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Original Research Article

Effect of Agro residues on Amylase activity of *Bacillus species*

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A B S T R A C T

Bacterial strains (B1, B2, B3, B4, B5 and B6) isolated from red soils of acharya nagarjuna University and were screened for Amylase production and tentatively identified as genus Bacillus by morphological, physical, physiological and biochemical characterization. α-amylase enzyme production was quantitatively characterized from Bacillus strains and optimized the culture conditions for bioprocessing under submerged fermentation with different agro residues for the isolate B6. The production of amylase was high under optimized conditions of 35°C, pH7.0 and 48 hrs of incubation. Out of the 5 oil seed cakes tested Soya bean oil seed cake induced maximum amylase production.

Keywords

Bacillus, α-amylase, bioprocessing, agro residues

Introduction

With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new areas have been opened for their utilization as raw materials for the production of value added fine products(Pandey *et al.*, 2000a). Application of these agro industrial residues in bioprocesses on one hand solves pollution problems which their disposal may otherwise cause (Pandey *et al.*, 2000b:2001). In addition, the utilization of these agro-industrial wastes, on the other, provides alternative substrates. Agricultural wastes are being used for both liquid and solid fermentations to reduce the cost of fermentation media. These wastes consist of carbon and nitrogen sources necessary for the growth and metabolism of organisms.

It is empherically known that higher yields of amylase can be obtained in media with complex raw material containing starch from maize, barley, wheat and malt (Burbidge and Collins1968).

Several oil cakes, because of their abundant availability and low price, are used as cattle feed (Norton1946)[1], fertilizer (Salgado, 1940), and, in rare cases after proper processing, food for humans [9] (Rastogi *et al.*, 1960). The use of complex starchy substances to achieve higher yields of α-amylase had been reported (Burbidge and Collins, 1968; Nyiri, 1971). Krishnan and Chandra (1982) investigated the effects of oilseed cakes on extracellular thermostable α-amylase production by *Bacillus*

licheniformis CUMC305 by growing the organism in a medium containing some essential minerals and a few oilseed cakes as the sole carbon and nitrogen sources. They reported that the oilseed cakes remarkably enhanced the production of thermostable amylase but the degree of enhancement, however, was relative to the concentration of the oilseed cake. Maximum increases were affected by a low concentration (0.5-1.0%) of groundnut or coconut, a high concentration (3.0%) of linseed or mustard, and an intermediate concentration (2.0%) of cotton, madhuca, or sesame.

This study was of interest because the cakes could completely replace peptone and beef extract, which are costly and impractical for the commercial production of the enzyme. Hence In present investigation an attempt was made to bioprocess amylase production from Bacilli and diversity by using agri waste materials under submerged fermentation

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).

Isolation, media and culture conditions

Red soil samples were collected from various sites of Acharya Nagarjuna University 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.

Screening for amylase producing *Bacillus* cultures

Screening for potent amylase producing Bacteria was carried out by Starch hydrolysis test (Aneja 2003). The selected isolates were screened for amylolytic activity on starch agar plates by Starch hydrolysis test [16]. 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 and 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.

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 (Amrutha *et al.*, 2012).

Assay of amylase

Unless otherwise stated, all experiments were carried out in triplicate. Amylase activity was assayed as described [18], 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.

Optimization of Amylase production

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.

Effect of incubation period, inoculum 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.

Bioprocessing of amylase production

Effect of different oilseed cakes 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 oil seed cakes on production of amylase activity in the above medium, 1% starch was replaced by different oil seed cakes as listed in Table-2. Similarly, for studying the effect of different combinations of oilseed cakes 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.

Effect of oil seed cakes on amylase production

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.

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 (B1, B2, B3, B4, B5 and B6) were found to be the producers of amylase [19] (amrutha and mounija 2016). These six isolates were tentatively identified as *Bacillus polymyxa, Bacillus pumilus, Bacillus megaterium, Bacillus acidocaldarius, Bacillus cereus and Bacillus subtilis.* Out of these, *Bacillus acidocaldarius (B6)* was found to be the promising producer of amylase. The morphological, physiological characteristics of the *Bacillus* culture (B6) were shown in Table 1.

Cultural conditions were optimized (Amrutha *et al.,* 2012) for the isolate B6 Amylase production by the strain B6 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 Amrutha *et al.,* 2016) and also reported in different species of *Bacillus i.e., B.subtilis, B.stearothermophilus, B.licheniformis and B.amyloliquefaciens*].

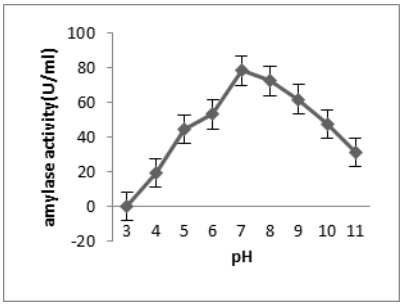

Fig.1 Effect of pH on amylase production from AVMB1

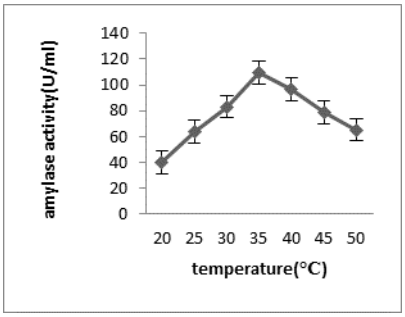

Fig.2 Effect of temperature on amylase production from AVMB1

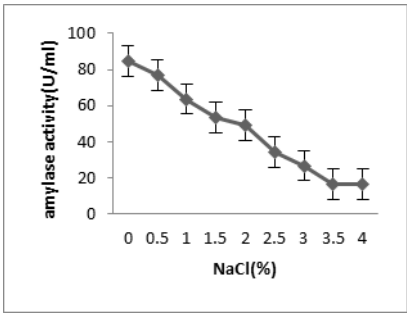

Fig.3 Effect of salinity on amylase production from AVMB1

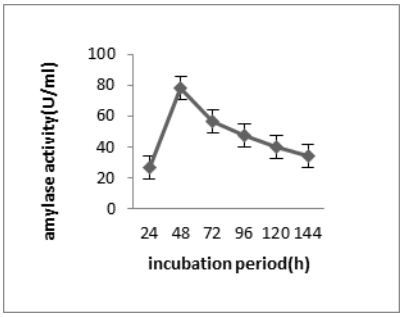

Fig.4 Effect of incubation period on amylase production from AVMB1

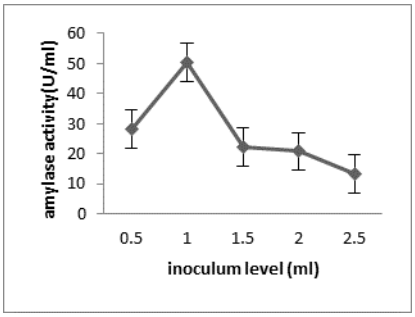

Fig.5 Effect of inoculums levels on amylase production from AVMB1

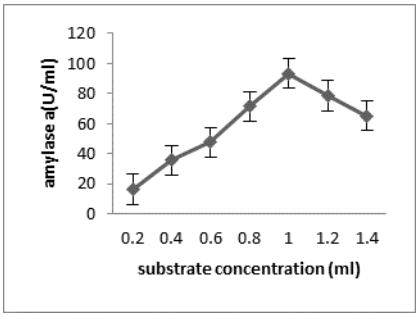

Fig.6 Effect of substrate concentration on amylase production from AVMB1

Fig.7 Effect of oilseed cakes on amylase production

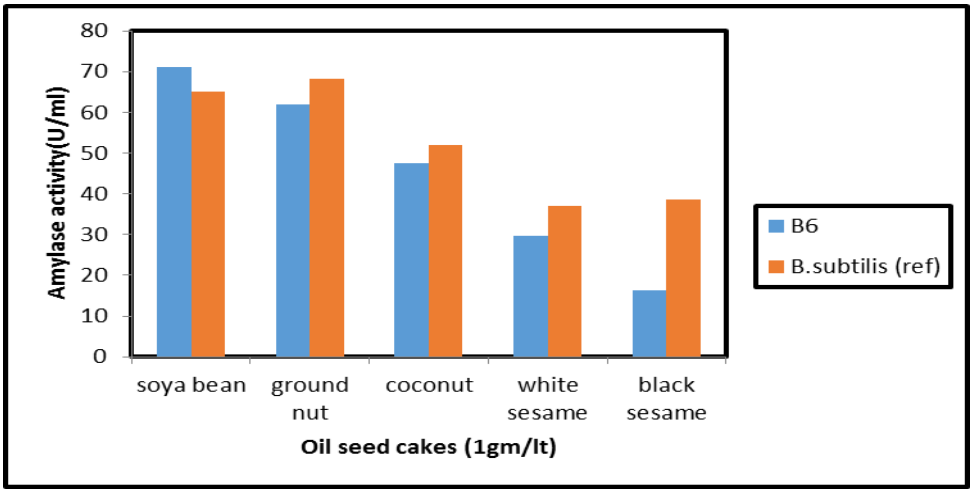

Fig.8 Effect of combination of oilseed cakes (3gm/lt) on amylase production

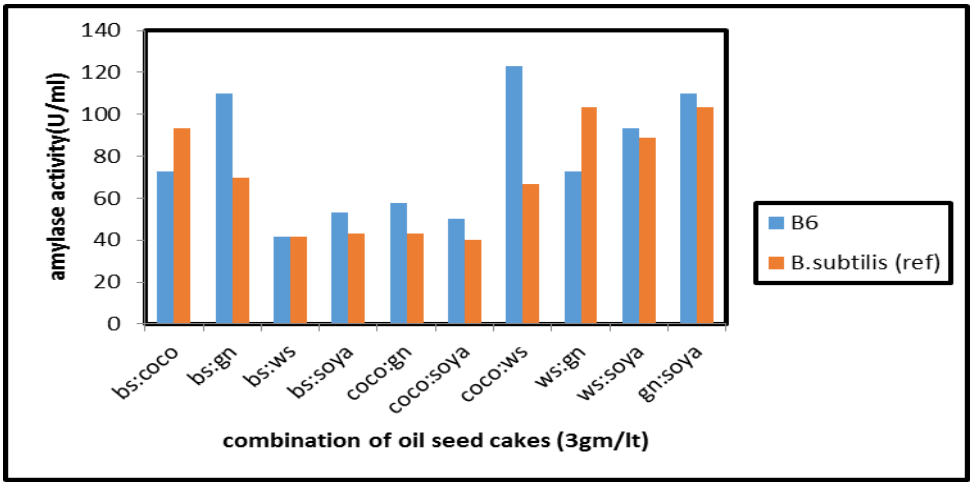

Fig.9 Effect of combination of oilseed cakes (4gm/lt) on amylase production

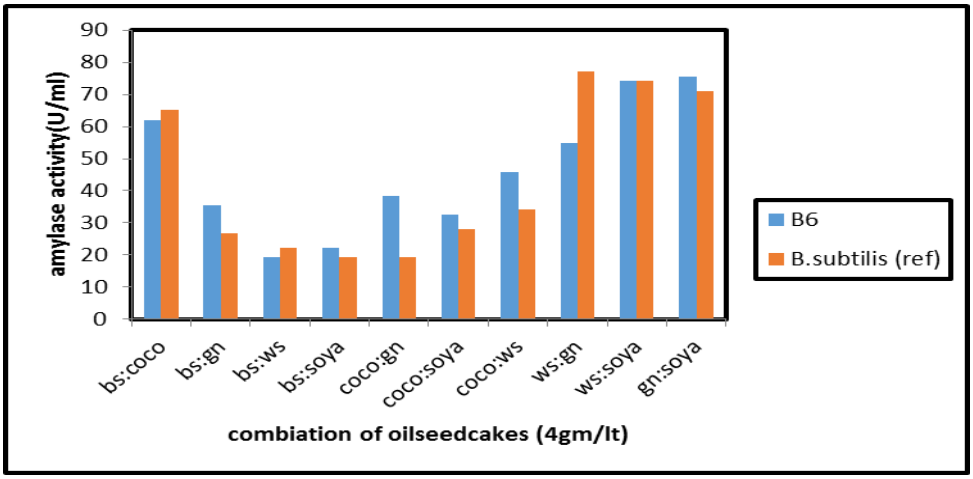

Table.1 Morphological and physiological characteristics of *B6*

Parameters	**Characteristics of B6**
a) Cellular characteristics	
Morphology	Straight, rod shaped
Staining	Gram positive, spore forming
b) Cultural characteristics	
Nutrient agar colonies	Round with lobate margins
c) Physiological characteristics	
Catalase	Positive
Amylase	Positive
Gelatinase	Positive
Caseinase	Positive
Lipase (olive oil)	Positive
Tween-80	Negative
Urease	Positive
Ammonia	Positive
Litmus	Acid
H2Sproduction	Negative
Indole	Negative
Methyl red	Negative
Voges proskauer	Negative
Citrase	Negative
Nitrate reductase	Negative
Lysozyme	Negative
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

Table.2 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 AVMB1 (U/ml)
Single Substrate	
Soya bean oilseed cake	71.11
Ground nut oilseed cake	62.01
Coconut oilseed cake	47.40
White sesame oilseed cake	29.62
Black sesame oil seed cake	16.29
Combination of Substrates (3gm/lt)	
Black sesame: Coconut	72.59
Black sesame: Ground nut	109.75
Black sesame: White sesame	41.48
Black sesame: Soya Bean	53.33
Coconut: Ground nut	57.77
Coconut: Soya Bean	50.37
Coconut: White Sesame	123.15
White Sesame: Ground Nut	72.86
White Sesame: Soya Bean	93.33
Ground nut: Soya Bean	109.75
Combination (4gm/lt)	
Black sesame: Coconut	62.01
Black sesame: Ground nut	35.55
Black sesame: White sesame	19.25
Black sesame: Soya Bean	22.22
Coconut: Ground nut	38.51
Coconut: Soya Bean	32.59
Coconut: White Sesame	45.92
White Sesame: Ground Nut	54.81
White Sesame: Soya Bean	74.07
Ground nut: Soya Bean	75.55

Our results were similar to that of optimum pH and temperature of amylase reported in a previous study (Pondey 2000). Considering the characteristics such as pH, temperature, salinity, incubation period, inoculums size, substrate concentration, SMF was carried out with different oil seed cakes under specified and optimized characteristics for the strain B6 and values were compared with reference (Table -4).

For efficient commercial production, a continuous effort is being made to find cheaper substrate sources. Available carbon and nitrogen sources are the decisive factors in the optimum production of enzymes, and these differ very much from substrate to substrate. Defatted or whole vegetable meals like sorghum, wheat bran, cotton seed meal, soybean meal, and alfa alfa meal are the

most commonly used fermentative additives if not the principal substrates[12] (Ratledge, 1977). Gangadharan *et al.,* (2006) worked to identify an effective agroresidue or their combinations as the substrate for the production of α-amylase from *Bacillus licheniformis* by solid state fermentation and reported that wheat bran and groundnut oilcake (GOC) in mass ratio of 1:1 was proved as best substrate source. An increased amylase production by *Bacillus subtilis*-159 was reported in the medium fortified with wheat bran as carbon source by Gayal *et al.,* (1989). Ajayi and Fagade (2003) reported that many *Bacillus* spp. isolated from local soil, waste water and food sources showed good enzymatic activity with the use of corn starch buffered substrate as substitute to determine its viability as a cheap carbon source.

In order 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-7). Earlier studies also reported that amylase production varies with strain and type oil and concentration of oil seed cake individually and in combination 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 (Gangadharan *et al.,* 2006;Krishnan and Chandra 1982).

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. whereas 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 beanoilseed cakes. While, in combinations at 4 gm/Lt, maximum production was noticed with combinations containing Soya bean and Ground nut oilseed cakes. From the above results it can be concluded that the agro residues are the cheap sources of amylase production and also increased amounts of amylase production was achieved under optimized conditions of 35°C, pH7.0. Soya bean oil seed cake induced maximum amylase production.

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