1.0 g), magnisium sulfate (0.05 g).

Medium (M-4): Arginine glycerol salts medium (AGS): Arginine monohydrocholoride (1.0 g), glycerol (12.5 g), dipotassium hydrogen phosphate (1.0 g), sodium choloride (1.0 g), magnesium sulphate (0.5 g), ferous sulphate (0.01 g), copper sulphate (0.001 g), zinc sulphate (0.001 g), mangenese sulphate (0.001 g), distilled water (1000 mL), pH of the medium (7.0).

After inoculating with the strains of actinomycetes the flasks were incubated at 37 ºC. After 48 h, the growth and amylase production was measured. Medium giving maximum production was selected for further study.

Quantitative test for amylase production: The method of Bernfeld[20] was used for amylase assay.

Assay medium: 1 % soluble starch in 0.02 phosphate buffer of pH 6.9. Dinitrosalcylic acid (DNS). **Reagent:** Dissolve 1 g of DNS in 20 mL 2.0 M sodium hydrochloride and 20 g of potassium sodium tararate in 100 mL distilled water.

Assay procedure: 1 mL of the enzymes extract was incubated with 1 mL of assay medium at 45 ºC for 1 h. The enzymes activity was measured on the basis of reducing sugar released during reaction. The amount of reducing sugar released was estimated by adding 1 mL of DNS reagent 1 mL filtrate starch reaction mixture and then determines the absorbance at 450 nm using aUV-120-01 spectrophotometer (Shimadzu). Control was prepared in the same way except the DNS reagent was added before incubation.

Unit of enzyme activity: One amylase unit, is amount of enzyme in 1 mL of filtrate which releases 1.0 mg of reducing sugar from 1.0 starch solution in 1 h at 45 ºC at pH 7.0.

Qualitative tests for amylase activity: The medium described by Hankin[21] was used to detect amylolytic activity. The medium contained nutrient agar 0.2 % soluble starch. The pH was maintained to 7.0. The plates were incubated with the isolated and purified actinomycetes. After 3-5 days of incubation the plates were flooded with an iodine solution. Yellow zone around the colony in a blue backgrounds shoes amylolytic activity. The diameter of the zones was measured. Only those actinomycetes were used for further study which showed amylolytic activity.

Enzyme production

Inoculum preparation: Arginine glycerol salts medium was used for inoculum development in broth culture. The medium of 150 mL was poured into 250 mL flask and pH was adjusted at 7.0 using 1 M NaOH and 1 M HCl. The medium was then autoclaved at 15 IBs psi and 121 ºC for 15 min. The medium was then inoculated by taking five loops full of strain each from the fresh culture plates under the sterilized conditions. Flasks were prepared in duplicate and kept in shaker incubator at 37 ºC and 150 rpm for 48 h. These inoculate were used in further study.

Shake flask fermentation: Batch culturing in shake flask was done for fermentation to produce extra cellular crude enzyme extract. Arginine glycerol salts medium 150 mL was poured into 250 mL flask for each strain. All the flasks were prepared in duplicate. The pH of medium in each was adjusted by using 0.1 M HCl and 0.1 M NaOH. The media was autoclaved at temperature of 121 ºC under 15 Ibs pressure for 15 min. Inoculums (10 %) were used and flasks were

incubated in shaker incubator at 37 ºC and 150 rpm for 96 h. Samples of 10 mL were collected from flask after 24 h for 6 days, centrifuged at 10,000 rpm filtered by filter paper and the filtrate was kept in refrigerator for further bioassay.

Optimization of different parameters for maximum enzymes production

Optimization of pH: pH of the arginine glycerol salts liquids medium in each flask having 150 mL of medium was adjusted to pH values of 3-10 by using 0.1 M NaOH and 0.1 M HCl. Cultures were inoculated as mentioned before. Incubation was at 37 ºC and 150 rpm in rotatory incubator shaker. 10 mL of samples taken after 48 h were analyzed for dry mass and enzyme activity. The pH of the samples was also noted after incubation.

Optimization of temperature: Production of extracellular amylase was carried out at a range of temperatures (30-80 ºC). Production medium was incubated at different temperatures.

Optimization of agitation speed: The effect of agitation speed, on the production of amylase was observed in the incubation rotatory shaker. The agitation speed was maintained at 120 to 190 rpm. For 48 h under optimized conditions.

Optimization of starch concentration: Growth and amylase production was optimized using different concentration of starch in the medium. 0.25, 0.50, 1.0, 1.25 and 1.50 % starch was added to the AGS medium. The amylolytic activity and dry cell mass was determined through amylase assay.

RESULTS AND DISCUSSION

Isolation and identification: Six strains of actinomycetes isolated from rhizosphere of a desert plant *Citrullus colocynth* and were identified on the basis of morphological and biochemical tests (Table-1).

TABLE-1
MORPHOLOGICAL CHARACTERIZATION OF ISOLATED STRAINS

Bacterial strain	Spore formation	Starch hydrolysis	Casein hydrolysis	Gram staining	Acid fast
AH-I	+	+	+	+	+
AH-II	+	+	+	+	+
AH-III	+	+	+	+	+
AH-IV	+	+	+	+	+
AH-V	+	+	+	+	+
AH-VI	+	+	+	+	+

Qualitative test for amylase production on starch agar media: The comparative measurement of amylase production by the six isolated strains of actinomycetes was carried out on starch agar media at pH 7.0. Results shown in Table-2 indicate that the strain AH-II gives maximum zone of hydrolysis *i.e.* 4.6 mm followed by (4.2, 3.4, 3.2, 2.3 and 2.1 mm) by AH-IV, AH-V, AH-I, AH-III and AH-VI), respectively. Strain AH-II indicating maximum zone of hydrolysis was selected for further study.

Quantitative test for amylase production by shake fermentation: Six strains of thermophilic actinomycetes were tested for their ability to produce amylase by shake fermentation experiments at temperature 37 ºC for 72 h at 150 rpm.

TABLE-2
ZONE OF HYDROLYSIS PRODUCED BY DIFFERENT STRAINS OF ACTINOMYCETES

Bacterial strains	Zone of hydrolysis (mm)
AH-I	3.2
AH-II	4.6
AH-III	2.3
AH-IV	4.2
AH-V	3.4
AH-VI	2.1

The amount of enzyme quantified according to the method of Bernfeld[20]. Strain AH-II produced maximum amylase while amylolytic activity of AH-IV was also good. Other strains AH-I, AH-III, AH-V and AH-VI showed low amylolytic activity.

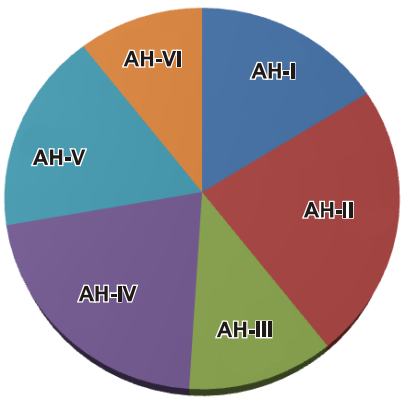

Fig. 1. Zone of hydrolysis showed by different strains of actinomycetes

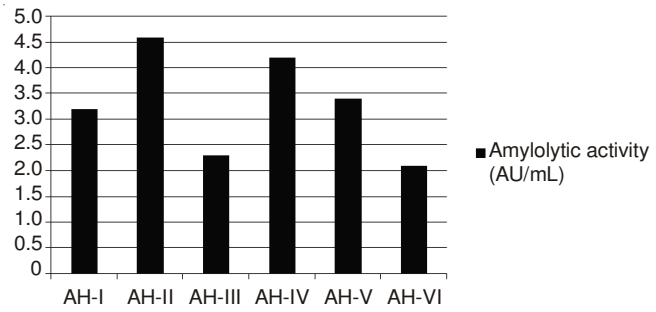

Fig. 2. Amylolytic activity of the thermophilic actinomycetes strains

Effect of metal ions on amylase production: Effect of metal ions on the production and activity of amylase in the crude enzyme extract was observed. Four metals in the form of chlorides (Fe^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+}) were added to the growth media in the concentration of 0.5 mg/100 mL. Slight increase in amylase activity was observed only in case of $MgCl_2$, No effect was observed in case of $CaCl_2$, $ZnCl_2$ and $FeCl_2$,

TABLE-3
EFFECT OF METAL IONS ON AMYLASE PRODUCTION

Metal ions (0.5 mg/100 mL)	Biomass (mg/mL)	Amylase activity (AU/mL)	Total protein (mg/mL)	Specific activity (AU/mg)
$FeCl_2$	1.60	22	1.38	15.94
$ZnCl_2$	1.60	22	1.38	15.94
$CaCl_2$	1.60	22	1.38	15.94
$MgCl_2$	1.83	23	1.38	16.66

Agro-industrial wastes and production of amylase

Soybean meal: Soybean meal was added to the basal medium in different concentrations as a substitute of starch and arginine. It was observed that growth of actinomycete and amylase production has been increased. Maximum production of 176.2 AU was observed after 72 h when 1.5 % soyabean meal was added to the medium (Fig. 3).

Wheat bran meal: Powdered wheat bran was added in the basal medium as a substitute of carbon and nitrogen sources. Maximum production of amylase (171.6 AU) was observed at 1.5 % wheat bran concentration after 72 h (Fig. 4).

Peanut meal: Powdered Peanut meal was added in the basal medium as a substitute of carbon and nitrogen sources. Maximum production of amylase (122.7 AU) was observed at 2 % peanut meal concentration after 72 h (Fig. 5).

Mustard meal: Powdered mustard meal was added in the basal medium as a substitute of carbon and nitrogen sources. Maximum production of amylase (121.0 AU) was observed at 1.00 % mustard meal concentration after 72 h (Fig. 6).

Sunflower meal: Powdered sunflower meal was added in the basal medium as a substitute of carbon and nitrogen sources. Maximum production of amylase (148.2 AU) was observed at 1.5 % sunflower meal concentration after 72 h (Fig. 7).

Linseed meal: Powdered linseed meal was added in the basal medium as a substitute of carbon and nitrogen sources. Maximum production of amylase (128.6 AU) was observed at 2 % linseed meal concentration after 72 h (Fig. 8).

Molasses meal: Molasses was added in the basal medium as a substitute of carbon and nitrogen sources. Maximum production of amylase (118.6 AU) was observed at 2.5 % molasses concentration after 72 h (Fig. 9).

Cotton seed meal: Powdered cotton seed meal was added in the basal medium as a substitute of carbon and nitrogen sources. Maximum production of amylase (132.4 AU) was observed at 1 % cotton seed meal concentration after 72 h (Fig. 10).

Corn meal: Powdered corn meal was added in the basal medium as a substitute of carbon and nitrogen sources.

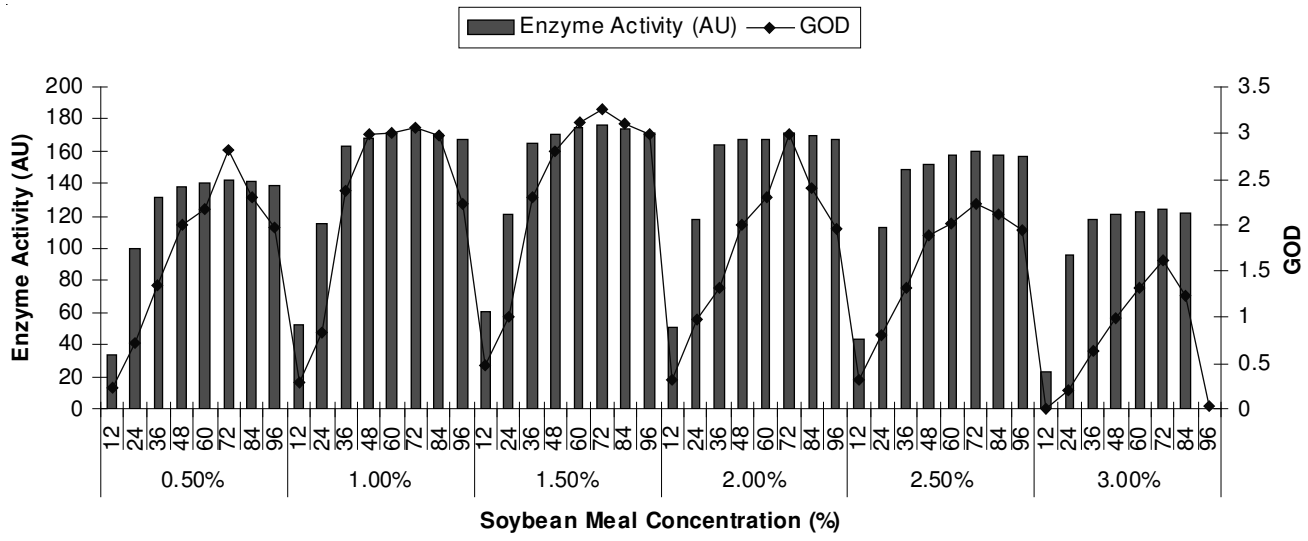

Fig. 3. Study on the growth and amylase production using soyabean meal

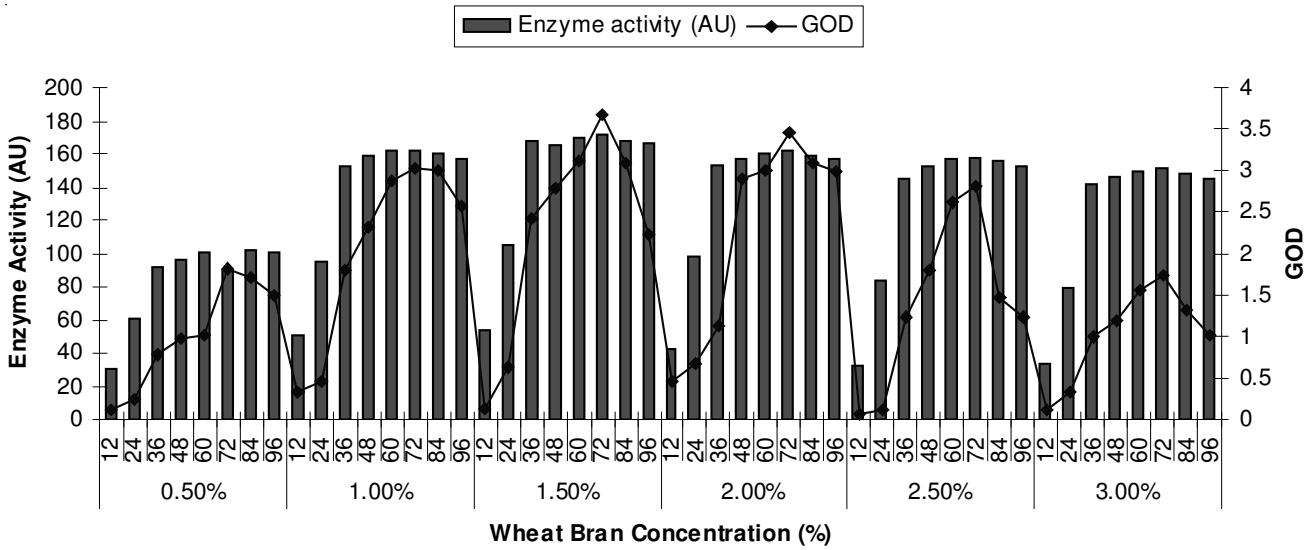

Fig. 4. Study on the growth and amylase production using wheat bran

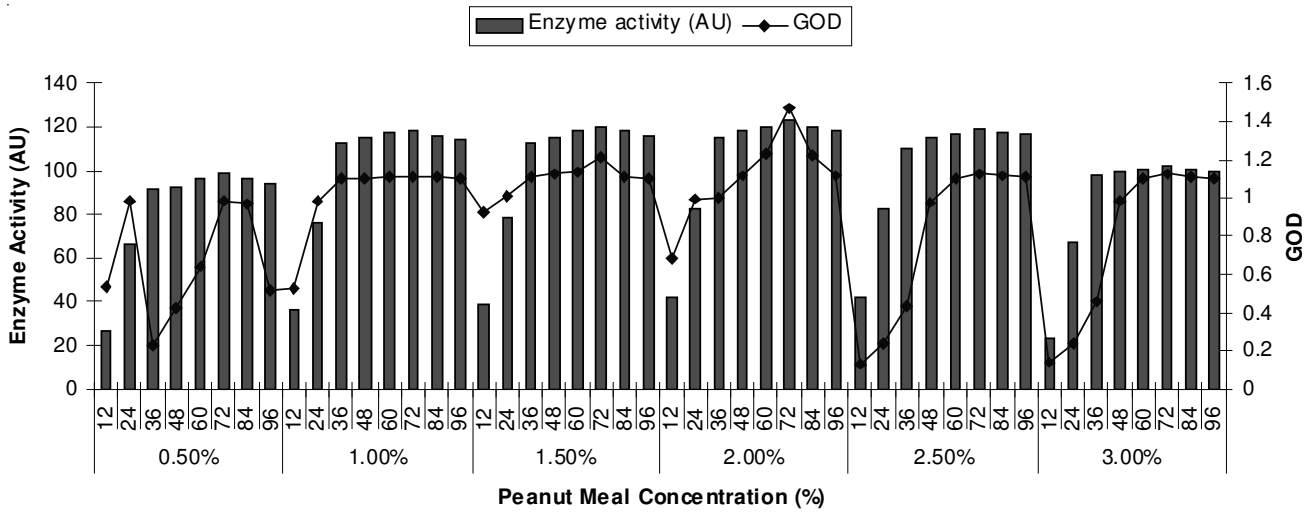

Fig. 5. Study on the growth and amylase production using peanut meal

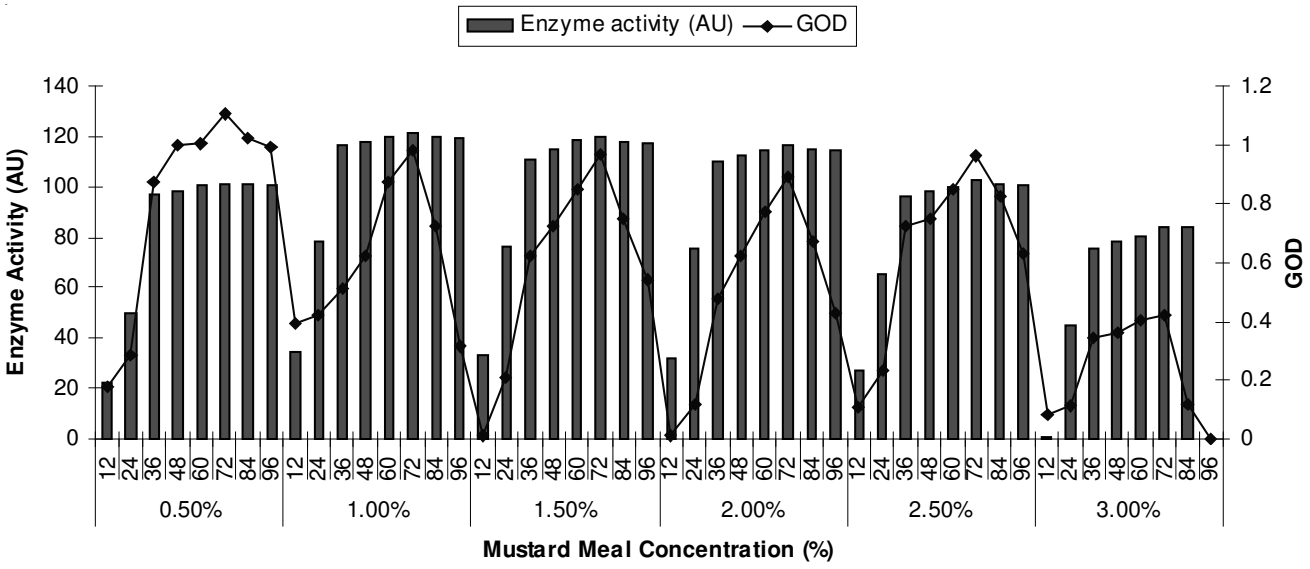

Fig. 6. Study on the growth and amylase production using mustard meal

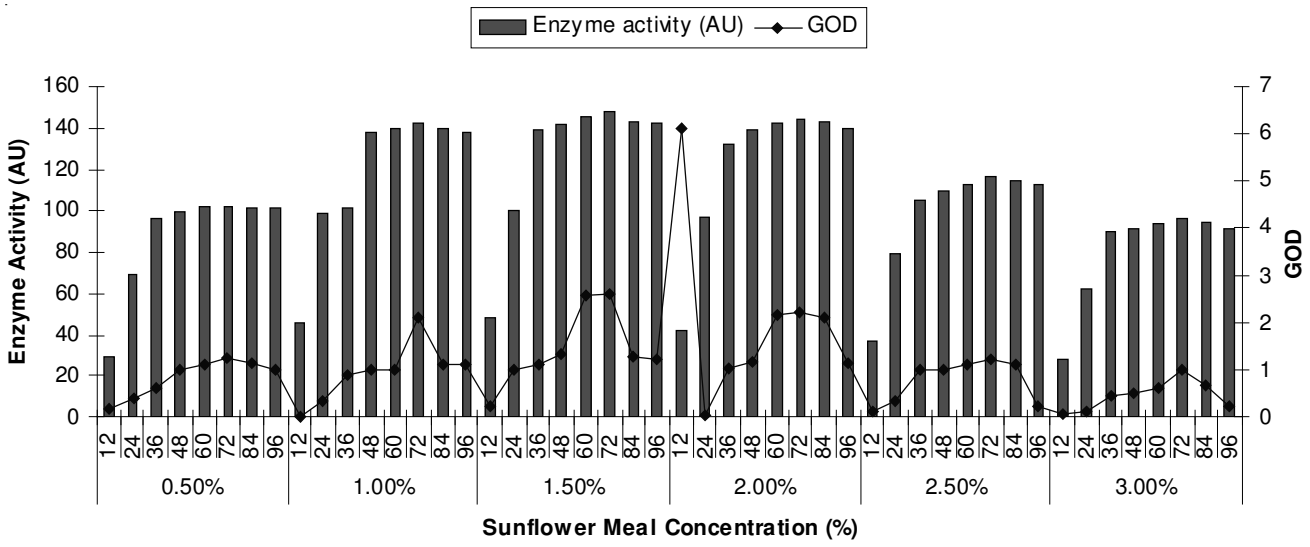

Fig.7. Study on the growth and amylase production using sunflower meal

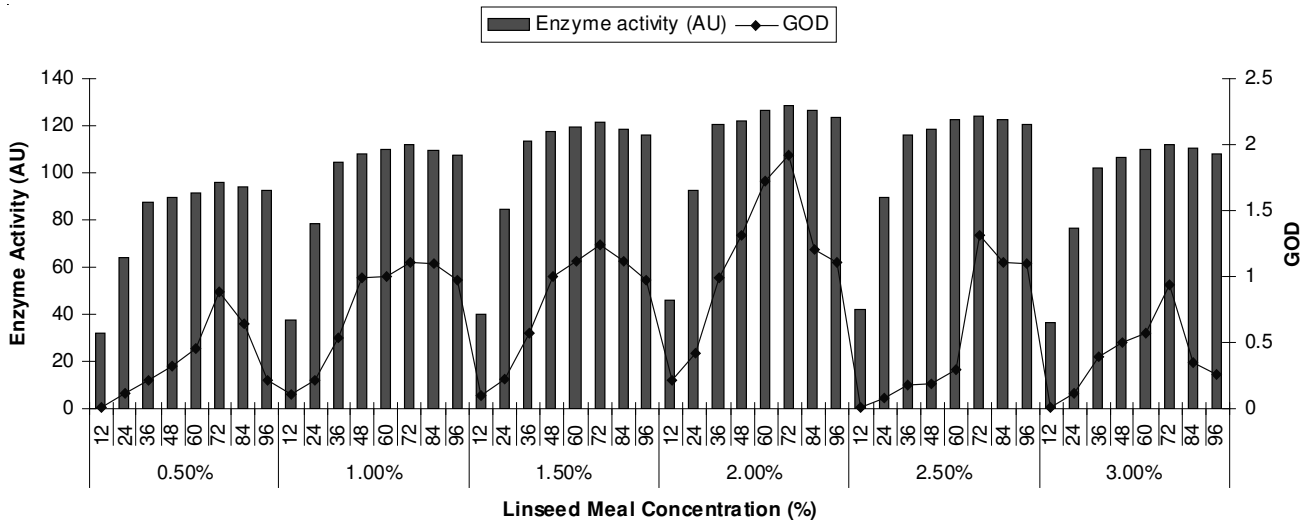

Fig. 8. Study on the growth and amylase production using linseed meal

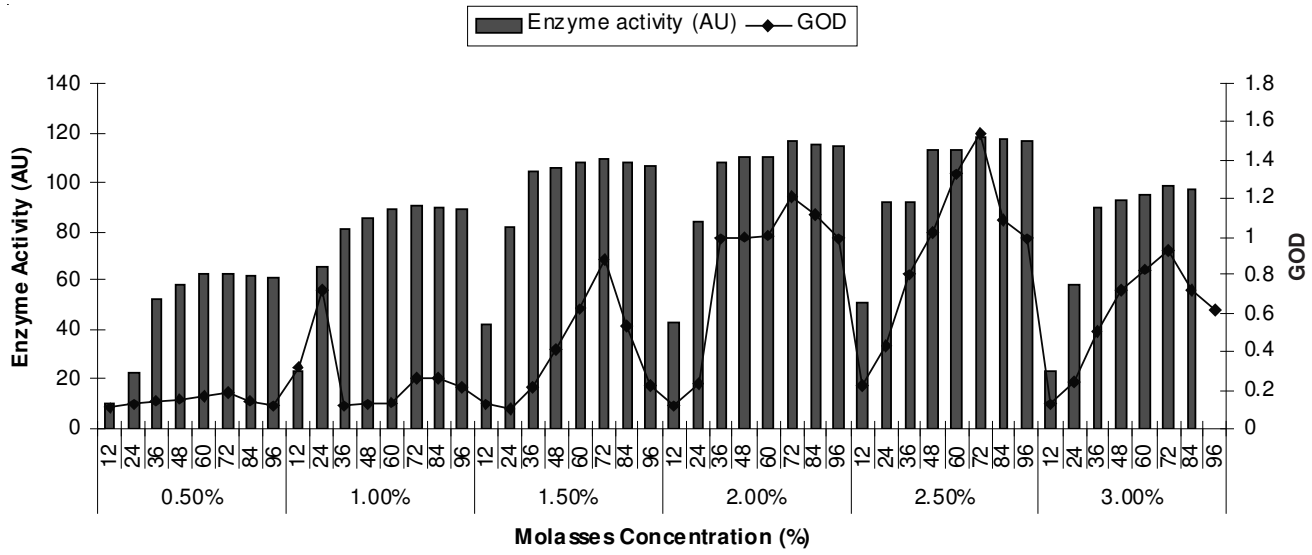

Fig. 9. Study on the growth and amylase production using molasses

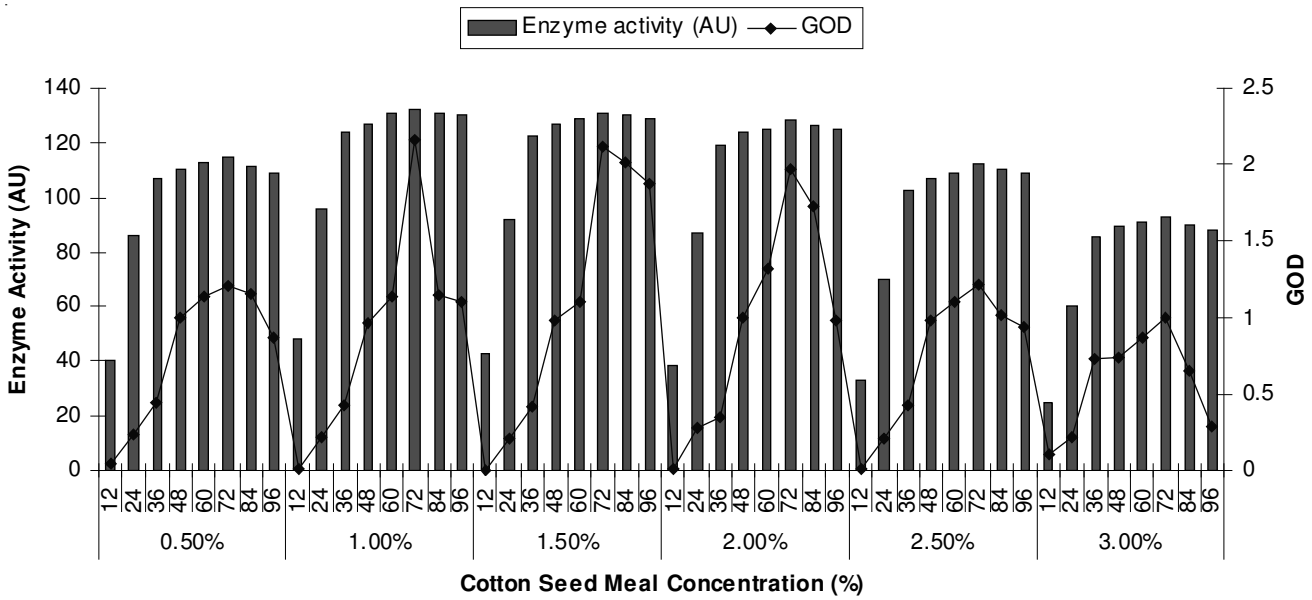

Fig. 10. Study on the growth and amylase production using cotton seed meal

Maximum production of amylase (151.2 AU) was observed at 1.5 % corn seed meal concentration after 72 h (Fig. 11).

Use of Agro-industrial wastes in combination

Molasses in combination with different Agro-industrial wastes: Molasses in combination with different agro-industrial wastes indicates that maximum amylase production was observed to be 3.992 and 198.6 AU, respectively after 72 h of incubation. Mustard meal in the ratio of 1:2 (Fig. 12).

Soybean meal in combination with other agro-industrial wastes: Soybean meal in combination with different agro-industrial wastes showed that maximum production was observed to be 3.826 and 230.6 AU, respectively, after 72 h of incubation by using soybean meal:molasses in the ratio of 1:2 (Fig. 13).

Wheat bran in combination with other agro-industrial wastes: Wheat bran in combination with corn meal, cotton seed meal, peanut etc were used as carbon and nitrogen sources. Maximum amylase production was observed to be 4.211 and 212.3 AU respectively after 72 h of incubation by using wheat bran. Molasses as 2:1 (Fig. 14).

Conclusion

(a) Thermophilic actinomycetes can be isolated from the desert plants where temperature is quit high in the day and low in the night. (b) Two strains of Actinomycetes AH-II and AH-IV possess great ability to produce thermophilic amylases. However, strain AH-II was selected for studies. (c) Actinomycete strain AH-II can be grown optimally at 60 ºC with optimum pH 7.5, agitation at 150 rpm and 1 % starch concentration. (d) Amylase production was maximum after 48 h of incubation. (e) Amylase is highly stable in the temperature range up to 60 ºC. (f) Actinomycete strains AH-II also produced protease and lipase along with amylase so I a valuable thermophilic industrial strain.

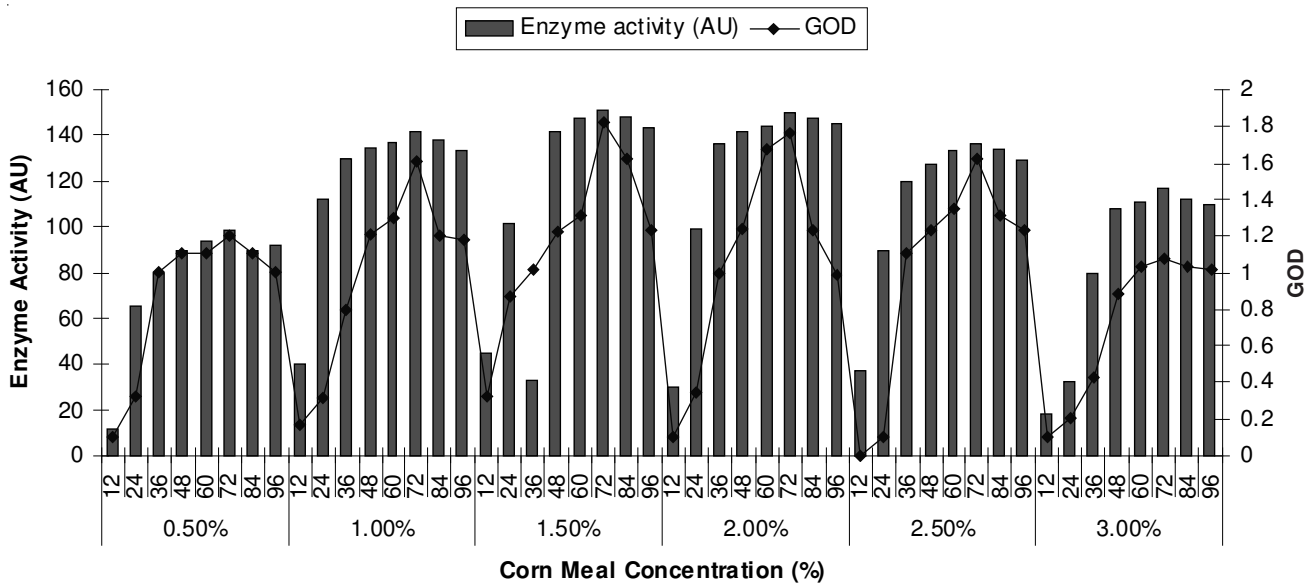

Fig. 11. Study on the growth and amylase production using corn meal

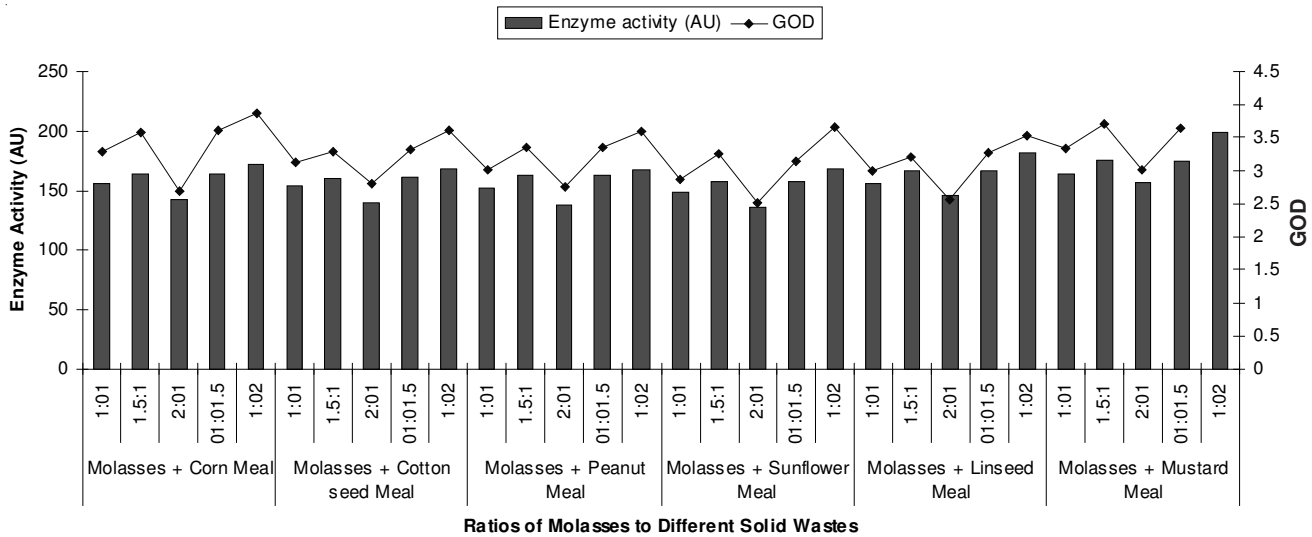

Fig. 12. Effect of molasses in combination with other agro industrial wastes

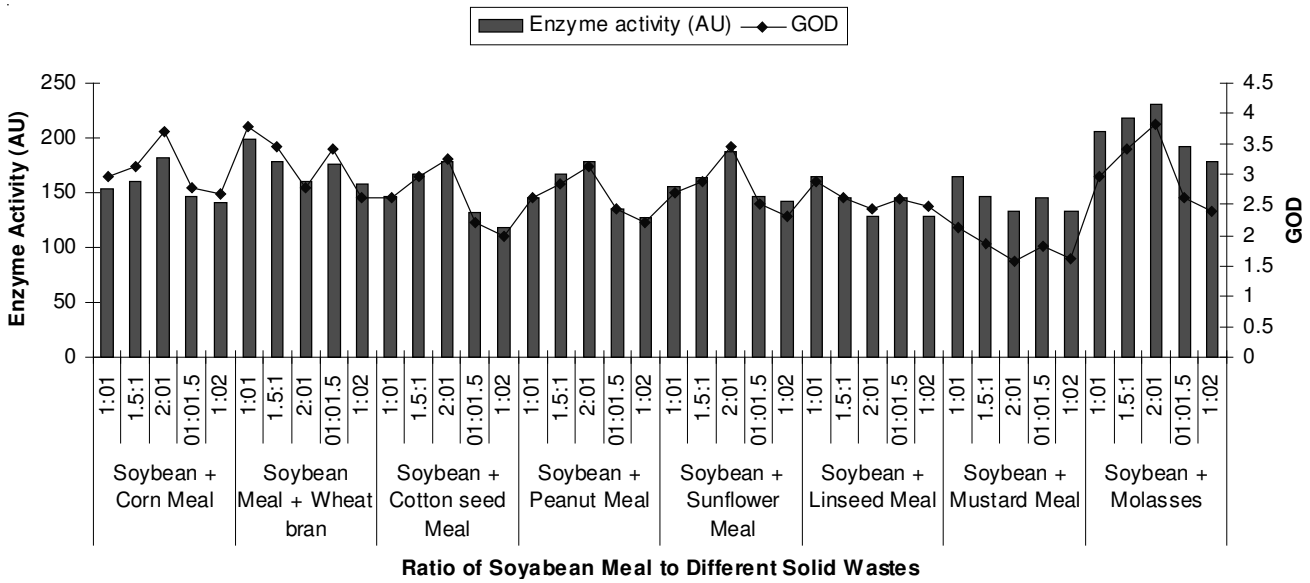

Fig. 13. Effect of combination of soybean meal with other agro-industrial wastes

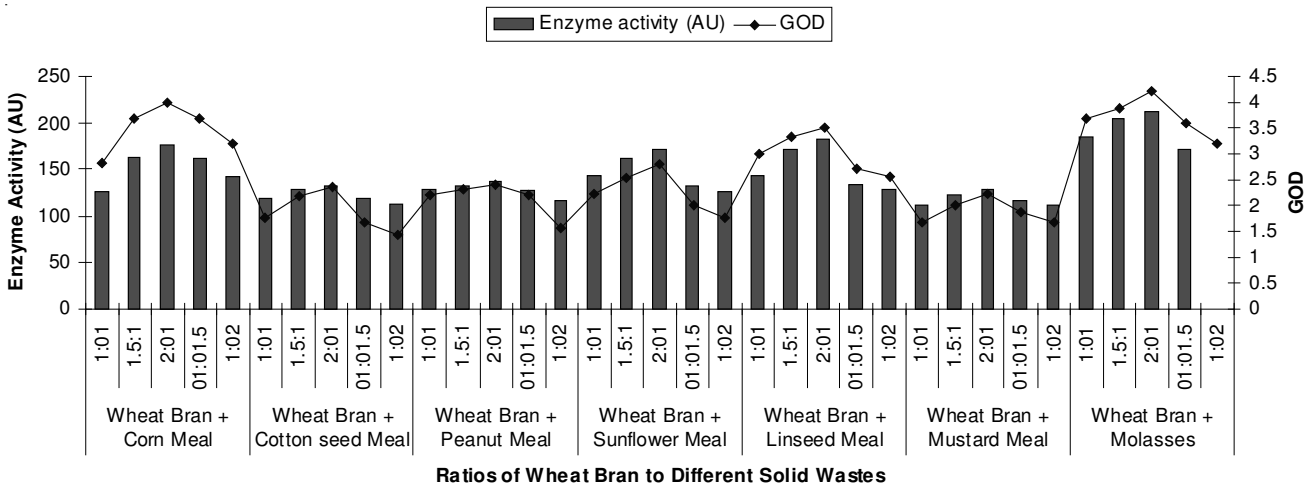

Fig. 14. Effect of combination of wheat bran other agro industrial wastes

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