SCREENING OF EFFICIENT STRAINS OF *BACILLUS* SPECIES FOR PRODUCTION OF AMYLASES

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Abstract: Twenty *Bacillus* isolates were studied for their ability and efficiency of production of amylases, the commercially important enzymes degrading the starch, using starch agar plates. Three *Bacillus species*, i.e. *Bacillus subtilis* 208, *Bacillus subtilis* 252 and *Bacillus subtilis* 288 showed the inhibition zones of diameter > 50 mm , starch degradation activity > 67% and higher amount of maltose production i.e. > 600µg/ml. Among these three cultures, *Bacillus subtilis* 252 showed maximum amylase activity with production of 900 µg/ml maltose in 1:10 diluted culture supernatant and proved to be the most efficient. Study of optimization of pH and temperature for maximum amylase production by this strain showed that, pH 7 and temperature 40^{o}C was found to be most suitable for maximum production of amylases. This *Bacillus species* may be used for commercial production of amylases. However, detail study regarding different parameters is essential to prove its practical feasibility and competence.

Keywords: *Bacillus* species, Amylases, Starch agar, Dinitrosalicylic acid.

Introduction: Screening and improvement of efficient cultures of microorganisms is the most important and prime essentiality of industrial production of valuable products. The enzymes are protein biocatalysts which constitutes a major part of commercial production of valuable products. The amylases (α 1-4, D-glucan glucano hydrolase, EC 3.2.1.1) have many industrial applications and constitute about 30% of the world enzyme production. It represents one of the three largest groups of industrial enzymes and account for approximately 60% of total enzymes sales in the world [1]. Commercial production of amylases is conducted by using solid state fermentation and submerged fermentation [2].

Amylases are used for preparation of sizing agents and removal of starch sizing from woven cloths, preparation of starch sizing paste for use in paper coating, liquefaction of heavy starch paste which form during heating steps in the manufacture of corn and chocolate syrups, production of bread, and removal of food spots in the dry-cleaning industry where the amylases function in conjugation with protease enzymes. In addition, the amylases are also employed as a replacement of malt for starch hydrolysis in the brewing industry. α -amylases are especially used for production of beer, alcohol and glucose syrup. It is also used as digestive aid in medicines and tonics [2].

The amylases are extracellular and inducible enzymes. The α-amylase is an 'endo' enzyme that break α-1,4 glycosidic linkages randomly within the starch molecule to produce maltose molecules and resistant cores called 'limit dextrins'. The β-amylase is an 'exo' enzyme that breaks alternate bonds from terminal ends. Both α and β amylase can not break α-1,6 linkages at branch points in amylopectin portion. They however, act on maltose to produce glucose [3]. The α-amylases are produced by using fungi *Aspergillus niger* and *A. oryzae* as well as the bacteria *Bacillus amyloliquefaciens*, *B. subtilis* and *B. licheniformis* [2]. *Bacillus subtilis* and related *Bacillus* strains are dominant enzyme producing microorganisms in applied and industrial microbiology. These organisms are an important source of industrial production of extracellular enzymes [4]. *Bacillus species* are advantageous for industrial production due to their characteristics- easy aerobic growth on simple laboratory media, normally difficult to get contaminated and do not need frequent

transfers due to the ability of endospore formation.

Estimation of enzyme activity- Activity of any enzyme can be calculated on the basis of either the amount of substrate utilized or the amount of product produced in unit time. In case of amylases, the substrate is starch and the product is the reducing sugar maltose [5]. Both the compounds are colourless and hence, cannot be estimated directly. However, they can be converted to coloured products by reacting with other chemical. The substrate starch reacts with iodine to give a blue colour and the product maltose reacts with alkaline dinitrosalicylic acid to give orange colour. However, the former reaction is not stoichiometric, i.e. the intensity of blue colour is not directly proportional to the starch concentration. Thus, it is only qualitative value and used to detect the diameter of zone of decolourization which indicates the amount of starch utilized. The estimation of maltose with dinitrosalicylic acid is stoichiometric and is used to estimate the amount of maltose produced. The intensity of orange colour is directly proportional to the amount of maltose produced which is estimated using colourimeter at 520nm [6].

The objective of present work is to study the amylase production ability of *Bacillus* cultures isolated from rhizosphere of crop plants and to select the most efficient amylase producing strains among these.

Materials and Methods:

Primary screening of amylase producing *Bacillus* cultures on starch agar plates-

Twenty cultures of *Bacillus* isolated from rhizosphere soil samples were studied for Gram nature, motility, ability of endospore formation and selective biochemical characters as oxidase, catalase, nitrate reduction and Voges-Proskauer test [4], [7]. The stock cultures of *Bacillus species* were revived and studied for their ability and efficiency of production of amylases, using starch agar plates and iodine the reagent [6],[8]. Total diameters of decolourization zone (T) and diameters of growth (G) of *Bacillus species* are noted and percent amylase activity was calculated. Eight isolates showing good amylase activity Percent amylase activity = (T – G)/T x 100.

Secondary screening of efficient amylase producing *Bacillus* cultures by colourimetric method-

A) Preparation of standard graph- (Maltose concentration verses Optical density)

Standard dilutions of maltose were prepared as 100, 200, 300, 400, 500, 600,700, 800, 900 and 1000 µg/ml. 2ml of DNSA reagent was added in 1ml of each maltose concentration. A standard graph of maltose concentrations verses optical density values at 520 nm was plotted [6].

B) Estimation of amylase activity of *Bacillus* cultures-

0.1ml active culture of each *Bacillus* strain was separately inoculated in 100 ml of starch broth [9] and incubated. The broth cultures were centrifuged and the supernatant samples were used as crude amylase source of respective *Bacillus* culture. A colorimetric assay of these samples was performed with DNSA and the amount of maltose produced by amylase activity of each culture. Cultures of *Bacillus species* producing higher maltose concentration (i.e. 650 mg/ml and more) were selected.

C) Optimization of pH and Temperature for amylase production by *Bacillus subtilis* 252 Optimum pH for maximum amylase production was determined by inoculating 0.1 ml active culture of *Bacillus subtilis*252 in 100 ml starch broths adjusted at different pH values i.e. 5, 6, 7, 8, 9 and 10. Optimum temperature for maximum amylase production was determined by inoculating 0.1 ml active culture of *Bacillus subtilis* 252 in 100 ml starch broth and incubating at different temperature values i.e. 30, 40, 50, 60, 70 and 80^{O}C. Amylase activity was determined by colourimetric assay using DNSA [6].

Results and Discussion:

The bacterial isolates were found to be aerobic, Gram positive, motile rods, characterized by endospore formation, oxidase positive, catalase positive, Voges-Proskauer test positive and nitrate reductase test positive [4], [7]-[8]. Among the twenty *Bacillus* cultures tested, eight cultures showed good starch degradation

activity with total diameter of decolorization minimum 25 mm. Among these, three isolates *Bacillus subtilis*208, *Bacillus subtilis*252 and *Bacillus subtilis*288 showed higher zones of starch degradation i.e. greater than 50 mm (table-1).

Table-1. Screening of amylase producing *Bacillus species*-

Sr. No.	*Bacillus species*	D (mm)	d(mm)	% amylase activity
1.	*Bacillus thuringiensis* 184	30	13	56.66
2.	*Bacillus subtilis*208	52	17	67.30
3.	*Bacillus thuringiensis*211	25	12	52.00
4.	*Bacillus cereus*220	28	14	50.00
5.	*Bacillus cereus*228	35	17	51.42
6.	*Bacillus subtilis*252	60	18	70.00
7.	*Bacillus thuringiensis*260	34	12	64.70
8.	*Bacillus subtilis*288	50	16	68.00

D=Total Diameter of starch degradation zone

d= Diameter of growth

This indicated that, these three *Bacillus* cultures were efficient in amylase production as compared to others

The three primarily screened isolates *Bacillus subtilis* 208, *Bacillus subtilis* 252 and *Bacillus subtilis* 288 were further tested for efficiency of amylase production. The amount of maltose produced by these cultures was respectively 650, 900 and 700 µg/ml. Total diameter of starch degradation zone more than 50 mm and percent amylase activity more than 67% were selected in primary screening (table-1).

In general, the results of colorimetric method were coincided with the amylase activity on starch agar plate. The *Bacillus subtilis* 252 showed maximum amylase activity (table-2).

Table-2. Screening of efficient amylase producing *Bacillus species* by colorimetric method

Sr. No.	Amylase positive *Bacillus spp*	O. D. values at 520nm	Amount of maltose produced (µg/ml)
1.	*Bacillus subtilis*208	0.19	650
2.	*Bacillus subtilis*252	0.26	900
3.	*Bacillus subtilis*288	0.20	700

The *Bacillus subtilis* 252 may be used for commercial production but still needs to study with respect to different parameters. It is because, the production of microbial amylases by bacteria is dependent on the composition of medium, method of cultivation, cell growth, nutrient requirements, incubation period, pH, temperature, metal ions and thermostability [10]. Angelo and Rangabhashiyam observed that, temperature 37°C and 2% starch concentration

was suitable for amylase production by *Bacillus species* [9]. *Bacillus* species play vital role in industrial production of amylases [11]. Indeed, 60% of commercially available enzymes are obtained from different species of *Bacillus* [12] . The 16S r-RNA sequence of the *Bacillus* culture showing maximum maltose production from starch, i.e. *Bacillus* -252 was determined using the standard method and identified as *Bacillus subtilis* [13].

Study of optimization of pH and temperature for maximum amylase production showed that, pH 7 and temperature 40°C was found to be most suitable for maximum production of amylase (Fig. 1 and Fig.2).

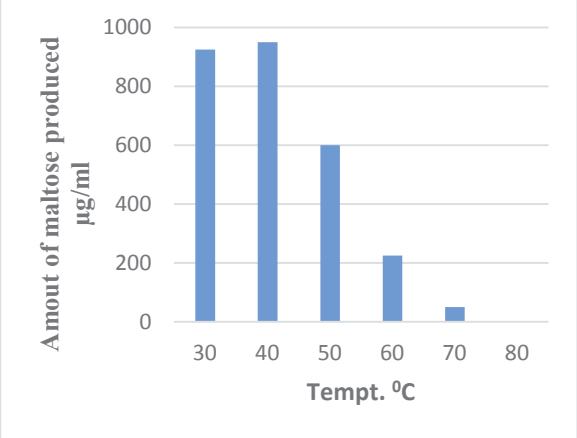

Fig.-1 Optimistion of Temp. for amylase production by *Baciilus subtilis*252

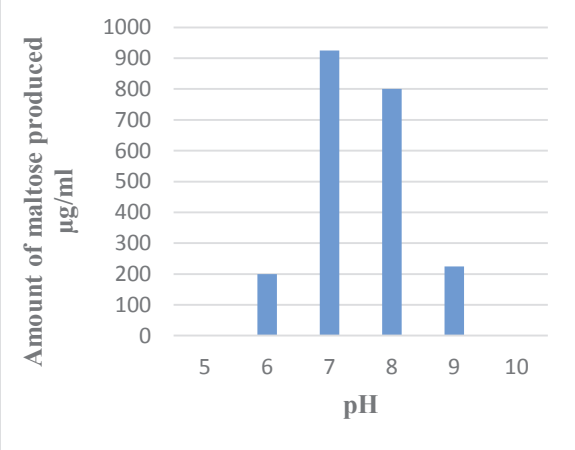

Fig. - 2 Optimistion of pH for amylase production by *Baciilus subtilis* 252

Conclusion: The isolate *Bacillus subtilis*252 is the most efficient amylase producer among the *Bacillus* isolates tested. However, study of efficiency of amylase production regarding different parameters is essential to decide practical feasibility and competence for its commercial use.

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