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Response Surface Methodology Optimization of Starch Isolation from Finger Millet (*Eleusine coracana*) through Solid State Fermentation

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Solid state fermentation was employed to isolate the starch from finger millet to improve its functional properties. This process was optimized by Box-Behnken response surface methodology by varying pH (4-7), innoculum concentration (10 -30), temperature (25-35°C) and time (2-6 d). *Aspergillus oryzae* (MTCC 3107) was obtained from National Chemical Laboratory, Pune and used for this study. Growth medium of 0.2% of KH_2PO_4, $MgSO_4 7H_2O$ and soluble starch each along with traces of $FeSO_4.7H_2O$ in 100 ml distilled water was used. Higher starch (0.64 mg/g) was isolated at pH 5.65, innoculum concentration of 28.57 per cent and temperature 35°C, time 3.8 days. A quadratic equation was fitted to develop a relationship between independent and dependant variables. The fitted model has high regression coefficient (R^2 - 0.99), adjusted regression coefficient (Adj.R^2 -0.99) with less standard deviation (SD- 0.005). This study concluded that SSF could be an effective method for isolation of starch from finger millet.

Key words: Starch isolation, Finger millet, Solid state fermentation, RSM, Modeling

Starch is a recyclable polymer present in the form of carbohydrate in all biomass (Pérez-Pacheco *et al.*, 2014) which is being obtained from many botanical sources such as tapioca, potato, oat, wheat and rice etc. Starch is an important factor affecting the physico-chemical and functional properties of flour and establishes the rheological and textural properties of foods (Adebowale *et al.*, 2005; Balasubramanian *et al.*, 2011). Millet contains starch in abundant at on par with other cereals, anti-nutrients of millet which affects the extraction of starch and it is being underutilized crop due to less commercial value. Additionally, the demand of starch is increased, so industries looking for alternate sources for starch isolation. Hence, starch isolation from the millets would increase the commercial value. Finger millet (*Eleusine coracana*) is one of the pseudo cereals extensively used in both native and processed form (Rao and Muralikrishna, 2001). Complexity of starch biosynthesis made difficult to isolate the starch from the botanicals and also functional properties of starch is varied based on the botanical sources (Morell and Myers, 2005; Tetlow *et al.*, 2004). So far starch has been extracted by mechanical, hydrothermal, chemical and microbiological methods. However, using of micro-organism in starch isolation has been increasing due to its action based on greener chemistry (Segura and Rosell, 2011). Microorganism can act as chiral catalyst to extract starch due to its high positional, specificity stereospecficity (Stanbury *et al.*, 2013). Solid state fermentation has been widely used for enzyme production, nutritional enrichment of substrate because of its ability of producing of enzyme consortium. However, best of our knowledge no study has been reported the effect of solid state fermentation on starch isolation. Hence, this study was framed to extract the starch from finger millet through solid state fermentation using *Aspergillus oryzae* and optimize the process using response surface methodology.

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Materials and Methods

Materials and chemicals

Finger millet (CO-15) was collected from the TNAU farm. *Aspergillus oryzae* (MTCC 3107) was purchased from National Chemical Laboratory, Pune and all the chemicals used in these studies were purchased from Hi Media Laboratory Pvt. Ltd., Mumbai.

Inoculum Preparation

Primary mother culture *Aspergillus oryzae* was grown and maintained in potato-dextrose agar medium at 30°C for seven d, stored at 4°C and sub-cultured fortnightly. For 100 ml of growth medium KH_2PO_4, $MgSO_4 7H_2O$ and soluble starch have been added at 0.2% each and traces of $FeSO_4.7H_2O$ was also added in 100 ml of distilled water in 500 mL conical flask and sterilized in an autoclave at 121°C for 15 min. The growth media were inoculated with loop full of culture containing 10^7 CFU and incubated at 30°C for 24 h.

Substrate preparation

The finger millet was cleaned thoroughly to remove all foreign matters and fine dusts. Cleaned finger millets were washed, weighed in a suitable container and soaked in distilled water for 12 h at room temperature to attain the equilibrium moisture content of 33 per cent and drained. Soaked finger millet was surface sterilized by treating with one per cent lime water for 10 min.

Solid state fermentation

One hundred gram of pre prepared finger millet was taken in a pre sterilized 100 mL glass beaker. The initial pH of the substrate was adjusted by adding 0.1N NaOH or 0.1N HCL to the substrate. The growth medium at the rate of 10-30% was added to the substrate and incubated according to the experimental design. Fermented grain was dried at 60°C in a tray drier to reduce moisture content less than 10 per cent and powdered using a pulverizer. Powdered fermented finger millet flour was stored in an air tight zip pack for further analysis.

Isolation of total starch

Starch was isolated from the fermented finger millet flour by alkaline steeping method (Wang and Wang, 2001) with slight modification. Finger millet flour and 0.1% NaOH were taken in the ratio of 1:2 and steeped for 18 h. Steeped slurry was blended thoroughly with stirrer and filtered through the muslin cloth and filtrate was centrifuged at 1300 G for 10 min. Supernatant was discarded and sediment starch was removed carefully. Sediment starch was washed with 0.1% NaOH for three times and with 0.1N HCl for neutralization. Neutralized starch was again washed with deionized water and centrifuged. Centrifuged starch was dried in an oven at 45°C for 48 h and expressed in mg.

Statistical analysis

Isolation of starch was optimized using Box-Behnken design by varying four process parameters

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} \sum_{j=1}^{4} b_{ij} x_{ij} + \sum b_{ii} x_i^2 \quad \ldots\ldots\ldots (1)$$

and their levels (Table 1). The Design expert statistical package 6 (State-Ease Inc., Minneapolis, MN, USA) was used for experimental design and regression analysis of data. A quadratic polynomial equation (1) was fitted to the data by multiple regression analysis and to predict the response. The quadratic equation is

Where, y is the predicted starch isolation; b_0 is the intercept; $b_{i=1-4}$ is linear coefficients, b_{ij} is interaction coefficient and b_{ii} is quadratic coefficient. The non linear regression analysis was performed to determine the significance of the regression coefficients.

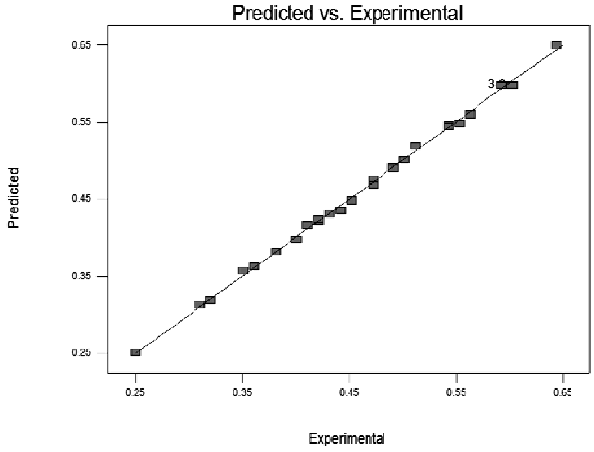

Fig. 1. Plot of experimental and predicted value for starch isolation

Results and Discussion

Response surface optimization

Both the experimental value and predicted value of starch isolation is shown in the table (2). The experimental values were fitted in to the linear, interactive, quadratic and cubic models. The suitability and adequacy of fitted model was assessed by the sequential sum of square analysis and the results are given in the model summary of statistic (Table 3). Higher statistic of quadratic model suggested that the quadratic model is best for fitting the experimental values (Table 3). Hence, quadratic model (Eqn.2) is used for further analysis.

Table 1. Range of process variables and their levels for design of experiment

Process parameters	Levels		
	-1	0	+1
A.Initial pH	4	5.5	7
B.Inoculum concentration, mL/100 g	10	20	30
C.Temperature ,°C	25	30	35
D.Time, d	2	4	6

Starch =+0.59+0.0008×A +0.091 × B+0.034 × C+0.054 ×D-0.099×A^2 -0.064× B^2-0.027 × C^2-0.11 ×D^2+0.023 × AB +0.013 ×AC+0.022×AD+0.018×BC-0.027×BD+0.0075×CD … (2)

Adequacy of fitted quadratic model was validated by plotting the randomly selected experimental and predicted values from the model developed (Fig.1).

Table 2. Box-Behnken design with experimental and predicted value of starch isoaltion

Initial pH	Inoculum Concentration, ml/ 100g	Temperature, ℃	Time (d)	Starch isolation, mg/ g	
				Experimental	Predicted
5.75	20	25	2	0.38	0.38
5.75	30	25	4	0.54	0.54
4.5	20	35	4	0.49	0.49
4.5	20	30	2	0.35	0.36
5.75	10	30	2	0.25	0.25
5.75	10	25	4	0.40	0.40
5.75	20	30	4	0.59	0.59
5.75	10	35	4	0.43	0.43
5.75	30	30	6	0.54	0.54
5.75	20	30	4	0.59	0.594
7	10	30	4	0.32	0.32
7	20	30	2	0.31	0.31
4.5	20	25	4	0.45	0.45
5.75	30	35	4	0.64	0.65
7	30	30	4	0.55	0.55
5.75	10	30	6	0.41	0.41
7	20	35	4	0.51	0.52
5.75	30	30	2	0.49	0.49
5.75	20	25	6	0.47	0.47
5.75	20	30	4	0.60	0.59
5.75	20	35	6	0.56	0.56
7	20	25	4	0.42	0.42
4.5	30	30	4	0.50	0.50
5.75	20	30	4	0.59	0.594
4.5	10	30	4	0.36	0.36
7	20	30	6	0.47	0.47
4.5	20	30	6	0.42	0.42
5.75	20	30	4	0.60	0.59
5.75	20	35	2	0.44	0.43

All the data are closely located to the diagonal line of the plot which implies that there was a good agreement between experimental and predicted value.

Analysis of quadratic model

Eventual analysis of quadratic model showed the suitablity of the model for starch isolation (Table 4). Non significane of lack of fit for the models implies that model is good. The R^2 and adjusted R^2 value of starch isolation were 0.999 and 0.997 indicated that all the process input variables were sufficent enoguh to represent the given ferementation condition. The regression coefficent implies that all

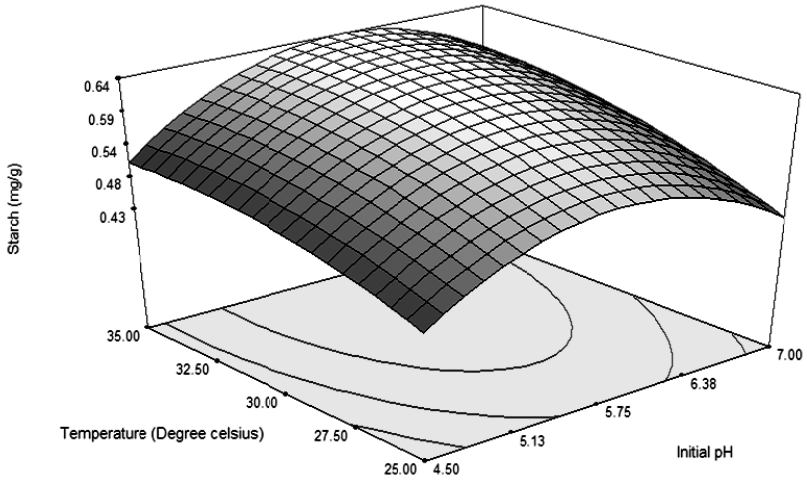

Fig. 2. Effect of initial pH and temperature on starch isolation

the three quadratic models explained the dependent variable with very less (0.005) varaiblity. The adjusted R^2 value is the R^2 value according to the sample size and number of terms used in the model (Swamy *et al.*, 2014). Both the adjusted R^2 value and predicted R^2 (0.993) value were in agreement, which represented a good concord between both experiemental and predicted value. An adequacy of starch isolation (100.274>4) pointed out that there was a low signal to noise ratio. Hence, this model could be used to lead the experimental plan gap. The coeffcient of variation (CV) and predicted sum of square (PRESS) of starch isolation was 1.162 and 0.002, respectivley which indicated that less varaibality of data points from the mean experimental and predicted values.

Table 3. Model summary statistics for starch isolation

Source	S.D.	R^2	Adjusted R^2	Predicted R^2	PRESS	Remarks
Linear	0.074	0.528	0.449	0.392	0.171	-
2FI	0.083	0.560	0.316	0.142	0.241	-
Quadratic	0.005	0.999	0.997	0.993	0.002	Suggested
Cubic	0.005	0.999	0.997	0.982	0.005	Aliased

S.D.-Standard deviation; PRESS-Predicted error sum of square

Effect of process variables

Effect of process variables and their interactions on isolation of starch is shown in the figures 2 and 3. Two way interactions was considered to construct the three dimensional graph by keeping one variable as constant. The optimum condition was 5.65 pH, 28.57 ml/100 g inoculum concentration, 35°C and 3.82 days for maximum starch isolation of 0.64 mg/g.

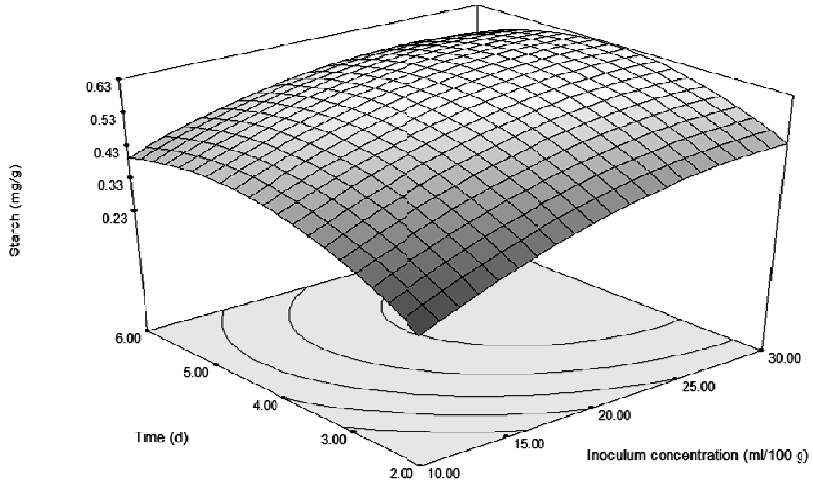

Fig. 3. Effect of inoculum concentration and time on starch isolation

Effect of pH on starch isolation

Starch isolation increased as increase in pH upto the optimum value of 5.65, further increase in pH decreased the isolation of starch (Fig.2). Enzyme assisted starch extraction was comparatively higher than conventional extraction (Zheng and Bhatty, 1998). Increased starch isolation was attributed to more enzyme complexes produced from the microorganism biomass which converted all non starch polysaccharides of the finger millet in to starch (Rao and Muralikrishna, 2001). Addition to that, enzymes produced during fermentation degraded the fibre bound phenolic compounds which increased the availability of starch (Meuser *et al.*, 1995). It was observed from the figure that starch isolation decreased as increased in pH because of higher pH affected the growth of microorganism and thus secreted less amount of enzyme and also due to suppression of no starch depolymerisation enzymes produced at higher pH.

Effect of temperature on starch isolation

Effect of tmeperature on starch isolation is shown in Fig. 2. It was observed that increasing the temperature constantly, increased the starch isoaltion and at lower temperaure less starch was obtained. This was because of the temperature dependance of the micro organism. *Aspergillus oryzae* has wide range of temperature adopatability (Hashemi *et al.*, 2010).

Effect of inoculum concentration on starch isolation

Higher the inoculum concentration, higher was the starch isolation (Fig.3). It may be due to more amount of enzymes from the biomass penetrating the substrates by attacking α-D-1-4 and α-D-1-6 linkages of cellulose and detaching the starch

Table 4. Analysis of variance for isolation of starch

Source	Coefficient Estimate	Sum of Squares	DF	Mean Square	F Value	Prob > F	Remarks
Model	0.594	0.280	14.0	0.020	667.92	< 0.0001	Significant
A	0.0008	0.000	1.0	0.000	0.28	0.6064	
B	0.0908	0.099	1.0	0.099	3300.28	< 0.0001	
C	0.0341	0.014	1.0	0.014	466.94	< 0.0001	
D	0.0541	0.035	1.0	0.035	1173.61	< 0.0001	
A2	-0.0990	0.063	1.0	0.063	2122.70	< 0.0001	
B2	-0.0640	0.026	1.0	0.026	887.93	< 0.0001	
C2	-0.0265	0.004	1.0	0.004	152.79	< 0.0001	
D2	-0.1065	0.073	1.0	0.073	2456.22	< 0.0001	
AB	0.0225	0.002	1.0	0.002	67.50	< 0.0001	
AC	0.0125	0.000	1.0	0.000	20.83	0.0004	
AD	0.0225	0.002	1.0	0.002	67.50	< 0.0001	
BC	0.0175	0.001	1.0	0.001	40.83	< 0.0001	
BD	-0.027	0.003	1.0	0.003	100.83	< 0.0001	
CD	0.007	0.0002	1.0	0.000	7.50	0.0160	
Residual		0.0004	14.0	0.000			
Lack of fit		0.0003	10.0	0.000	1.00	0.5484	Not significant
Pure error		0.0001	4.0	0.000			
Cor total		0.2809	28.0				
Mean	0.471						
C.V.	1.162						
PRESS	0.002						
Adeq Precision	100.274						

C.V-Coefficient of variation

component from the anti-nutritional factors such as phytic acid and tannin. Low inoculum concentration produced less amount of biomass which resulted in a lower bioconversion (Yan *et al.*, 2014).

Effect of time on starch isolation

Increased ferementation time increases the starch isolation, this could be as a consequence of conversion of non starch polysacchrides into starch by the enzymes produced during fermentation. Maximum starch isolation was observed at fourth day of fermentation. Further increasing time decreased the starch isolation could be caused by degradation of starch by amylopectin enzymes (Nirmala and Murlikrishna, 2002). Amylase and protease enzymes of *Aspergillus oryzae* helped in the conversion of polysaccharides into monosaccharides with increase in fermentaion time (Hashemi *et al.*, 2010; Yu *et al.*, 2012).

Conclusion

Isolation of starch from finger millet by solid state fermentation could be attractive for commercial starch producing industries in environmentally affiable way. Isolated starch can be used as gelling and thickening agent in the food. On the other hand, starch can also be used for manufacturing adhesive, biodegradable polymers, and pharmaceuticals. Hence, this research work recommends that solid state fermentation could be used for isolation of starch as environment benign process and desired modification could be achieved by using suitable organism.

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