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Optimization of α-amylase production on agriculture byproduct by *Bacillus cereus* MTCC 1305 using solid state fermentation

Ravi Kant Singh*, S.K.Mishra, Narendra Kumar

Department of Biotechnology, IMS Engineering College, Ghaziabad, U.P. India

ABSTRACT

Most of enzymes are produced by submerged fermentation (SmF) at industrial scale. However, in the past decade, solid state fermentation (SSF) is becoming popular for producing enzymes due to its inherent advantages, e.g. higher yield, improved oxygen circulation, less energy requirement, minimum efforts in downstream processing. This paper describes the production of α-amylase from agricultural byproducts by *Bacillus cereus* MTCC 1305 in solid state fermentation (SSF). Substrates used are wheat bran (WB), corn flour (CF), rye straw (RS), wheat straw (WS) and rice bran (RB). Wheat bran has been found to yield maximum production of α-amylase among five substrates. Effects of process variables, namely incubation time, temperature, initial moisture content, pH of medium, supplementary carbon source, supplementary nitrogen source, and inoculum level on production of α-amylase have been studied, and accordingly optimum conditions have been determined. It has been found that the α-amylase production is the highest at 80 hr incubation period, 55 ^{0}C incubation temperatures, substrate: moisture ratio 1:1, pH of 5.0 and 10% inoculum level. Glucose (0.05 g/g) has been found the best supplementary carbon source. Supplementation of different nitrogen sources (0.02 g/g) showed decline in enzyme production.
Key words: solid state fermentation, *Bacillus cereus,* wheat bran, α–amylase, optimization, supplementary carbon source, supplementary nitrogen source.

Corresponding author
Email: rksingh.iitr@hotmail.com

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INTRODUCTION

Amylases have been reported to occur in microorganisms, although they are also found in plants and animals. Two major classes of amylases have been identified in microorganisms, namely α-amylase and glucoamylase. Among various extracellular enzymes, α-amylase ranks first in terms of commercial exploitation [1]. Spectrum of applications of a-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industry [2].

The production of α-amylase by submerged fermentation (SmF) using synthetic media has been reported by many workers [3-5]. The contents of synthetic media are very expensive and uneconomical. Therefore, they need to be replaced by the more economically available substrates to reduce the cost. In this regard, agricultural byproducts are generally taken as low cost substrate for the production of α-amylases. The use of agricultural wastes makes solid state fermentation attractive alternative method [6]. Thus the use of solid state fermentation (SSF) for α-amylase production is better than submerged fermentation (SmF) due to its simple techniques, low capital investment, lower levels of catabolite repression and better product recovery [7].

Amylases are a group of enzymes that have been found in several microorganisms like bacteria [8-12] and fungi [13- 23]. The most effective amylases are those that are thermostable [24]. They are generally preferred as their application minimizes contamination risk and reduces reaction time, thus enabling considerable energy saving. Thermostable α-amylases are used for the liquefaction of starch at high temperature and thermolabile α-amylases are used for the saccharification of starch in baking [25]. Babu and Satyanarayana [1] have reported production of α-amylase by a thermophilic *Bacillus* sp. and optimization of culture conditions for maximum enzyme production. Suitability of thermophilic *Bacillus coagulans* for α-amylase production by solid-state fermentation in flasks, reactor and trays has been reported [26].

The present investigation dealt with the optimization of cultivation parameters for maximum production of α- amylase by *Bacillus cereus* in solid state fermentation system, and the effect of end product of starch hydrolysis (Glucose) on amylase synthesis.

MATERIAL AND METHODS

Microorganism

Bacillus cereus MTCC 1305 used in the present study was obtained from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained on nutrient agar (NA) slants containing 1 % starch at 4 °C.

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Screening of agriculture byproducts as substrates for fermentation process

All four agriculture byproducts are not available in completely dried form. Prior to utilize them in bioprocess, it is necessary to dry these solid substrates. Therefore, in the present study the amount of wet solid substrate was kept in the oven at 80 ^{0}C for 12 h to remove the moisture from the agriculture byproducts. After drying, the mass of these substrates were measured. In these experiments, four agriculture byproducts; wheat bran (WB), corn flour (CF), rye straw (RS), wheat straw (WS) and rice bran (RB) were taken as solid substrate. The content of the flasks were mixed thoroughly and sterilized in the autoclave at 121 ^{0}C temperature and 1 atmospheric pressure for 15 minutes and then cooled at room temperature. Each flask was incubated with 2 ml of inoculum and subsequently rotated in a rotary incubator shaker at 37 ^{0}C. Further optimization of process parameters was studied using wheat bran as substrate for solid-state fermentation.

Enzyme production

The culture was transferred from stock to 100 ml nutrient broth and the inoculated flasks were incubated overnight at 37 ^{0}C and 150 rpm. Cells were harvested from the broth and the biomass concentration was checked at 620 nm. 10% inoculum (volume per mass) was taken in each set of experiments of α-amylase production. Production media contained 10 gm of solid substrate (agro-waste) with 1:1 moisture in 500 ml Erlenmeyer flasks and were inoculated with the above inoculum. Inoculated production media were incubated under static conditions at 37 ^{0}C and amylase production was checked after every 24 hrs for 5 days.

Enzyme extraction

α-amylase enzyme was extracted in 50 ml of 0.1 M phosphate buffer (pH=7) on a rotary shaker at 200 rpm for 25 min. The content was filtered through muslin cloth, filtrate was centrifuged at 10,000 rpm for 10 min and clear brown supernatant was used as the enzyme source.

Enzyme assay

α-amylase activity was determined by incubating a mixture of 0.5 ml of aliquot of each enzyme source and 1 % soluble starch dissolved in 0.1 M phosphate buffer, pH=7, at 50 ^{0}C for 20 min [27]. The reaction was stopped by adding 1 ml of 3, 5-dinitrosalicylic acid, and then followed by boiling for 10 min. The final volume was made up to 12 ml with distilled water and the reducing sugar released was measured at 540 nm [28]. One unit (U) of a-amylase activity was defined as the amount of enzyme that releases 1 mmol of reducing sugar as glucose per minute, under assay conditions and expressed as U/g of dry substrate. All the experiments were performed in triplicates and the standard deviation has been reported.

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Determination of Km and Vmax

The initial reaction rate of α-amylase was determined at different starch concentration ranging from 0.5% to 4% (w/v) (pH 5.0) and after incubating at 50 ^{0}C for 10 min enzyme activity per unit time was determined in each substrate concentration. Value of K_m and V_{max} were determined by plotting Lineweaver–Burk plot.

Optimization of cultural parameters

Inoculum size was varied as 5, 10, 15 and 20 % (volume per mass) of inoculum, where 1 % (volume per mass) corresponds to cells with OD_{620nm} = 0.1 of inoculum size added to 10 g of substrate. Substrate: moisture ratio was maintained as 1:0.5, 1:1, 1:1.5 and 1:2 and the enzyme production was checked using wheat bran as substrate.

Effect of supplementary carbon & nitrogen source on α-amylase production

Carbon sources (0.05 g/g dry substrate) as glucose, soluble starch, maltose and sucrose, and nitrogen sources (0.02 g/g dry substrate) as casein hydrolysate, NH_4Cl, yeast extract and $NaNO_3$ were supplemented as individual components to the production media to check their effect on enzyme production.

RESULT AND DISCUSSION

In solid state fermentation process, the selection of suitable solid substrate is a critical factor, so that the screening of agricultural byproducts is essential steps for the study of amylase production. We have taken four agriculture byproducts as a solid substrate to find out suitable substrate for optimum production of α- amylase. Wheat bran (WB), corn flour (CF), rye straw (RS), wheat straw (WS) and rice bran (RB) were selected under the screening system. The order of substrate stability was found as wheat bran > corn flour > wheat straw > rice bran > rye straw (Fig. 1). The maximum enzyme was produced in presence of wheat bran alone as a substrate. Therefore, in subsequent experiments, wheat bran was used as the substrate for α-amylase production. In our previous study wheat bran was found to be the best substrate for α-amylase production by a thermophilic fungus *Humicola lanuginose* [18].

The critical importance of moisture level in SSF media and its influence on the biosynthesis and secretion of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles. An increase in moisture level is believed to reduce the porosity of the wheat bran, thus limiting oxygen transfer [29]. Low moisture content causes reduction in the solubility of nutrients of the substrate and a low degree of swelling [30]. Substrate: moisture ratio was maintained as 1:0.5, 1:1, 1:1.5 and 1:2 and the enzyme production was checked using wheat bran as substrate. A high enzyme titer was attained when the Substrate: moisture ratio was 1:1 (Fig. 2). Varying inoculum size of bacterial cells during the

fermentation indicated 10 % (volume per mass) inoculum as optimum for the enzyme production (Fig. 3). Increase in inoculum size was found to adversely affect the enzyme production.

Wheat bran and two waste products obtained while processing of rice to rice flakes, coarse waste and medium waste were evaluated for a-amylase production by solid-state fermentation. Among the three substrates tested highest enzyme production was observed with wheat bran 128 U/g) (Table 1). Maximum enzyme production was observed after 96 h, which decreased with further incubation.

The influence of supplementary carbon & nitrogen sources was studies. Supplementation of carbon sources in the form of monosaccharides, disaccharides and polysaccharides resulted in marginal increase in α-amylase production by *B. cereus* during solid-state fermentation using wheat bran. Highest production was observed with glucose 152 U/g) (Fig. 4). Earlier researchers reported soluble starch as the best carbon supplement for α-amylase production in *M. thermophila* D14 [31] and *H. lanuginosa* [18]. Previous finding have shown that peptone, sodium nitrate & casein hydrolysate are good nitrogen supplements for α-amylase production in *A. fumigates* [32], *A. niger* [33], *A. oryzae* [34]. In my study, addition of organic nitrogen sources such as casein hydrolysate, yeast extract and inorganic nitrogen source such as NH_4Cl, $NaNO_3$ to the medium resulted in considerable decrease in α-amylase production by *B. cereus* (Fig. 5). V_{max} of α-amylase for starch was calculated as 56.18 mg/ml/ min and K_m as 9.79 mg/ml from the Lineweaver–Burk plot of amylase activity on starch at 50 ^{0}C (Fig. 6).Therefore, in subsequent experiments inoculum level of 10% (v/w), incubation time 96 hr & Substrate: moisture ratio 1:1 along with wheat bran as the solid substrate for maximum α-amylase production.

CONCLUSION

The use of solid state fermentation for production of α-amylase using *Bacillus cereus* is an economical process and is very simple to apply. All the solid substrates wheat bran (WB), corn flour (CF), rye straw (RS), wheat straw (WS) and rice bran (RB) can be used for supported biosynthesis of α-amylase using *B. cereus* under solid state fermentation system. However, these substrates did not cause enzyme productions as high as wheat bran. Therefore, wheat bran has been superior to other solid substrates for the synthesis of α-amylase from *B. cereus* by solid state fermentation. The maximum productivity of α-amylase (152 U/g) was achieved by utilizing wheat bran as the solid substrate with glucose as an additional carbon source in 96 h at temperature 37 ^{0}C and substrate: moisture ratio 1:1, pH of 5, and inoculum level of 10 % (w/v) with glucose as supplement.

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Fig 1: Effect of solid substrate on α- amylase (U/g) production by *B. cereus* MTCC 1305 under solid sate fermentation using agro-waste as substrate

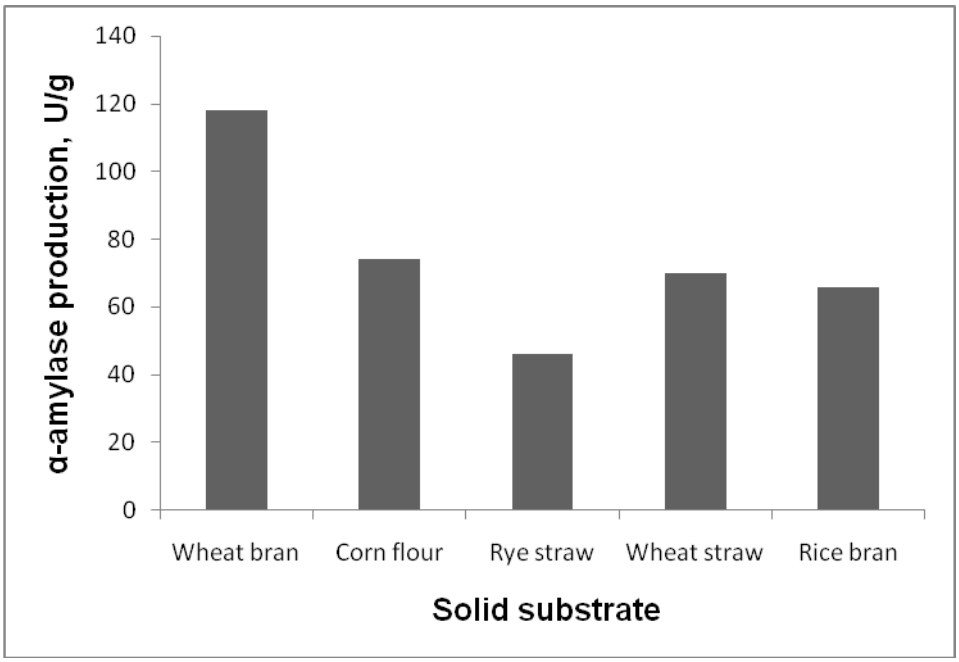

Fig 2: Effect of initial moisture content of the medium on α-amylase (U/g) production by *B. cereus* MTCC 1305 under solid sate fermentation using wheat bran as substrate

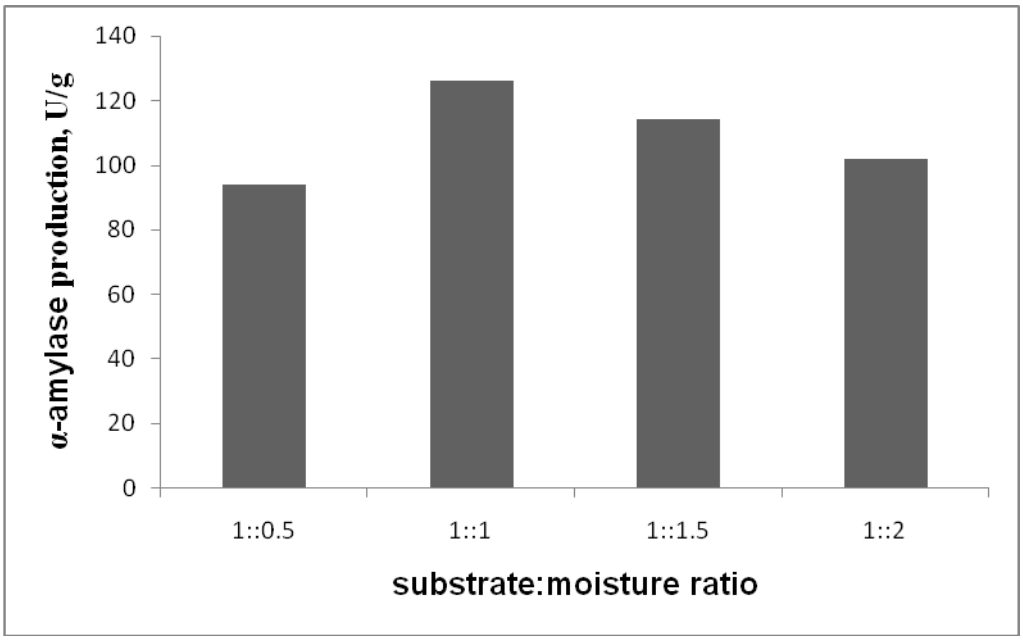

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Fig 3: Effect of incubation size on the production of α-amylase (U/g) by *B. cereus* MTCC 1305 under solid state fermentation using wheat bran as substrate

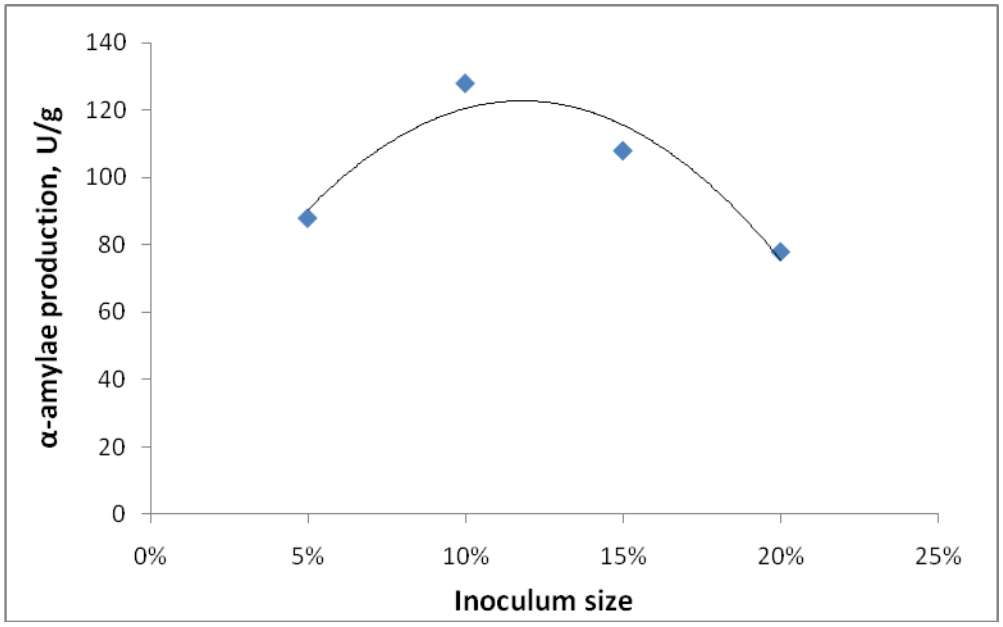

Fig 4: Effect of carbon source (0.05 g/g) supplementation on α-amylase (U/g) production by *B. cereus* MTCC 1305 under solid sate fermentation using wheat bran as substrate

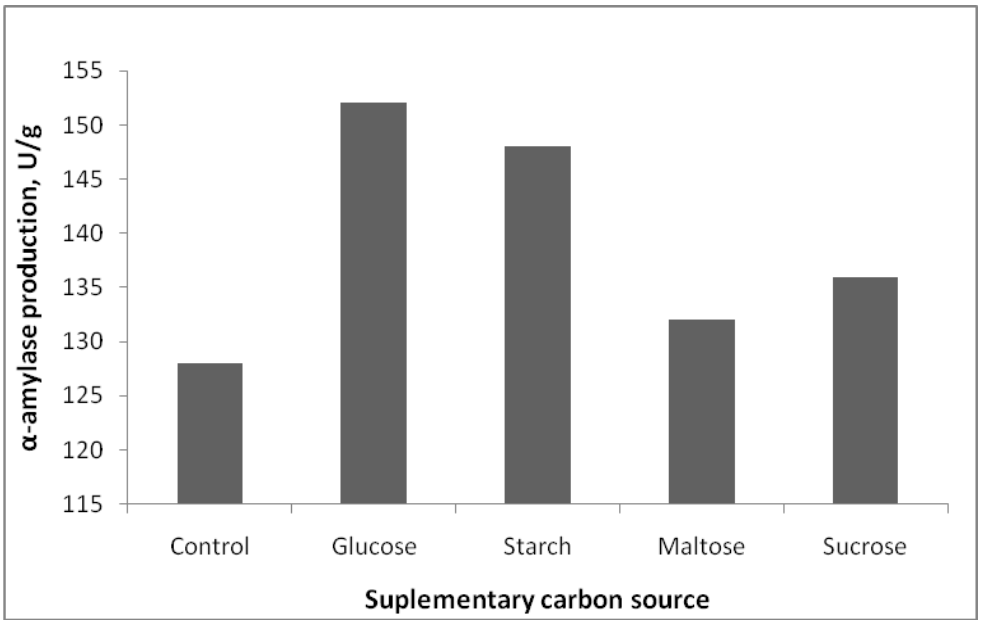

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Fig 5: Effect of nitrogen source (0.02 g/g) supplementation on α-amylase (U/g) production by *B. cereus* MTCC 1305 under solid sate fermentation using wheat bran as substrate

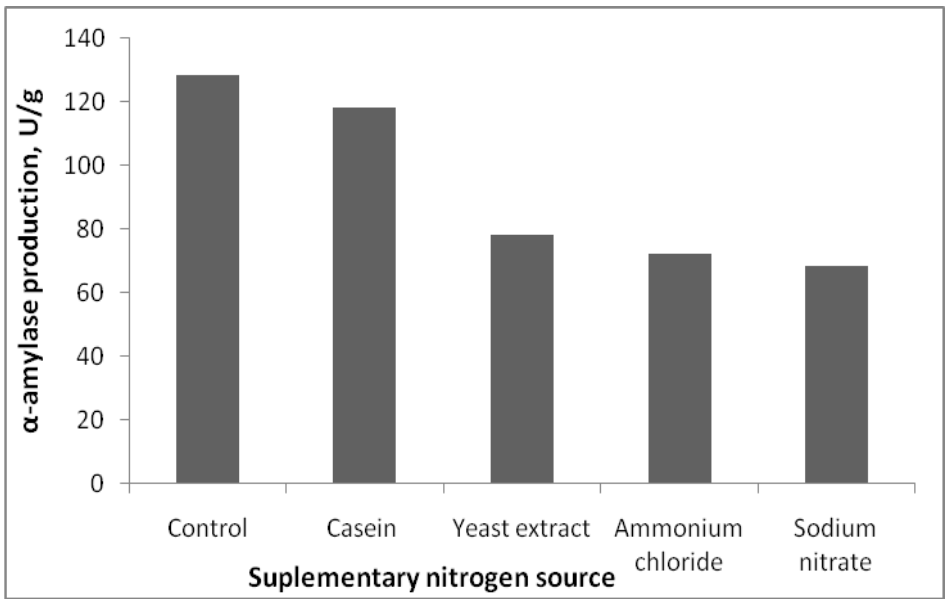

Fig 6: Lineweaver–Burk plot of α-amylase activity

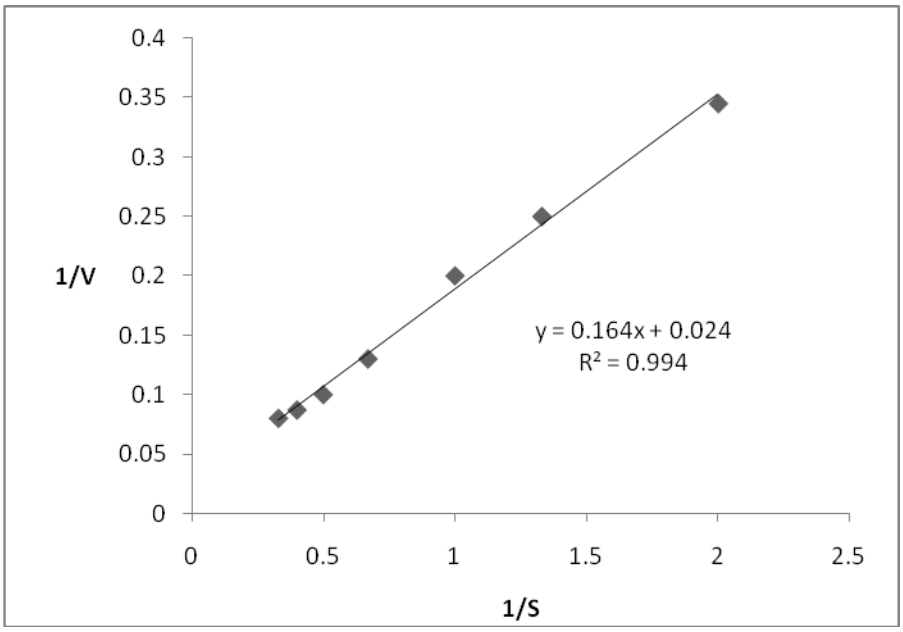

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Table 1: Production of α-amylase (U/g) by *Bacillus cereus* MTCC 1305 on different substrates by solid state fermentation

Incubation time in hr	Solid substrate				
	WB	CF	RS	WS	RB
	Enzyme production (U/g)				
24	26	18	10	22	15
48	72	48	26	46	35
72	98	68	38	64	52
96	128	74	46	70	66
120	76	42	14	38	34

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