



## Original article

Solid state fermentation of amylase production from *Bacillus subtilis* D19 using agro-residues

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## ABSTRACT

The selected bacterial strain, *Bacillus subtilis* D19 was inoculated into the solid residues such as, wheat bran, banana peel, orange peel, rice bran and pine apple peel. Among the tested solid wastes, wheat bran showed enhanced the production of amylase (640 U/g) than other tested substrates. The carbon and nitrogen sources were initially screened by traditional method using wheat bran medium. Amylase activity was high in the wheat bran substrate supplemented with starch (670 U/g). The tested nitrogen sources enhanced amylase activity. Among the selected nitrogen sources, yeast extract stimulated maximum production of amylase (594 U/g). In two level full factorial experimental design, maximum activity (1239 U/g) was obtained at pH 9.0, 70% (v/w) moisture, 1% (w/w) starch, 1% (w/w) yeast extract and 5% (v/w) inoculums. Central composite design and response surface methodology was used to optimize the required medium concentration (pH, moisture content of the medium and starch concentrations) for the maximum production of amylase. All three selected variables enhanced amylase production over 3 fold in optimized medium.

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## 1. Introduction

Amylases are the glucosidal linkage of complex polysaccharides (Pandey et al., 2000). These enzymes plays very important role in various industrial processes. The fungi such as, *Penicillium* sp., *Rhizopus* sp., *Cephalosporium* sp. and *Aspergillus niger* are the important producer of  $\alpha$ -amylase (Suganthi et al., 2011; Valsalam et al., 2019; Arokiyaraj et al., 2015). Moreover, the synthetic media is highly expensive and almost not suitable for commercial production of industrial enzymes. Traditional optimization method is time consuming and it frequently ignores the interactions between factors. However, statistical optimization method has various advantages than traditional optimization method. Statistical optimization method such as, Placket and Burman design has been

frequently employed to screen large number of factors. Response surface methodology (RSM) including, fractional factorial design, full factorial design, regression analysis were useful to find the significant factors and helps to build models to evaluate the interaction between medium components and to select suitable conditions of factors for a optimum response (Reddy et al., 2008). RSM has been used for optimizing process parameters to enhance the production of enzymes for various applications (Saran et al., 2007; Mohandas et al., 2010). In statistical method optimization strategy, contour plots and 3D response surface plots can provide clear analysis of interactions between factors. Hence, optimization of enzyme production by statistical approach is frequently employed for predicting optimum response and to enhance enzymes production (Mullai et al., 2010). In this study, *B. subtilis* D19 was used for the production of amylase in Solid state fermentation for various industrial processes.

## 2. Materials and methods

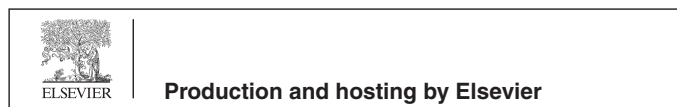
## 2.1. Bacterial strain

Food sample was used for the isolation of bacteria for the production of amylase in SSF.

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## 2.2. Characterization of amylase producing bacterial isolate

16S rDNA was sequenced using universal forward and reverse primers (Arasu et al., 2013). The 16S rDNA gene sequence was analyzed and sequence similarity was assessed and sequences were submitted to GenBank database.

## 2.3. Solid state fermentation

In our study, various agricultural wastes were collected locally. The agricultural residues such as, wheat bran, banana peel, orange peel, rice bran and pine apple peel, were screened initially. These agricultural by-products was not readily available as dried form, hence, the collected substrates were incubated for 12 h at 80 °C to eliminate moisture content of the solid substrates. The agro-residue was weighed (5.0 g) and desired moisture level was maintained (75%, v/w) and sterilized. It was rotated several times in order to mix the inoculums with substrate.

## 2.4. Amylase extraction and enzyme assay

Enzyme assay was performed as described by Miller (1959). Soluble starch (1%, w/v) was used as the substrate. Sample (0.1 ml) was incubated with 1 ml substrate for 30 min. Then one ml DNS reagent was added and boiled for 10 min. Finally the optical density of the sample was read at 540 nm using a UV-visible spectrophotometer.

## 2.5. Screening of carbon and nitrogen source on amylase production

In this study, production of amylase by *B. subtilis* was optimized by traditional method in SSF. The nutrient factors (carbon and nitrogen source) were selected and screened by this method. These nutrient sources were supplemented with 5 g wheat bran and amylase assay was carried out.

## 2.6. *Bacillus subtilis* D19 enzyme optimization

Two factors were selected at two different levels. Inoculum level positively influences on enzymes production. Yeast extract (nitrogen source) and starch (carbon source) were selected based on initial screening experiments. The 2<sup>5</sup> factorial experimental designs consisted of a total of 32 runs. Experiment was performed in 100 ml Erlenmeyer flasks containing 5.0 g substrate (wheat bran) with suitable media components.

Experiment was conducted in duplicate and an average was considered as amylase activity (response Y). From the analyzed results, three factors were selected to optimize the concentration of variables by central composite design and response surface methodology (p < 0.05). The selected variables were, pH, moisture and starch. These variables used were tested at five different levels (-α, -1, 0, +1, +α). Central composite design consists of 20 experimental runs for three selected variables. Finally, the second-order polynomial equation was used to fit the experimental results. For a three-factor system, the second order polynomial equation is as follows (2):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_{ij}$$

$$I = 1 \quad i = 1 \quad ij = 1$$

where Y is the response; b<sub>0</sub> is the offset term; and b<sub>i</sub>, b<sub>ii</sub>, and b<sub>ij</sub> are the coefficients of linear terms, square terms, and coefficients of interactive terms, respectively. X<sub>i</sub>s are A, B, and C; X<sub>ij</sub>s are AB, AC, and BC (A = pH; B = moisture; C = starch). Amylase analysis was performed in duplicates and the mean value was taken as response Y.

## 3. Results

### 3.1. Screening and characterization of amylase producing *B. subtilis* D19

The bacterium *B. subtilis* D19 showed considerable activity on starch agar plates. This organism produced more than 20 mm zone on the substrate agar plate, which showed more hydrolytic zone than the other tested bacterial strains. The selected bacterial isolate was this organism hydrolyzed starch, casein and negative for gelatine hydrolysis. It was identified based on these biochemical characters and 16S rDNA sequencing. The 16S rDNA sequence (KF 638634.1) showed more than 99% similarity with *Bacillus subtilis* D19.

### 3.2. Effect of solid substrates

In this study, the selected bacterial strain, *B. subtilis* was inoculated into the solid residues such as, wheat bran, banana peel, orange peel, rice bran and pine apple peel. Among the tested solid wastes, wheat bran enhanced the production of amylase (640 U/g) than other tested substrates. Also, the other substrates such as, rice bran and orange peel, banana peel and pine apple peel supported amylase production (Fig. 1).

### 3.3. Optimization

Amylase activity was high in the wheat bran supplemented with starch (670 U/g) (Fig. 2a). The tested nitrogen sources enhanced amylase activity (594U/g) (Fig. 2b).

### 3.4. Screening and optimization of variables by statistical approach

In *B. subtilis* D19, amylase production was screened by a two-level full factorial design and the concentration was optimized by central composite design. In two level full factorial design enzyme yield ranged between 145 and 1239 U/g (Table 1). In this experimental design, maximum activity (1239 U/g) was obtained at pH 9.0, 70% moisture, 1% starch, 1% yeast extract and 5% inoculums. ANOVA of the experimental data was evaluated and the regression model was statistically significant and described in Table 2. In our study, the “F” value of the CCD model was 9.30 and this model is statistically significant. The p-value of this model is 0.0052. In this model case A, B, CE, DE, ABD, ACD, ACE, ABCD, ABDE, BCDE, ABCDE are significant model terms (Tables 3 and 4). The interactive effect between pH, moisture content and starch for amylase production by *B. subtilis* D19 were described in Fig. 3(a–c). In our study, amylase increased higher starch concentrations up to optimum level and decreased.

## 4. Discussion

Carbon sources in the culture medium stimulated amylase production and has been described previously. In *Bacillus cereus*, supplemented carbon sources positively regulated amylase production (Tamamura et al., 2014; Arasu et al., 2019; Babu and Satyanarayana, 1993). In *Bacillus thermooleovorans*, amylase activity was enhanced by starch, lactose, glucose and maltose (Narang and Satyanarayana, 2001; Arasu et al., 2017).

In our study, amylase production was enhanced by the supplementation of yeast extract as nitrogen source (594 U/g). However, these required nutrient sources may differ from species to species. In *Bacillus* sp. amylase production was enhanced by the addition of ammonium nitrate with the culture medium (Hashemi et al., 2010). After initial screening, the fermentation bioprocess was

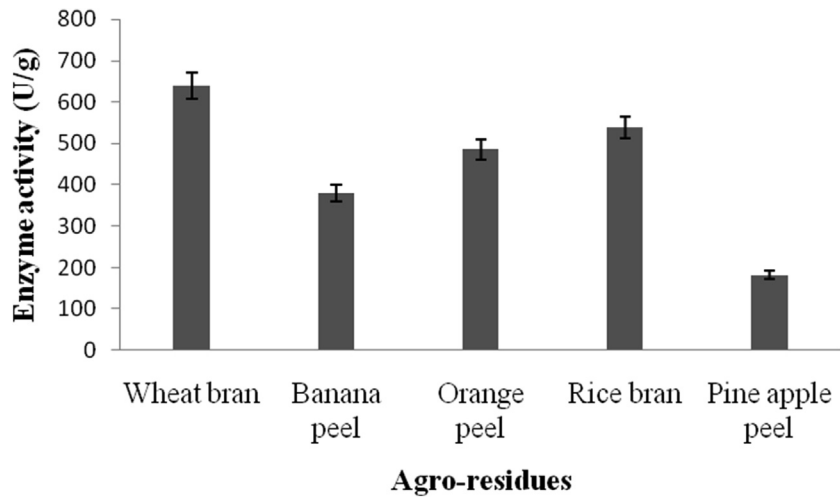


Fig 1. Effect of agro-residues on amylase enzyme production by *B. subtilis*.

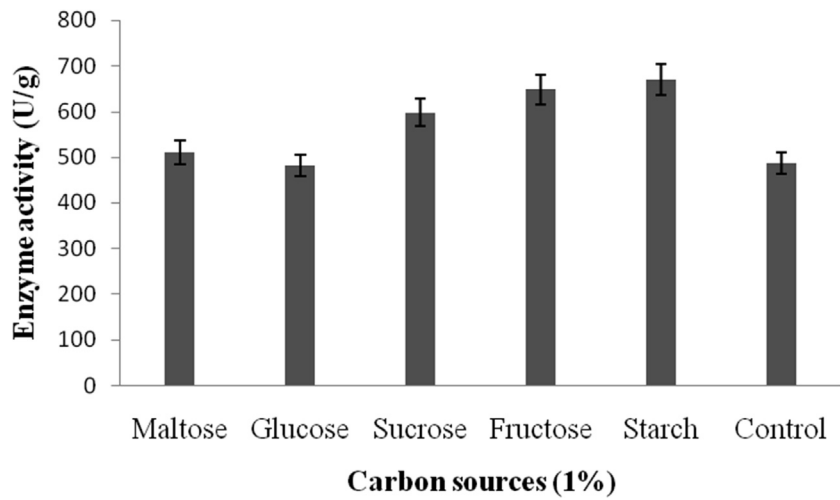


Fig. 2. a. Effect of carbon on amylase enzyme production by *B. subtilis*.

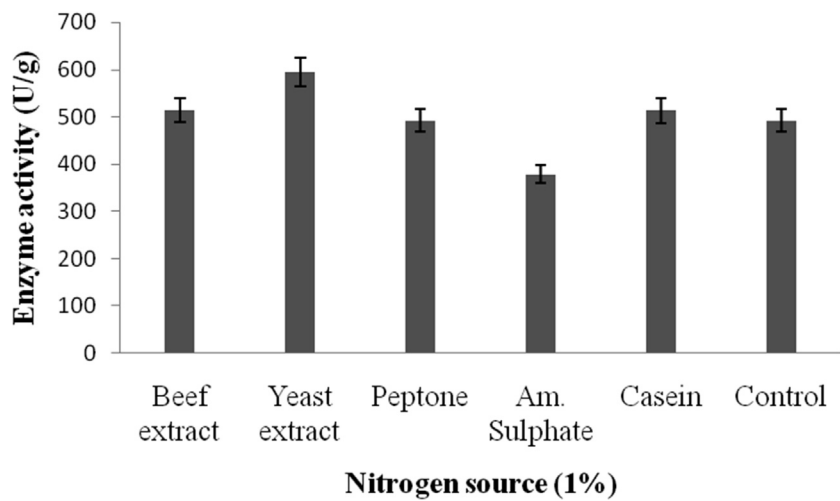


Fig. 2. b. Effect of nitrogen on amylase enzyme production by *B. subtilis*.

**Table 1**  
Two level full factorial designs for amylase production by *B. subtilis*.

Run	A:pH	B:Moisture %	C:Starch	D:Yeast extract %	E:Inoculum %	Enzyme activity U/g
1	9	70	0.1	0.1	5	325
2	9	70	1	1	1	540
3	6	40	0.1	1	1	299
4	9	40	1	1	1	605
5	6	40	1	1	1	340
6	6	40	0.1	1	5	755
7	9	70	0.1	1	1	721
8	9	40	0.1	1	1	729
9	6	40	1	1	5	425
10	6	70	0.1	1	5	440
11	6	70	0.1	0.1	1	540
12	9	40	0.1	1	5	145
13	9	70	0.1	0.1	1	672
14	9	40	1	0.1	1	376
15	9	40	1	0.1	5	941
16	9	70	1	0.1	1	546
17	9	40	0.1	0.1	1	1163
18	9	40	1	1	5	912
19	6	40	1	0.1	5	471
20	6	70	1	1	5	514
21	9	70	0.1	1	5	935
22	6	40	0.1	0.1	5	181
23	6	70	0.1	1	1	481
24	6	40	0.1	0.1	1	321
25	6	70	1	1	1	270
26	9	70	1	0.1	5	676
27	9	70	1	1	5	1239
28	6	70	1	0.1	1	826
29	6	70	1	0.1	5	550
30	9	40	0.1	0.1	5	710
31	6	70	0.1	0.1	5	890
32	6	40	1	0.1	1	381

**Table 2**  
ANOVA table for 2<sup>5</sup> factorial experimental design for amylase production by *B. subtilis*.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	2.213E+06	25	88537.11	9.30	0.0052
A-pH	3.941E+05	1	3.941E+05	41.38	0.0007
B-Moisture	62216.28	1	62216.28	6.53	0.0431
C-Starch	56907.03	1	56907.03	5.92	0.0506
AB	50007.03	1	50007.03	5.25	0.0618
AC	9975.78	1	9975.78	1.05	0.3456
AD	34650.28	1	34650.28	3.64	0.1051
BD	6300.03	1	6300.03	0.6615	0.4471
BE	13081.53	1	13081.53	1.37	0.2856
CD	4394.53	1	4394.53	0.4614	0.5223
CE	1.784E+05	1	1.784E+05	18.73	0.0049
DE	66703.78	1	66703.78	7.00	0.0382
ABC	8224.03	1	8224.03	0.8635	0.3886
ABD	4.007E+05	1	4.007E+05	42.08	0.0006
ABE	36113.28	1	36113.28	3.79	0.0994
ACD	1.034E+05	1	1.034E+05	10.86	0.0165
ACE	3.513E+05	1	3.513E+05	36.89	0.0009
BCD	17344.53	1	17344.53	1.82	0.2259
BCE	41112.78	1	41112.78	4.32	0.0830
BDE	34914.03	1	34914.03	3.67	0.1040
ABCD	1.078E+05	1	1.078E+05	11.32	0.0152
ABCE	17437.78	1	17437.78	1.83	0.2248
ABDE	1.226E+05	1	1.226E+05	12.88	0.0115
ACDE	5751.28	1	5751.28	0.6039	0.4666
BCDE	84769.03	1	84769.03	8.90	0.0245
ABCDE	59254.03	1	59254.03	6.22	0.0469
Residual	57141.69	6	9523.61		
Cor Total	2.271E+06	31			

**Table 3**  
Experimental design and results of the CCD for amylase production by *B. subtilis*.

Std	A:pH	B:Moisture		C:Starch		Enzyme activity U/g
			%		%	
7	6		75		1	1520
15	7.5		55		0.6	2980
14	7.5		55		1.27272	2500
2	9		35		0.2	435
3	6		75		0.2	1098
5	6		35		1	1240
11	7.5		21.3641		0.6	110
17	7.5		55		0.6	2970
1	6		35		0.2	1012
6	9		35		1	1019
18	7.5		55		0.6	3019
19	7.5		55		0.6	2890
16	7.5		55		0.6	3010
9	4.97731		55		0.6	92
13	7.5		55		-0.0727171	1900
4	9		75		0.2	1892
12	7.5		88.6359		0.6	3092
10	10.0227		55		0.6	2742
8	9		75		1	1780
20	7.5		55		0.6	2982

**Table 4**  
Results of the regression analysis of the CCD on amylase production by *B. subtilis*.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	1.724E+07	9	1.916E+06	5.31	0.0077
A-pH	1.626E+06	1	1.626E+06	4.51	0.0598
B-Moisture	4.228E+06	1	4.228E+06	11.71	0.0065
C-Starch	3.325E+05	1	3.325E+05	0.9213	0.3598
AB	4.287E+05	1	4.287E+05	1.19	0.3013
AC	3960.50	1	3960.50	0.0110	0.9186
BC	31500.50	1	31500.50	0.0873	0.7737
A <sup>2</sup>	5.874E+06	1	5.874E+06	16.27	0.0024
B <sup>2</sup>	4.738E+06	1	4.738E+06	13.13	0.0047
C <sup>2</sup>	1.884E+06	1	1.884E+06	5.22	0.0454
Residual	3.610E+06	10	3.610E+05		
Lack of Fit	3.599E+06	5	7.198E+05	343.27	<0.0001
Pure Error	10484.83	5	2096.97		
Cor Total	2.085E+07	19			

further optimized by statistical approach. The variables were screened by a two level full factorial design and further optimized by central composite design. RSM has been employed previously to optimize the variables (Rahman et al., 2004). Low cost medium and culture medium optimization is very important (Chauhan and Gupta, 2004; Dash et al., 2015; Rajagopalan and Krishnan, 2008). Hence, optimization of any new bacterial isolate is pre request for any industrial processing. In our study, this organism preferred alkaline pH, and starch is preferable for the synthesis of amylase. The influence of nutritional and physical factors was reported by Sahnoun et al. (2015). In a study, Tanyildizi et al. (2007) reported that pH 7.0 is optimum for the production of amylase from *Bacillus amyloliquefaciens*. Most of the bacteria from the genus *Bacillus* use pH between 7.0 and 10.0 for the production of amylase (Saxena et al., 2007; Goyal et al., 2005). The designed CCD model in this study is statistically significant for *B. subtilis*. RSM has been used for the production of enzymes and coefficient estimate was analyzed (Olivera et al., 2004; Adinarayana and Ellaiah, 2002). The pH of the substrate (wheat bran) also positively influenced on amylase production in *B. subtilis* D19. The influence of pH on enzyme production has been reported earlier by Pandey et al. (2000). In enzyme bioprocess, physical parameters influenced on

amylase production (Tamilarasan et al., 2010; Agrawal et al., 2005; Ikram-ul-Haq et al., 2003; Vijayaraghavan and Vincent, 2012). In this study amylase activity was 3 fold in optimized medium than unoptimized culture medium. Generally, RSM mediated optimization procedures enhanced enzyme production. In a study, *Bacillus* sp. RKY3 was used to optimize enzyme production and achieved 2.3 fold enzyme activities (Anbu et al., 2008)

## 5. Conclusions

In conclusion, production of amylase at optimum pH and moisture content and supplementation of starch in the wheat bran media seemed to enhance the enzyme yield. This study revealed the influence of physical factors and carbon source on amylase production.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

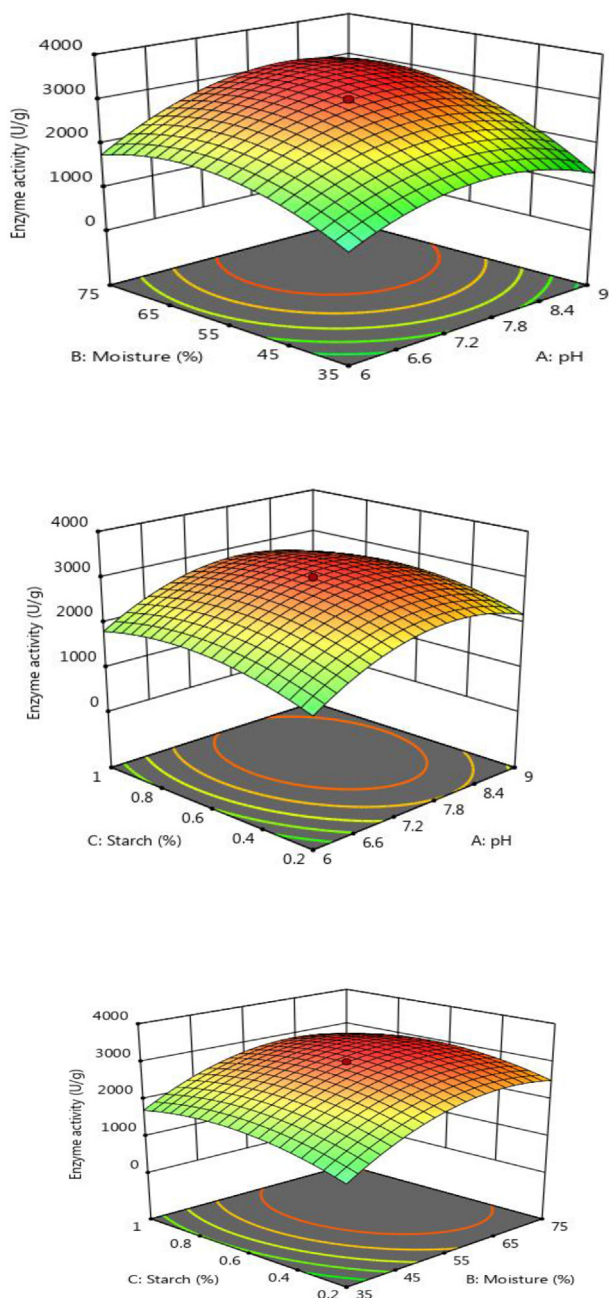


Fig. 3. Response surface plot for amylase production by *B. subtilis*.

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