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Effect of inducers and enhancers on bioprocessing of α-amylase produced by *Bacillus* sp AVMB2 isolated from chilli Rhizosphere

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ABSTRACT

Bacterial strain (AVMB2) isolated from Chilli rhizosphere were screened for Amylase production and tentatively identified as genus Bacillus by morphological, physical, physiological, biochemical and molecular characterization by partial 16S r RNA gene sequencing. α-amylase enzyme production was quantitatively characterized from Bacillus strains and optimized the culture conditions for bioprocessing under submerged fermentation with different organic sources. The production of amylase was increased by 35-60% under optimized conditions of 35°C, pH7.0 with starch as carbon sources and peptone as nitrogen sources respectively after 48 hrs of incubation. Soya bean oil seed cake induced maximum amylase production.

Key words: *Bacillus*, Amylase, Chilli rhizosphere, Carbon, Nitrogen, Soya bean, Oil seed cake.

INTRODUCTION

Microbial enzymes present a wide spectrum of characteristics that make them useful for specific application. Microbial α-amylases are among the most important hydrolytic enzymes and have been extensively studied. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry [1]. Each application of α-amylase from microbial source requires unique properties with respect to specificity, stability, temperature and pH dependence [2].

Amylases contribute major class of industrial enzymes constituting approximately 25% of the enzyme market [3,4]. Now a days, amylases (α-amylases, β-amylases and gluco amylases) represent one of the most important enzyme groups within the field of Biotechnology [5]. α-amylases (EC 3.2.1.1- 1,4- α-glucanglucano hydrolase, endoamylase) is a classical calcium containing enzyme that catalyze the hydrolysis of starch and related carbohydrates by randomly cleaving internal α-D-(1,4) glycosidic linkage, yielding glucose, maltose, malto triose and other oligosaccharides [6]. Further more their exquisite specificity keeps unwanted side reactions to a minimum and maximizing the yield. α-amylases so far known are not sufficient to meet most of the industrial demands [7]. Hence screening of micro organisms with higher α-amylases could therefore facilitate the discovery of novel α-amylases suitable for new industrial applications such as bread and baking industries [8].

More over, microbial amylases have a broad spectrum of industrial applications, as they are more stable with great genetic diversity and, high enzymatic activity under wide range of environmental conditions (extreme pH, temperature, salinity, pressure etc.,). The major advantages of using micro organisms for production of amylases are simple, cost effective and easily standardized to obtain enzymes of desired characteristics [9,10] more over able to

produce in bulk and the ease at which it can be processed for desired products [11]. The increased uses have placed greater stress on increasing indigenous α-amylase production and search for more efficient processes [12]. The production of microbial amylases from bacteria is dependent on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, incubation period, pH, temperature, metal ions and thermostability [1]. Such industrially important micro organisms are in fact found in the genus *Bacillus* and can be exploited commercially for their rapid growth rate leading to short fermentation cycles, capacity to secrete proteins into the extracellular medium and safe handling.

Bacillus is endowed with the production of thermostable α-amylase and also large quantities of other enzymes. Indeed, 60% of commercially available enzymes are obtained from different species of *Bacillus i.e., B.subtilis, B.stearothermophilus, B.licheniformis and B.amyloliquefaciens*[13]. Though, different *Bacillus* species have similar growth patterns and enzyme profiles, but their optimized conditions vary, depending upon the strain. In the present study, bioprocessing of α amylase of *Bacillus* strains (AVMB2) isolated from chilli rhizo sphere and the effect of inducers and enhancers on enzyme production was characterized under submerged fermentation (SmF).

MATERIALS AND METHODS

For the studies on isolation, characterization, growth and quantification of amylase produced by *Bacillus* isolates, the chemicals used were analytical grade chemicals and reagents purchased from NSP (Mumbai, India).

2.1. Isolation, media and culture conditions

Soil samples were collected from various sites of Chilli rhizosphere located in Achampeta of Guntur district, A.P. The samples were collected from a depth of 5-6 cm after scrapping the top layer. The samples were brought to the laboratory in sterile zip lock covers and stored in refrigerated conditions if not used immediately.

The isolation of soil bacteria was performed by serial dilutions. After sieving, the soil sample collected, 1 gram of the soil was dissolved in 80% saline solution and mixed thoroughly for 10 minutes and subjected to heat shock treatment in a water bath set at 80°C for 10 minutes to ensure only the microbial spores behind. The serially diluted sample suspensions ranging from 10^{-5} to 10^{-7} were plated onto the Nutrient Agar Medium (NAM) using spread plate technique and incubated for 24 to 48 hrs at 37°C.

2.2. Screening for amylase producing *Bacillus* cultures

Screening for potent amylase producing Bacteria was carried out by Starch hydrolysis test. The selected isolates were screened for amylolytic activity on starch agar plates by Starch hydrolysis test [14]. The microbial isolates were streaked on starch agar plates and incubated at room temperature (37°C) for 48 hrs. After incubation, the plates were flooded with iodine solution with a dropper for 30 seconds. Presence of a clear zone around the growth indicate +ve result where as the presence of blue colour around the growth indicate –ve result. The isolates that produce clear zones of hydrolysis were considered as amylase producers and were further investigated.

2.3. Morphological, biochemical and physiological characterization

Colony characters like colour, diameter, shape, configuration, margin, elevation and mucilage production, endo spore staining and gram staining were studied as per the standard procedures. Biochemical and physiological characterization were studied [15].

2.4 Molecular characterization of bacterial isolate

Pure cultures of potential fungal antagonistic CEG was grown until log phase and genomic DNA were isolated essentially according to Bazzicalupo [43]. The amplification of 16S rRNA gene was done by using universal bacterial primer 1492R (5´-TACGGYTACCTTGTTACGACTT-3´) and 27F (5´ AGAGTTTGATCMTGGCTC AG-3´) as per the conditions described by Pandey [16]. The PCR product was sequenced at Indian Institute of Horticulture Research, Hasserghat, Bangalore. The sequences obtained were compared with those from the GenBank using the BLAST program [16] and Phylogenetic trees reconstructions were obtained by the Neighbor joining method 1000 bootstrap replicates were performed to assess the statistical support for each branch in the tree [17,18].

2.4. Assay of amylase

Unless otherwise stated, all experiments were carried out in triplicate. Amylase activity was assayed as described [19], with some modifications. Briefly, the 0.5ml of 1% starch in 0.1M phosphate buffer (pH6.5) + 0.5 ml of enzyme were incubated for 30 min at room temperature (37°C). The reaction was arrested by adding 1.0 ml of dinitrosalicysilic acid reagent and kept on boiling water bath for min and 10 ml of distilled water was added. Absorbance was measured at 540nm against blank. Blank was the same as above without incubation. One unit of the amylase activity was defined as the amount of enzyme that liberated one µ mole of reducing sugar (maltose equivalent) under assay conditions.

2.5. Optimization of Amylase production

2.5.1. Effect of pH, temperature and NaCl on amylase

Effect of pH was studied from pH 2.0- pH 11.0 in phosphate buffer. Effect of temperature was studied from 20°C-50°C and salinity was studied by using 0%-4% of NaCl in same buffer.

2.5.2. Effect of incubation period, inoculums level and substrate concentration

Effect of incubation period was studied from 24-144 hrs in phosphate buffer. Effect of inoculum level was studied using 0.5-4.0% and substrate concentration was studied by using 0.2-1.4% in the same buffer.

2.6. Bioprocessing of amylase production

2.6.1. Effect of carbon and nitrogen sources on amylase production

For optimization of cultural conditions, amylase production medium (Peptone, 6.0 g l^{-1} ; Potassium chloride, 0.5 g l^{-1} ; Magnesium sulfate, 0.5 g l^{-1} ; Starch, 1.0 g l^{-1} ; Distilled water, 1000.0 ml and pH 7.0) was used. To study the effect of carbon source on production of amylase activity in the above medium, 1% starch was replaced by different carbon sources as listed in Table-2. Similarly, for studying the effect of nitrogen source in the same above medium, peptone was replaced by different organic and inorganic nitrogen sources listed in Table-3.

Submerged fermentation process

After optimization of temperature, pH, NaCl, carbon source and nitrogen sources, bioprocessing of amylase was assayed by using different oil seed cakes as listed in Table-4 under submerged fermentation process. Inoculum was prepared by transferring one loop- full of cells from slant culture to the inoculum (50 ml/250 ml Erlenmeyer flask) and incubating the flask at room temperature in a rotary shaker at 120 rpm for 48 h. Fermentation medium (total volume 100 ml in 250 ml Erlenmeyer flask) was inoculated with 0.1% inoculum and incubated for 72 h under the same conditions. After 48 h of fermentation, broth was centrifuged at 6000 rpm for 15 min at 4°C and the crude extract was taken and used for amylase activity.

2.6.2. Effect of oil seed cakes

2.6. 2.1. Effect of different concentrations of oil seed cakes

In order to evaluate the ideal concentration of various oil seed cakes (Black sesame oil seed cake, Coconut oil seed cake, Ground nut oil seed cake, Soya bean oil seed cake and White sesame oil seed cake) required for effective amylase production, the *Bacillus* isolates were grown in the production medium with different concentrations of each oil seedcake ranging from 1.0 -5.0 gm/litre. After incubation, the amylase activity was estimated by the procedure detailed.

2.6.2.2. Effect of combination of oil seed cakes

The impact of combinations of the oil seed cakes on amylase production, (in equal proportions) on the amylase production, was determined by inoculating the production medium containing 3 and 4 gm of different oil seed cakes in combination later amylase activity was estimated as per the procedure detailed.

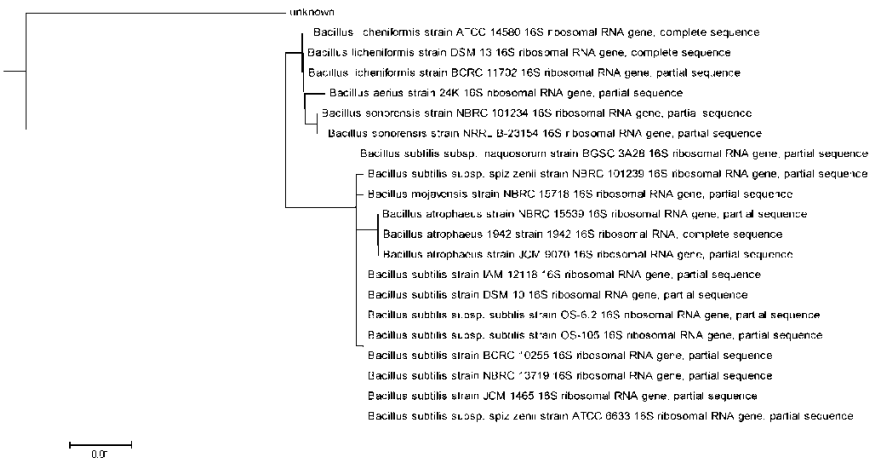
RESULTS AND DISCUSSION

In the present study, thirty one cultures (B1, B2, B3,……B31) were isolated, screened for amylase production quantitatively and six isolates ($AVMB_1$, $AVMB_2$, $AVMB_3$, $AVMB_4$, $AVMB_5$ and $AVMB_6$) were found to be the producers of amylase where as the isolate $AVMB_2$ was promising amylase producer. The morphological, physiological characteristics of the *Bacillus* culture were shown in Table 1.

Table-1 Morphological and physiological characteristics of Bacillus culture AVMB2

Parameters	Characteristics of AVMB2
Cellular characteristics	
Morphology	straight, rod shaped
Staining characteristics	
gram positive, spore forming	
a) Cultural characteristics	
Nutrient agar colonies	round with smooth margins
b) Physiological characteristics	
Growth factor	optimum growth at 35°C
range 10 to 55°C	
Catalase	positive
Amylase	positive
Gelatinase	positive
Caseinase	positive
Lipase (olive oil)	positive
Tween -80	negative
Urease	negative
Ammonia	positive
Litmus milk	alkaline with proteolysis
H_2S production	negative
Indole production	negative
Methyl red test	negative
Voges proskauer test	positive
Citrase	positive
Nitrate reductase	positive
Lysozyme	positive
Deamination of phenyl alanine	negative
Sugar fermentation	
Acid from	
(a) Glucose	positive
(b) Arabinose	negative
(c) Xylose	negative
(d) Mannitol	negative
Gas from glucose	negative

Fig 1 Phylogenetical analysis of *Bacillus* AVMB2 (Unknown) isolate based on 16S rDNA base sequence



The bacterial isolate AVMB2 (Unknown) was characterized by 16S r DNA partial sequence analysis. A 1342bp PCR product of 16S r DNA gene was amplified from genomic DNA of Bacillus AVMB2 strain. Sequence similarity showed the Bacillus AVMB2 stain 16S r DNA sequence had 99% similarity 16S r-DNA to B.lichniformis stain

and 86% similarity to 16S r-DNA gene sequence of *B.subtilis* a phylogenetic analysis revealed that it is closely related Bacillus licheniformis. The sequence was submitted in GEN BANK NCBI (ID No. 1653770) with a name *Bacillus* AVMB2 (Fig 1).

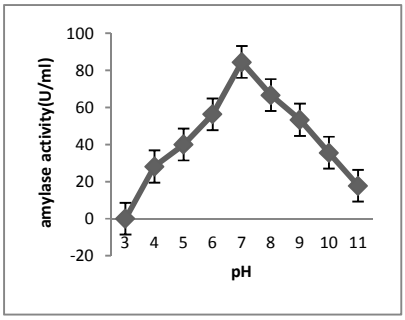


Fig.2-Effect of pH on amylase production from AVMB2

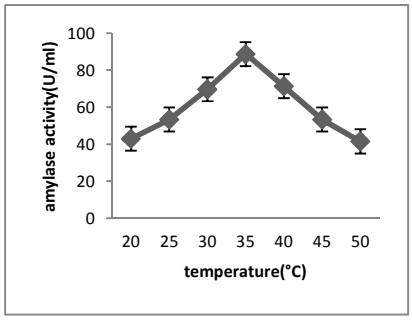


Fig.3-Effect of temperature on amylase production from AVMB2

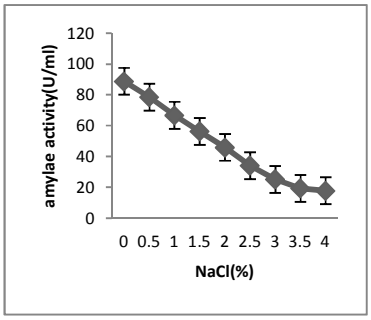


Fig.4-Effect of salinity on amylase production fromAVMB2

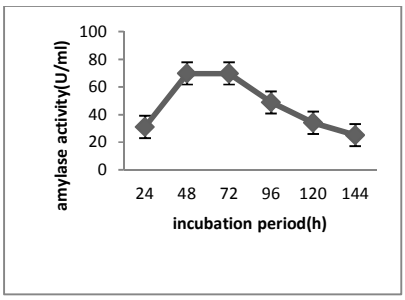


Fig. 5-Effect of incubation period on amylase production from AVMB2

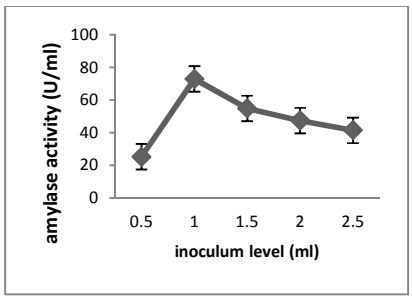


Fig. 6-Effect of inoculums levels on amylase production from AVMB2

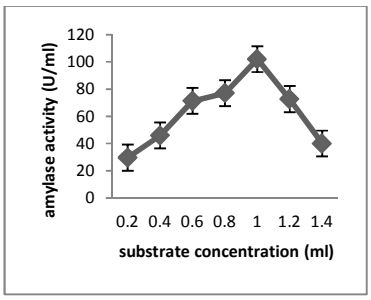


Fig. 7-Effect of substrate concentration on amylase production from AVMB2

Amylase production by the strain $AVMB_2$ showed maximum activity at neutral pH (7.0) and thermophilic temperature (40°C) as shown in Fig. 1 and 2. And hence pH and temperature were optimized at pH7.0 and 40°C. Most of the *Bacillus* strains used commercially for the production of α-amylases by SmF have an optimum pH between 6.0 and 9.0 for the growth and enzyme production [13,20,21] and also reported in different species of *Bacillus i.e., B.subtilis, B.stearothermophilus, B.licheniformis and B.amyloliquefaciens*[13].Our results were similar to that of optimum pH and temperature of amylase reported in a previous study [22,23,24,9,25,26].

Table 2- Effect of carbon source on the production of amylase from *Bacillus sp*. AVMB2
[Data are average of triplicates]

Carbon source	amylase activity of AVMB2 (U/ml)
Arabinose	81.48
Galactose	40.00
Fructose	72.86
Sorbose	93.33
Glucose	31.11
Mannose	50.37
Xylose	72.86
Ribose	50.37
Lactose	81.48
Maltose	97.92
Starch	105.12
Mannitol	40.00

Jana *et al*. [27] reported an extracellular, thermostable salt tolerant amylase producing *Bacillus* sp.MD-124, isolated from municipal garbage. In fact, some isolates can also be grown at neutral pH in the presence of sodium chloride

[28]. The effect of salinity on amylase production of the strain AVMB2 was studied and found that the salinity inhibits the growth of AVMB2 (fig. 3) and hence the strain AVMB2 were identified as sensitive to NaCl in contrast to the previous report. Similarly the effect of incubation period, inoculums level and substrate concentration on amylase production was also studied. The optimum conditions 48 hrs of incubation, 1.0 % inoculum level and 1.0% substrate concentration were found to be optimized conditions for maximum amylase activity for the strain AVMB2 (fig. 4, 5 and 6). The results showed coincidence with the earlier reports [29,30,31].

Effect of carbon and nitrogen on amylase production by the strain AVMB2 was studied using different carbon sources as listed in table -2 and nitrogen sources listed in table- 3.

In strain AVBM2 starch induced maximum amylase activity (105.12 U/ml), followed by maltose, sorbose, arabinose and lactose and very low amylase activity was observed in galactose, mannitol and glucose (Table-2, fig-7). Several reports revealed carbon source utilized for highest production of α amylase is highly specific to the species of *Bacillus*. Different species of bacillus showed maximum enzyme production at different carbon sources such as starch, lactose, maltose [31,32,33,34,35,36,37,38,39,40]. Our results revealed the strain AVMB2 showed varied growth response at different carbon sources. Based on the results starch for the strain AVMB2 was selected as carbon sources for inducing maximum amylase activity in bioprocessing.

Table 3- Effect of nitrogen source on the production of amylase from Bacillus sp.
[Data are average of triplicates]

Nitrogen source	amylase activity of AVMB2 (U/ml)
Organic sources	
Peptone	115.26
Yeast	66.66
Beef	40.00
Inorganic sources	
Urea	29.62
Potassium nitrate	56.29
Sodium nitrate	42.96
Sodium nitrite	63.56
Ammonium oxalate	75.55
Ammonium chloride	48.88
Ammonium nitrate	53.33
Ammonium sulphate	22.22
Asparagine	71.11

Hiller *et al*[42] demonstrated the effect of lactose and nitrogen on cell physiology and α-amylase production. The strain AVMB2 produced higher amounts of amylase with the complex nitrogen sources, than the simple nitrogen sources (table-3). Among the complex nitrogen sources, peptone produced maximum amylase, followed by yeast and beef in strain AVMB2 (table- 3, fig-8). Earlier, yeast extract was found to be good nitrogen source for the production of α-amylase in *B.alkalophilus, Bacillus* strains NCIN 1120, IMD 37024 [43] and MID 435 [24] and *B. amyloliquefaciens* [23,45,46,47].Bajpai and Bajpai reported peptone was found to be the best nitrogen source followed by beef extract and yeast extract for maximum production of α amylase from *B.licheniformis* TCRDC-B13 [22]. Inorganic nitrogen source such as ammonium chloride induced maximum α-amylase production by *B.subtilis* DM-03 [47,48,49] where as ammonium sulfate or ammonium acetate inhibited the enzyme production in *Bacillus cereus50*. In our results, among the simple nitrogen sources, ammonium oxalate and sodium nitrite for the strain AVBM2 induced highest amylase production. Urea resulted in low amylase production in the strain. Our results reported that that C/N ration of 1:1 with starch and peptone as carbon and nitrogen sources for AVMB2, respectively (fig-9).

Considering the characteristics such as pH, temperature, salinity, incubation period, inoculums size, substrate concentration, carbon source and nitrogen source as important inducers and effectors for bioprocessing, SmF was carried out with different oil seed cakes under specified and optimized characteristics for the strain AVMB2 (Table -4).

Table 4- Effect of oil seed cakes on the production of amylase from *Bacillus sp.*
[Data are average of triplicates]

Oil seed cakes (1 gm/lt)	amylase activity of AVMB2 (U/ml)
individual	
Soya bean oilseed cake	68.21
Ground nut oilseed cake	47.40
Coconut oilseed cake	47.40
White sesame oilseed cake	17.77
Black sesame oil seed cake	13.33
Combination (3gm/lt)	
Black sesame: Coconut	74.07
Black sesame: Ground nut	71.31
Black sesame: White sesame	63.56
Black sesame: Soya Bean	50.37
Coconut: Ground nut	32.59
Coconut: Soya Bean	38.51
Coconut: White Sesame	47.40
White Sesame: Ground Nut	74.07
White Sesame: Soya Bean	96.39
Ground nut: Soya Bean	90.27
Combination (4gm/lt)	
Black sesame: Coconut	63.56
Black sesame: Ground nut	17.77
Black sesame: White sesame	22.22
Black sesame: Soya Bean	16.29
Coconut: Ground nut	23.70
Coconut: Soya Bean	19.25
Coconut: White Sesame	23.70
White Sesame: Ground Nut	56.29
White Sesame: Soya Bean	82.96
Ground nut: Soya Bean	66.66

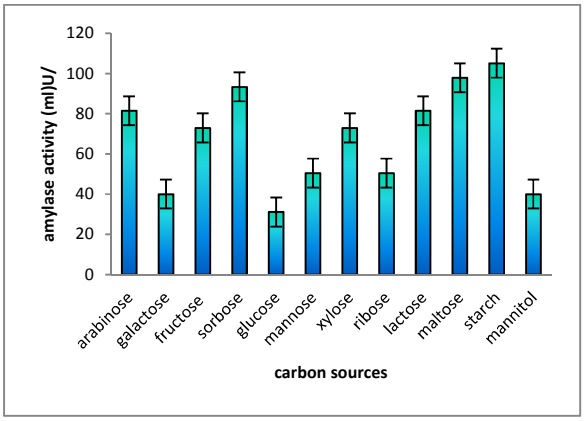


Fig.8- Effect of carbon sources on amylase production byAVMB2

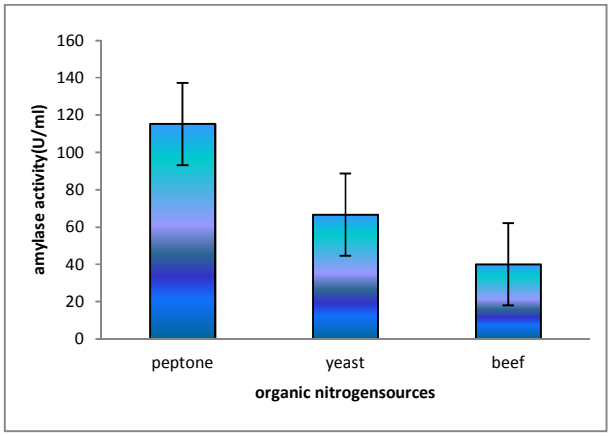


Fig.9- Effect of organic nirogen sources on amylase production byAVMB2

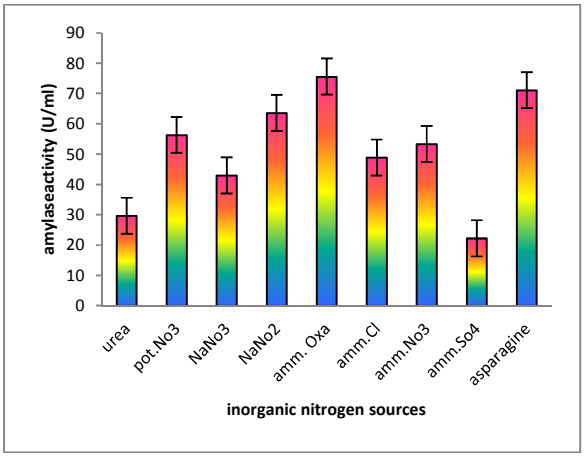


Fig.10- Effect of inorganic nitrogen sources on amylase production byAVMB2

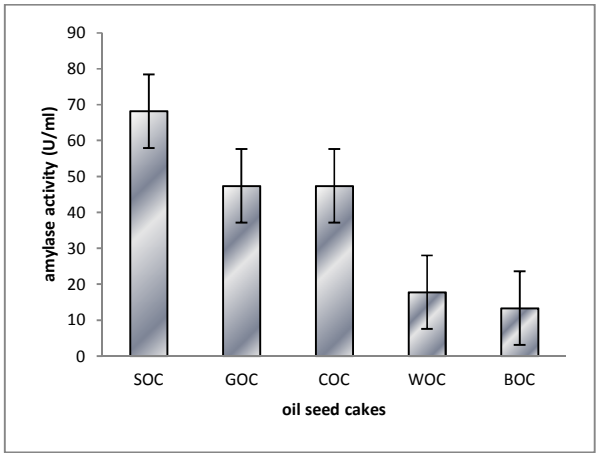


Fig.11- Effect of oil seed cakes on amylase production byAVMB2

In an attempt to choose a potential substrate which supports amylase production, various agro residues (soya bean, coconut, ground nut, black sesame and white sesame) were screened for the replacement of starch. Different patterns of the enzyme induction was observed when different oil seedcakes were used. α-amylase was maximally expressed in the presence of soya bean oil seed cake followed by coconut oil seed cake (Table-4, fig-10). Earlier studies also reported that amylase production varies with strain and type oil and concentration of oil seed cake individually and in combination [29,51]. wheat bran and groundnut oilcake (GOC) in mass ratio of 1:1 was proved as best substrate source for the production of alpha amylase from *Bacillus lichensiformis* by solid state fermentation [29].

In present results the ideal concentration for maximum amylase production for all the oilseed cakes expect for coconut oil seed cake was found to be 3gm/lt followed 4 gm/lt. where as for coconut oil seed cake it was found to be 2gm/lt followed by 3 gm/lt. The amylase production was found to be more when 3 gm/Lt quantity of oilseed cakes was used in combination than 4 gm/Lt. When 3 gm/Lt quantity of oilseed cakes were used in combination, maximum amylase production was noticed in the combinations containing Coconut, Ground nut and Soya bean oilseed cakes. While, in combinations at 4 gm/Lt, maximum production was noticed with combinations containing Soya bean and Ground nut oilseed cakes.

CONCLUSION

From the above results it can be concluded that 35-60% increased amylase production was achieved under optimized conditions of 35°C, pH7.0 with maltose and starch as carbon sources, yeast extract and peptone as nitrogen sources respectively after 48 hrs of incubation. Soya bean oil seed cake induced maximum amylase production.

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