Optimization of the use of fungal enzymes amylase and glucoamylase to enhance the nutritional and functional properties of rice flour

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ABSTRACT

Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial applications. Amylase and glucoamylase were produced using *Aspergillus oryzae* under solid state fermentation. The enzymes obtained were partially purified using isopropanol and rice flour was treated with these hydrolysing enzymes. Enzymatic pretreatment conditions were optimized using Response Surface Methodology (RSM). Effect of different parameters such as slurry concentration, enzyme concentration, time and temperature was studied. Enriched rice flour was obtained by treating 7 % slurry with 1 ml of enzyme at 55 °C for 40 min. This enriched rice flour was analysed for its nutritional, functional, toxic residues and microbiological parameters. Protein content of the enzymatically treated rice flour increased from 8.03 % to 20.79 % (2.5 fold enhancement) and ash from 0.69 % to 1.40 %. Fat content of both, untreated and treated rice flours, differed insignificantly but crude fiber was hydrolysed from 2.50 to 0.42. Moisture content of the treated rice flour decreased from 12.40 % to 11.85 %. The digestibility coefficient of in vitro digestibility also increased from 644.63 to 1351.67 in treated rice flour. Microbiological count of treated rice flour decreased whereas contaminating organisms (coliforms and salmonella) and toxic residues (aflatoxins) were not detected.

Key words: Rice flour, enzymes, *Aspergillus oryzae*, nutritional and functional properties, RSM

1. Introduction

Rice is one of the major staple food grains in the world. Rice is composed largely of carbohydrates 80%, with little protein 6-7%, but the protein they contain is particularly nutritious, rich in essential amino acid lysine and hypoallergenic for use in foods. In addition to this, some unique functional properties of rice, such as flavor carrying capability, low fat etc. make it a grain for use in many value-added products [1]. The application of rice in value-added products could give food industries a new avenue of use, thus increasing its demand. There is a need to study and improve the quality and quantity of rice protein. However, to enhance the use of rice proteins, it is desirable to develop effective methods to separate or concentrate the protein component from these rice co-products. Moreover, infants, young children and elderly cannot eat a sufficient amount of rice to obtain a satisfactory protein intake. If the protein concentration is increased to about 20-25%, it would indeed be a good nourishing food for them.

The traditional process for rice protein separation from starch is tedious and costly. On the other hand use of artificial materials such as sodium hydroxide, acids and surfactants is undesirable for use in food production. The enzymatic process is one alternative which uses only natural materials and is therefore advantageous for food production. Enzymes are produced by various micro-organisms including bacteria, fungi and yeasts and are considered as important products obtained for human needs through microbial sources. The advantage of using micro-organisms for the production of enzymes is that bulk production is economical and microbes are easy to manipulate to obtain enzymes with desired characteristics. Fungal enzymes are preferred over other microbial sources owing to their widely accepted Generally Regarded As Safe (GRAS) status [2]. A large number of industrial processes pertaining to the areas of industrial, environmental and food biotechnology employ such enzymes at some stage or the other.

Amylases are universally distributed throughout the animal, plant and microbial kingdoms. Among various extracellular enzymes, alpha amylase ranks first in terms of commercial exploitation [3]. Spectrum of application of amylases 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 industries and distilleries [4]. Glucoamylases are industrially important hydrolytic enzymes of biotechnological significance and are currently used for dextrose production, confectionary, baking and in pharmaceuticals [5].

Studies on fungal amylase especially in the developing countries have concentrated mainly on *Aspergillus* species probably because of ubiquitous nature and non-fastidious nutritional requirement of this organism [6] , [7]. The exclusive production of glucoamylases is achieved by *Aspergillus niger* [8] and *Aspergillus oryzae* in enzyme industry [9]. *Aspergillus oryzae*, a GRAS strain is a filamentous fungus which does not produce any toxin and its enzymes are permitted for use in food products. This justifies its use for protein enrichment of agricultural wastes and by-products. It does not reveal any adverse effects based upon its lack of toxicity and the fact that the enzyme produced by it was considered to be acceptable for use in food [10].

Cereal brans and flours, potato residue and other starchy waste materials have been utilized as fermentation substrate for amylase and glucoamylase production by filamentous fungi [9]. Wheat bran, paddy husk, rice processing waste and other starch containing wastes have gained importance as supports for growth during enzyme production and wheat bran as the most promising substrate has been reported by Anto *et al* [11]. Large quantities of broken rice and rice bran are generated as by-products during the milling of rice. Rice bran and to a lesser extent, broken rice are under-utilized. Hence, the objective of the present study was to use these enzymes to enhance the nutritional and functional properties of rice flour under optimized conditions.

2. Materials and Methods

2.1. Large scale production of fungal enzymes

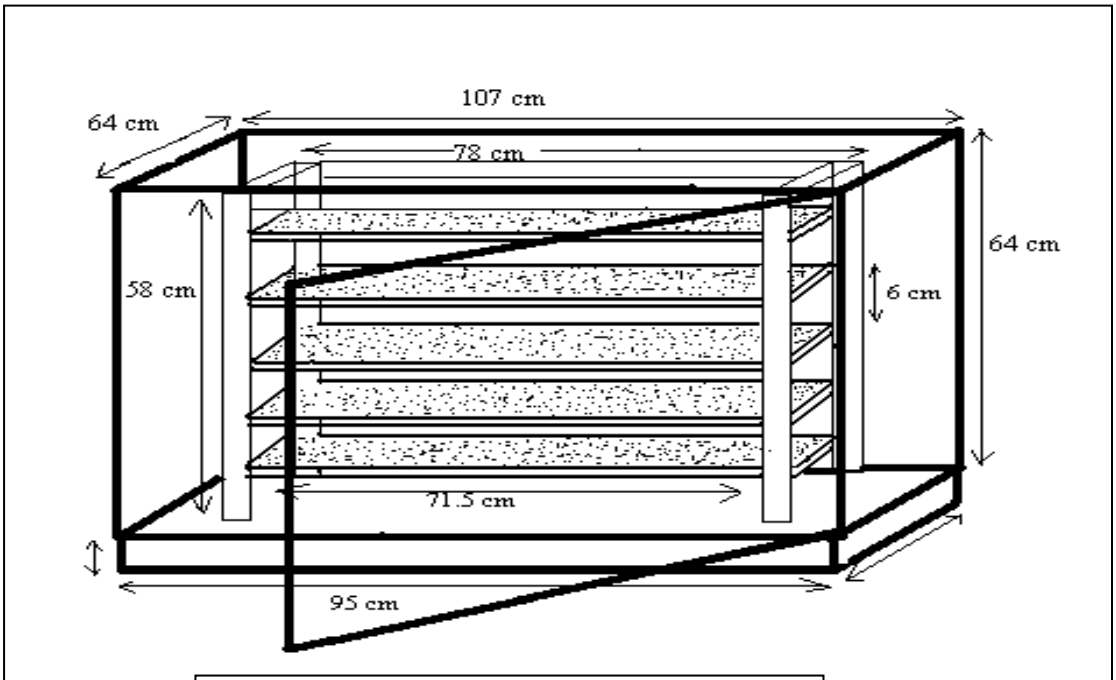
2.1.1.1. Inoculum preparation

Non-toxic fungi, *Aspergillus oryzae* (MTCC 3107) was procured from MTCC (Microbial Type Culture Collection), Institute of Microbial Technology, Chandigarh, Punjab. Amylase and Glucoamylase are produced by this organism and are Generally Recognized As Safe (GRAS). A spore inoculum was prepared by adding 10-15 ml of sterile Tween 80 (0.8%) to the 3 day old culture slants and shaken vigorously. One ml of the inoculum was used per flask containing 5g substrate with 20 ml mineral media (pH 5) to carry out solid state fermentation. The flasks were incubated at 30 °C for 5 days.

2.1.2. Solid state fermentation under optimized cultural conditions using a self designed fermenter

A rectangular box measuring 107 cm length × 64 cm breadth × 64 cm height, made up of fibre glass sheets and with working capacity of 10kg (wet wt.) was used as a fermenter vessel (Figure 1). An airtight door was provided in the front side of rectangular fermenter to ensure closed fermentation. It not only helps in controlling contamination but also prevents the exit of biological molecules for safety hazards. Fermentation was carried out in five stainless steel screen bottom trays of size 71.5 cm length × 31 cm width × 3.5 cm height and the diameter of perforation was 0.3 cm. Before fermentation, the trays were surface disinfected with a swab dipped in 100% ethanol. These specifications were selected keeping in view

the type of substrate used and to provide adequate environment for the growth of fungi during the course of fermentation. Rice bran, 500 g was moistened with 1.5 L distilled water. This moist bran was taken in polypropylene bags and autoclaved at 15 pounds (lbs) per square inch (psi) for 45 minutes. After cooling, rice bran was spread uniformly up to a height of two cm in each tray. Culture of *Aspergillus oryzae* was added and mixed well under sterilized conditions. Trays were placed one above the other with the gap of 6 cm in between and stacked on an aluminium stand of size 78 cm length × 38 cm width × 58 cm height. A GI sheet box measuring 95 cm length × 52 cm width × 12 cm height, filled with water was attached at the base of the rectangular fermenter. Steam was generated in the fermenter on heating the water to maintain 95% relative humidity in the chamber. Forced air circulation does not exist in the space between two successive trays or through the substrate bed itself



and oxygen transfer occurs primarily by diffusion. Since no special measures were available for dissipation of heat during the metabolism, the main mechanism of heat removal used was conduction through the walls of the trays and via. the latent heat of vaporization of the moisture. Fermentation was carried out for five days at 30±2°C.

2.1.3. Enzyme extraction and its partial purification

After five days of fermentation, 50 g (wet weight basis) of the fermented substrate was taken from each tray in a 250 ml Erlenmeyer flask. To each flask 500 ml of citrate buffer (pH 5.0) was added and the mixture was vigorously homogenized at 250 rpm for 30 min. The solid biomass residue was separated from the suspension by filteration and then the filterate was centrifuged at 10,000 rpm for 20 mins. The filterate was again filtered through Whatman's filter paper No.1 so as to obtain a cell free supernatant which was used as source of crude enzyme [12]. Further alpha amylase and glucoamylase enzymes were isolated and purified with isopropanaol [13] . Mold filterate (50 ml) was chilled at 4°C in a refrigerator and 50 ml of chilled isopropanol was added to it. The precipitate was separated by centrifugation at 10,000 rpm for 15 mins. The precipitates were dissolved in 12.5 ml of 0.05M citrate buffer having pH 5 [14] to obtain partially purified enzyme, which was assayed for its activity.

2.2. Milling process for rice flour prepartion

Paddy (10 kg) procured from department of plant breeding and genetics, Punjab Agricultural University was first cleaned by using a Satake Pre-cleaner and the stones and extraneous matter were removed. Then, husk was removed from the paddy by using a Crompton Greaves Mini Rice Mill and brown rice was obtained after the process. Brown rice was polished to obtain white rice with the help of Satake rice polisher machine. White rice obtained was then graded into head rice and broken rice using an Indosaw Seed Grader machine. Broken rice obtained as a by-product of milling process was ground using grinding machine and rice flour was obtained (Fig 2).

2.3. Pretreatment of rice flour

Rice flour slurry (flour: water = 5g: 100 ml) was heated at 75°C for 20 minutes to gelatinize the rice starch. After gelatinization, slurry was cooled and treated with amylase and glucoamylase to digest the gelatinized starch and was centrifuged at 6000 rpm for 30 minutes to obtain precipitates which were dried to obtain high protein rice flour.

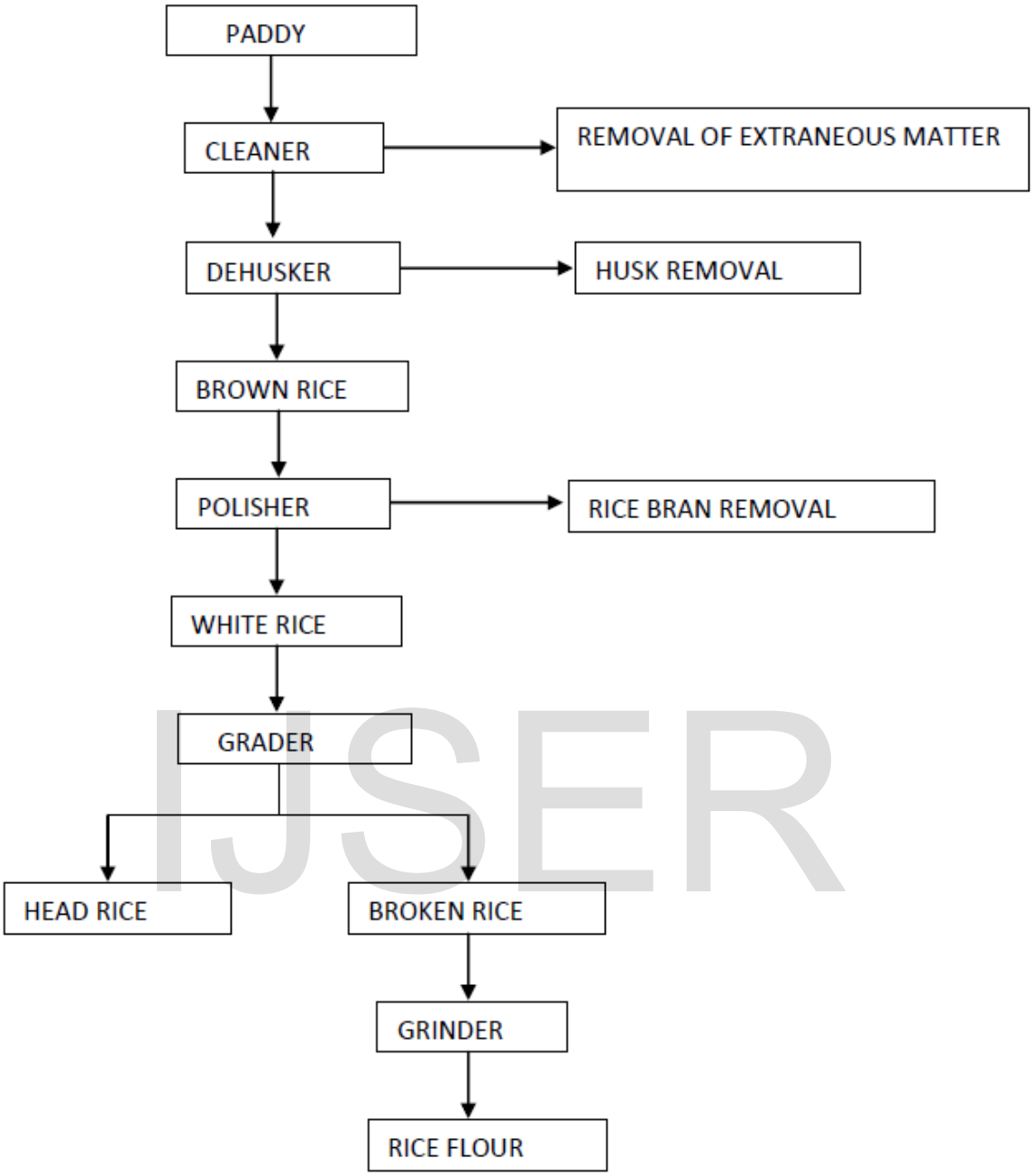


Fig 2 : Milling of Rice flour

2.4. Optimization of process parameters for enzymatic pretreatment

2.4.1. Enzyme concentration

For the production of high protein rice flour different enzyme concentrations such as 1ml, 2ml, 3ml, 4ml and 5ml were used to hydrolyze rice starch in the rice flour slurry.

2.4.2. Length of starch digestion period

Optimal time length for starch digestion at an optimal enzyme concentration was determined by incubating the rice flour slurry with enzymes for 10, 20, 30 and 40 minutes.

2.4.3. Temperature

Rice flour starch digestion in the slurry was carried out at varying temperatures such as 30, 40, 50 and 60°C for 10, 20, 30 and 40 minutes to determine optimal reaction temperature for enzymes to hydrolyze starch from the rice flour slurry.

2.4.4. Rice flour slurry concentration

Rice flour slurry was made at different concentrations i.e. 5g: 100ml, 6g: 100ml and 7g:100ml and was gelatinized at 75°C for 20 minutes. These were cooled and treated with 1ml, 2ml, 3ml, 4ml and 5ml of enzyme at 30, 40, 50 and 60 °C for 10, 20, 30 and 40 minutes to determine optimal conditions for the production of high protein rice flour. The protein content was estimated through Micro-Kjeldhal [15] method and the flour with highest protein content was used for further studies.

2.5. Production of protein enriched rice flour

Protein enriched rice flour was obtained by making a slurry concentration of 7g: 100ml and heating at 75 °C for 20 minutes to gelatinize the rice flour starch. Then the slurry was cooled and treated with 1ml of the enzyme at 55 °C for 40 minutes. The treated slurry was then centrifuged at 6000 rpm for 20 minutes to obtain the treated precipitates. The precipitates were dried in hot air oven at 50 °C for 6 hours. The dried mass obtained was ground to yield high protein rice flour (Figure 3).

2.6. Quality evaluation of treated and untreated rice flour

2.6.1. Physico-chemical and functional evaluation

2.6.1.1. Moisture content

The moisture content in the rice samples was determined by the Hot Air Oven Single Stage Method [15] .

2.6.1.2. Protein

Protein content was estimated by using Micro-Kjeldhal method [15].

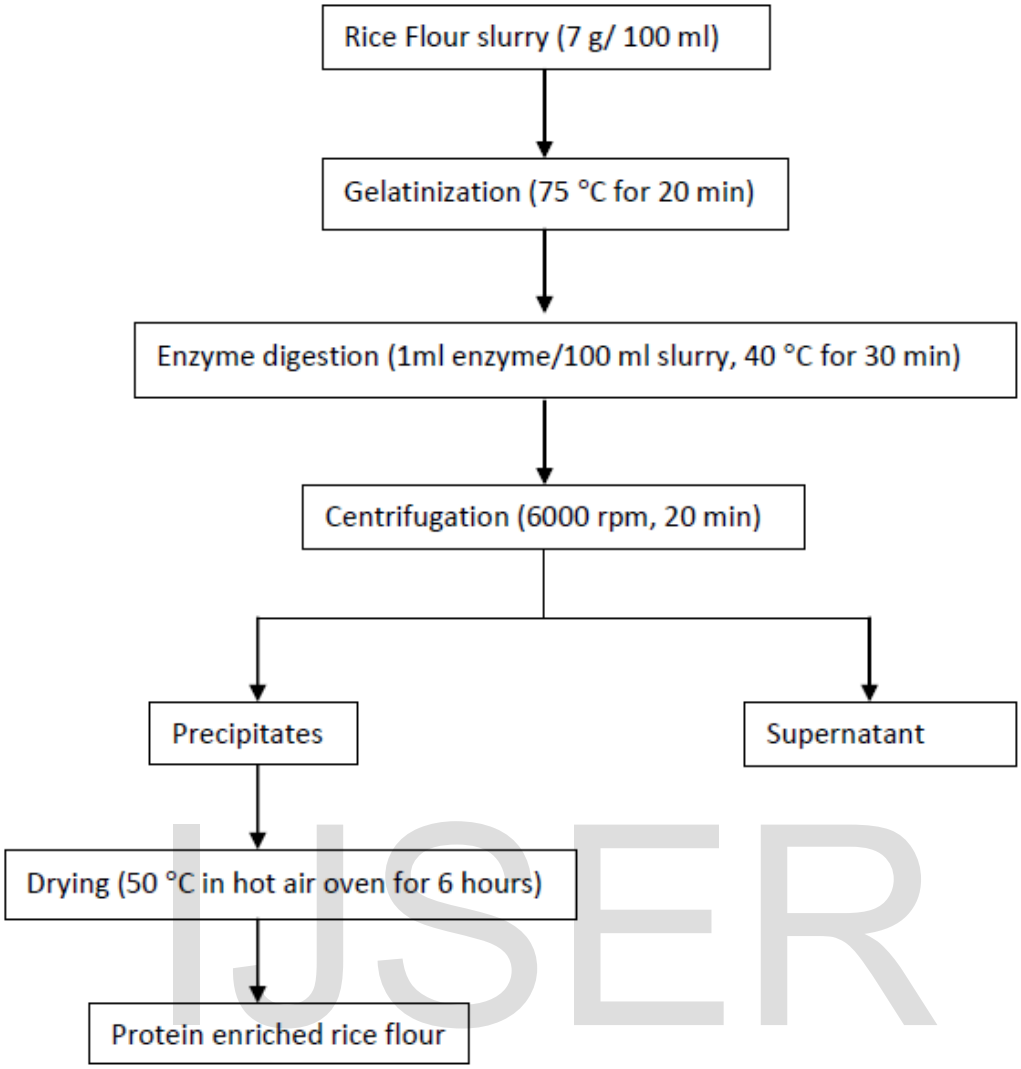


Fig 3: Schematic diagram for the production of protein enriched rice flour

2.6.1.3. Fat

For the estimation of crude fat content Soxlet method [15] was used.

2.6.1.4. Crude fiber

Crude fibre was estimated using the method of AOAC [15].

2.6.1.5. Ash

Five grams of the sample was taken in a previously weighed crucible. Crucibles were then placed in a muffle furnace at 550 °C for 4 hours or until light grey ash resulted [15]. The residue left was weighed after cooling it to room temperature in a dessicator.

2.6.1.6. Mineral content

Mineral content was analyzed using inductively coupled argon plasma (ICAP) machine.

2.6.1.7. Protein digestibility (in vitro)

The in-vitro protein digestibility was estimated using method given by Akeson and Stachman [16].

2.6.2. Microbiological evaluation

2.6.2.1. Total plate count

Total plate count in the sample was done using plate count agar of Hi-Media company by pour plating technique.

2.6.2.2. Yeast and mold count

Yeast and mold count in the sample was done using potato dextrose agar (PDA) of Hi-Media company by pour plating technique.

2.6.2.3. Coliforms

Coliform count in the sample was estimated using Eosine Methylene Blue (EMB) agar of Hi-Media company by pour plating technique.

2.6.2.4 *Salmonella*

Salmonella count of the sample was estimated using *Salmonella* Differential Agar (Raj Hans media) of Hi-Media company by pour plating technique.

2.6.3. Toxic residue studies

2.6.3.1. Aflatoxin residue

Aflatoxin residues were analyzed qualitatively by using Pressure Minicoloumn.

2.7. Statistical analysis

The regression analysis for fitting the data in the following second order polymonial according to response surface methodology (RSM) was done in the Design expert software'(DXT trial 8.01).

$$y' = \beta_0 + \sum_{i=1}^{n}\beta_i x_i + \sum_{i=1}^{n}\beta_i x_i^2 + \sum_{i=1}^{n-1}\sum_{j=1}^{n-1}\beta_{ij} x_i x_j + \varepsilon$$

Where β_o, β_i, β_{ij} are the constant coefficients usually determined by least square method and ε is the error involved in estimating the coefficient β from the experimental data. The analysis of variance (ANOVA) for the splitting of model and overall effect of variables and non-linear regression for the fitting of data was carried out in 'Design expert software'.

2.7.1. Optimization of process parameters for enzymatic pretreatment of rice flour using response surface methodology (RSM)

For optimization firstly the Analysis of Variance for the overall effect of four factor variables on the response variable according to the fitted model was done and least significant factor affecting the response variables was selected. The three dimensional plots and contour plots according to fitted model and fixed variable was drawn in the 'Design expert software' (DXT trial 8.01) to localize an optimum treatment condition for rice flour treatment, the numerical optimization technique was employed for optimization of different processes of Response Surface Methodology.

3. Results and Discussion

3.1. Large scale production of fungal enzymes

The optimized conditions were selected based on their effect on enzyme production. From each tray containing 2 kg wet substrate, 50g was taken out after five days of incubation and assayed for enzyme activities. Both alpha amylase and glucoamylase activities from each tray showed only slight difference between each other as shown in Fig 4.

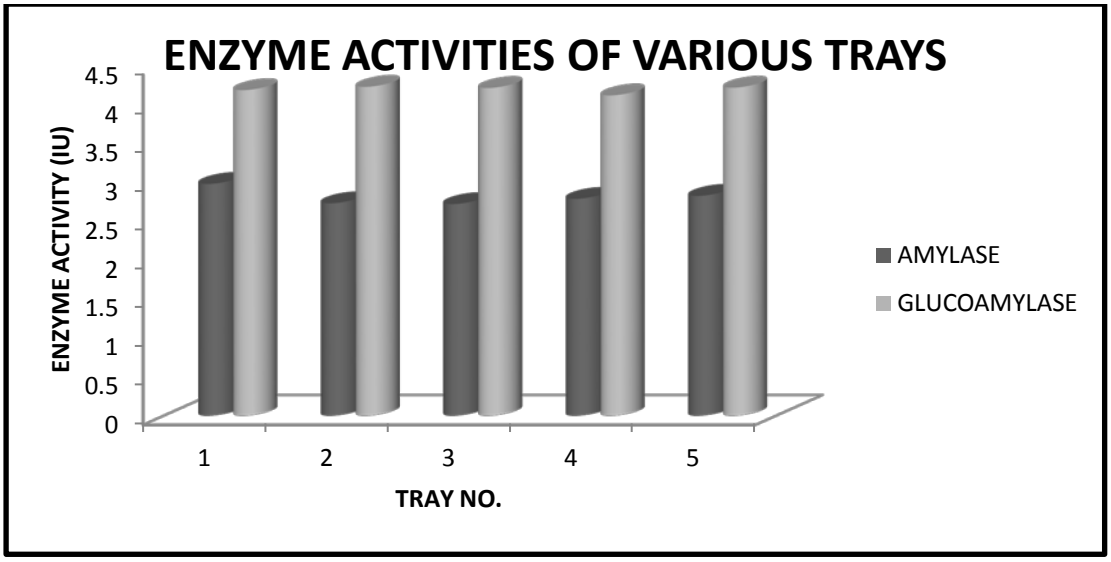


Fig: 4 Enzyme activities of five trays of fermenter

3.2. Effect of different parameters on the production of enriched rice flour

Effect of different parameters such as slurry concentration (5-7%), enzyme concentration (1-5 ml), time (10-40 min) and temperature (30-60 °C) has been studied. Treatment of 7% slurry concentration with enzyme concentration (1ml) at 55 °C for 40 min resulted in production of rice flour which has been modified and protein content was enhanced approximately 3 fold from 8.08% to 20.79%. Similarly, enzymatic pretreatment of oat bran using viscozyme L have been studied to enhance protein extraction [17]. The optimum conditions of enzymatic pretreatment were, viscozyme L concentration (30 FBG/10 g of oat bran), incubation time (2.8 hrs), pH (4.6) and incubation temperature (44 °C). Under such conditions, the predicted maximum protein extracted was 55.69%, which coincided with the experimental values.

3.3. Optimization of the conditions for the enzymatic pretreatment of rice flour

Response Surface Methodology (RSM) software DXT trial 8.01 version was used to optimize conditions for the enzymatic pre-treatment of rice flour. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. Box-Behnken design of RSM was used. The independent variables included slurry concentration (5-7 %), enzyme concentration (1-5 ml), time (10-40 min) and temperature (30-60 °C) and response variable was Protein Content (PC). The experimental runs suggested by RSM were 29 and these experiments were performed (Table 1). For appropriate fitting of model, the Analysis Of Variance (ANOVA) was carried out for the production of enriched rice flour. It indicated that the developed model for the response has a good lack of fit (Table 2). The value for determination coefficient R^2 was 0.966 for the production of enriched rice flour. This gives a good correlation between the factors and the response. Experimental results are close to the predicted value (Figure 5).

Table 1: Experimental data for four factors using Response Surface Analysis

Std	Run	A:Sc %	B:Ec ml	C:Time Min	D:Temp C	PC % N
3	1	5	5	25	45	13.3
7	2	6	3	10	60	12.4
6	3	6	3	40	30	14.2
12	4	7	3	25	60	18.6
28	5	6	3	25	45	13.8
26	6	6	3	25	45	13.8
14	7	6	5	10	45	12.8
9	8	5	3	25	30	11.8
13	9	6	1	10	45	14.5
15	10	6	1	40	45	15.4
4	11	7	5	25	45	18.6
20	12	7	3	40	45	18.3
24	13	6	5	25	60	13.2
18	14	7	3	10	45	16.9
1	15	5	1	25	45	12.5
16	16	6	5	40	45	13.6
19	17	5	3	40	45	12.4
22	18	6	5	25	30	14.8
2	**19**	**7**	**1**	**25**	**45**	**20.5**
29	20	6	3	25	45	13.8
8	21	6	3	40	60	14.5
21	22	6	1	25	30	15.2
5	23	6	3	10	30	12.8
27	24	6	3	25	45	13.8
11	25	5	3	25	60	12.5
23	26	6	1	25	60	14.3
25	27	6	3	25	45	13.8
10	28	7	3	25	30	18.5
17	29	5	3	10	45	12.9

Table 2: ANOVA for Response Surface Quadratic for enriched rice flour

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	140.94	14	10.07	28.56	< 0.0001	Significant
A-Sc	108	1	108	306.43	< 0.0001	
B-Ec	3.1	1	3.1	8.8	0.0102	
C-Time	3.1	1	3.1	8.8	0.0102	
D-Temp	0.27	1	0.27	0.77	0.3962	
AB	1.82	1	1.82	5.17	0.0392	
AC	0.9	1	0.9	2.56	0.1319	
AD	0.09	1	0.09	0.26	0.6212	

BC	#######	1	#######	#######	0.9341
BD	0.12	1	0.12	0.35	0.5649
CD	0.12	1	0.12	0.35	0.5649
A2	18.29	1	18.29	51.89	< 0.0001
B2	2.88	1	2.88	8.18	0.0126
C2	0.72	1	0.72	2.04	0.1746
D2	0.033	1	0.033	0.092	0.7657
Residual	4.93	14	0.35		
Lack of Fit	4.93	10	0.49		
Pure Error	0	4	0		
Cor Total	145.87	28			

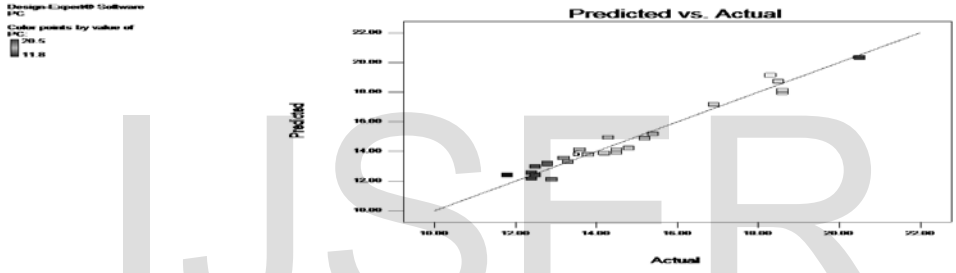


Fig 5 Experimental vs predicted results of maximum protein content

Both the linear and quadratic terms had significant effect in explaining the data at 5 % level of significance, but quadratic model was suggested for optimization of parameters (Table 3). It has been observed that coefficients of linear and square terms of slurry concentration (A), enzyme concentration (B), time (C) and temperature (D) and their interactions AB, A^2, B^2 and D^2 were found significant ($P< 0.05$) which indicate that small increase and decrease in their value leads to variation in production of enzyme treated flour while insignificant terms such as AC, AD, BC, BD, CD and C^2 had no effect on the treatment (Table 2). The multiple regression analysis was used to fit the second order polynomial to the experimental results by least square method (Table 4). The regression coefficients obtained for the measured response has also been represented in the table.

Contour and 3-D surface plots were drawn taking two factors into consideration and keeping other factors as constant. A direct correlation has been found between slurry concentration and enzyme concentration and graphs have been plotted to understand their effect on increase in protein content. It has been seen that with the increase in slurry

Table 3: Analysis of Variance (ANOVA) for model fitting for enriched rice flour

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F

Mean vs Total	6184.56	1	6184.56			
Linear vs Mean	114.47	4	28.62	21.87	< 0.0001	
2FI vs Linear	3.06	6	0.51	0.32	0.9156	
Quadratic vs 2FI	23.4	4	5.85	16.6	< 0.0001	Suggested
Cubic vs Quadratic	4.85	8	0.61	45.03	< 0.0001	Aliased
Residual	0.081	6	0.013			
Total	6330.43	29	218.29			

Table 4: Regression coefficients from quadratic model

Coefficient Factor	Estimate	Standard df	95% CI Error	95% CI Low	High	VIF
Intercept	13.8	1	0.27	13.23	14.37	
A-Sc	3	1	0.17	2.63	3.37	1
B-Ec	-0.51	1	0.17	-0.88	-0.14	1
C-Time	0.51	1	0.17	0.14	0.88	1
D-Temp	-0.15	1	0.17	-0.52	0.22	1
AB	-0.67	1	0.3	-1.31	-0.038	1
AC	0.48	1	0.3	-0.16	1.11	1
AD	-0.15	1	0.3	-0.79	0.49	1
BC	-0.025	1	0.3	-0.66	0.61	1
BD	-0.18	1	0.3	-0.81	0.46	1
CD	0.18	1	0.3	-0.46	0.81	1
A^2	1.68	1	0.23	1.18	2.18	1.08
B^2	0.67	1	0.23	0.17	1.17	1.08
C^2	-0.33	1	0.23	-0.83	0.17	1.08
D^2	-0.071	1	0.23	-0.57	0.43	1.08

concentration and decrease in enzyme concentration, protein content of the flour increases (Figure 6a and Figure 6b). It has also been observed that as the time period of the treatment increases, there is an increase in concentration of protein (Figure 6c and Figure 6d). A similar trend was found in the case of temperature which is shown in Figure 6e and Figure 6f. The interrelation between other factors depicted in figures from 6g to 6l also showed the similar trend.

The most desired combination has been selected for the treatment of enzyme on rice flour for the production of enriched rice flour which has been shown in Figure 6m and Figure 6n. Conditions found optimum for the production of enriched rice flour were 7% slurry concentration, 1 ml of enzyme concentration for 38.21 min at 54.26°C. Similarly, rice flour was enzymatically modified using lipase pancreatic and amyloglucosidase to obtain resistant starch [18]. For this, RSM was used to determine the best operating conditions for each enzyme. For lipase pancreatic, the highest value for resistant starch (45%) was achieved within 2 hours reaction at pH 7 using an enzyme/substrate ratio of 4% (w/w) and Dp= 100/200 tyler. For amyloglucosidase optimum conditions corresponded to an enzyme/substrate ratio of 0.006 ml/g and Dp= 100/200 tyler at 45 C yielded 57% of resistant starch in 2 hour reaction. These results showed the potential of using both enzymes to modify

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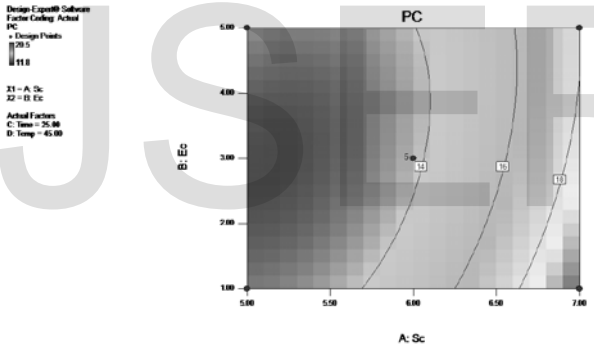

Fig 6 (a) Contour plot for enriched rice flour (Sc vs. Ec)

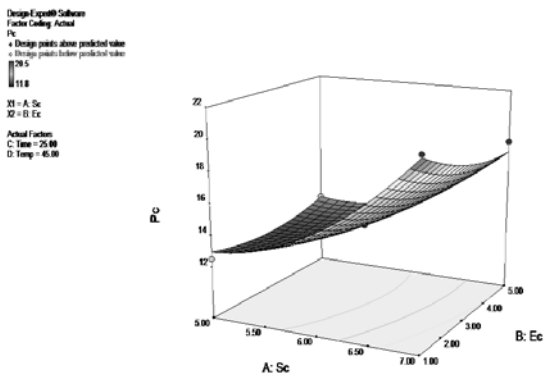

Fig 6 (b) Surface plot for enriched rice flour (Sc vs. Ec)

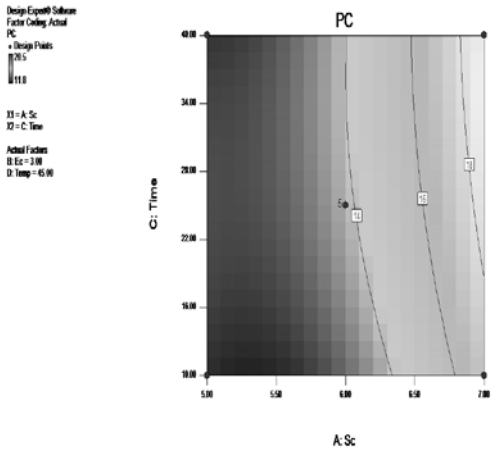

Fig 6 (c) Contour plot for enriched rice flour (Sc vs. time)

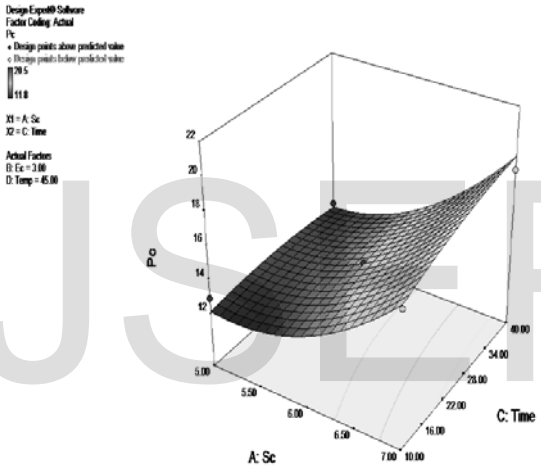

Fig 6 (d) Surface plot for enriched rice flour (Sc vs. time)

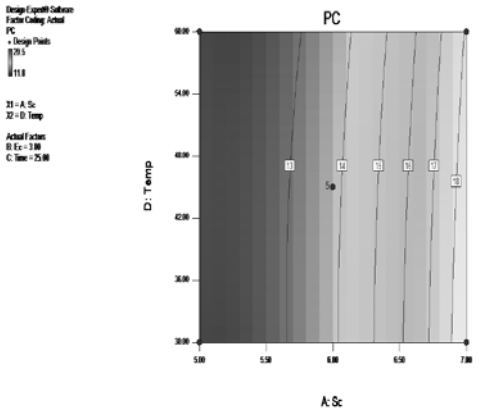

Fig 6(e) Contour plot for enriched rice flour (Sc vs. temp.)

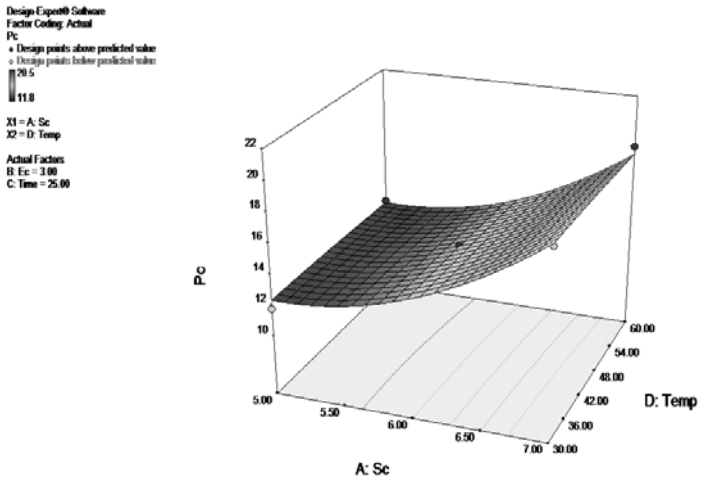

Fig 6 (f) Surface plot for enriched rice flour (Sc vs. temp.)

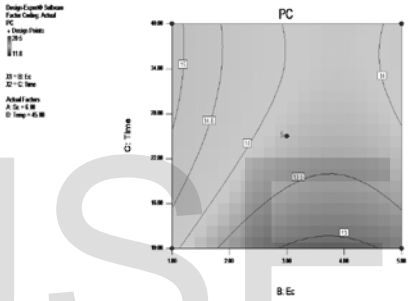

Fig 6 (g) Contour plot for enriched rice flour (Ec vs. time)

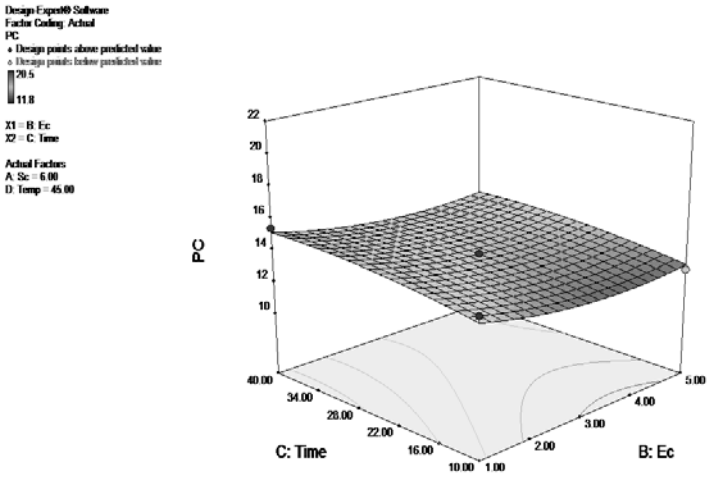

Fig 6 (h) Surface plot for enriched rice flour (Ec vs. time)

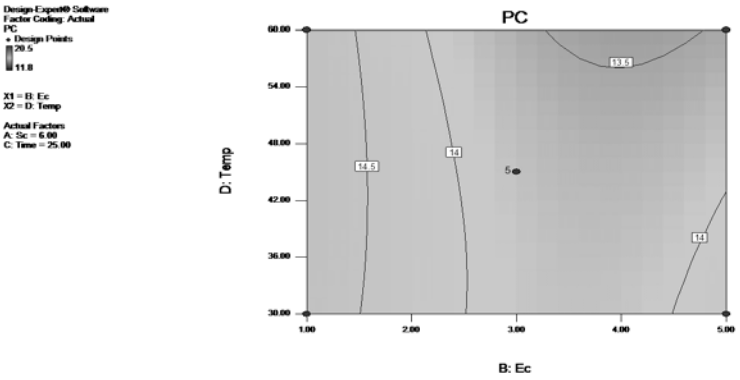

Fig 6 (i) Contour plot for enriched rice flour (Ec vs. temp.)

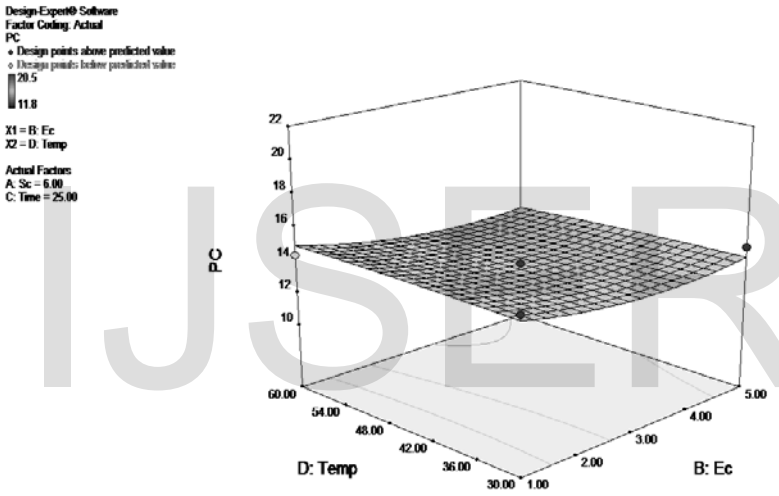

Fig 6 (j) Surface plot for enriched rice flour (Ec vs. temp.)

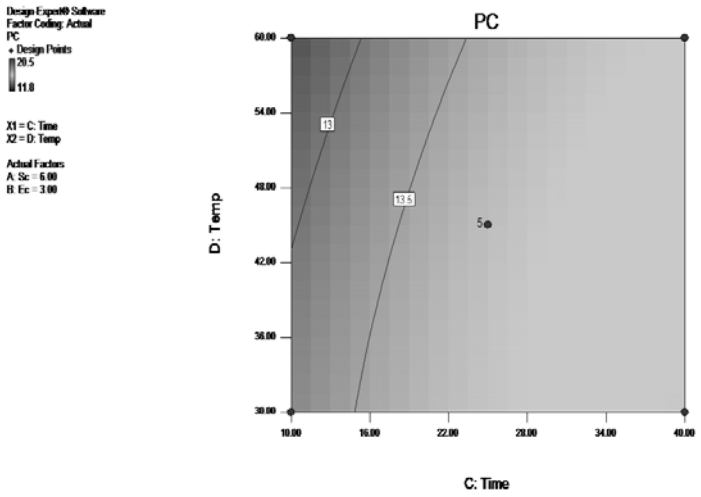

Fig 6 (k) Contour plot for enriched rice flour (Time vs. temp.)

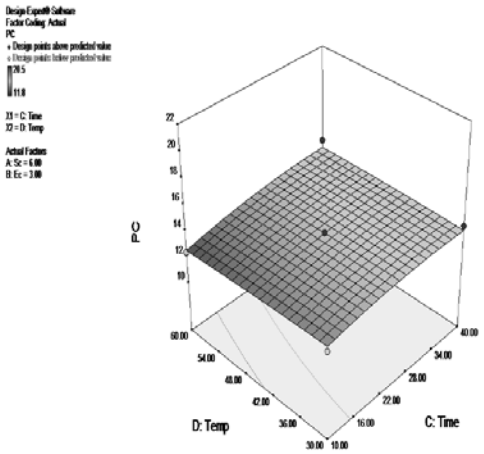

Fig 6 (l) Surface plot for enriched rice flour (Time vs. temp.)

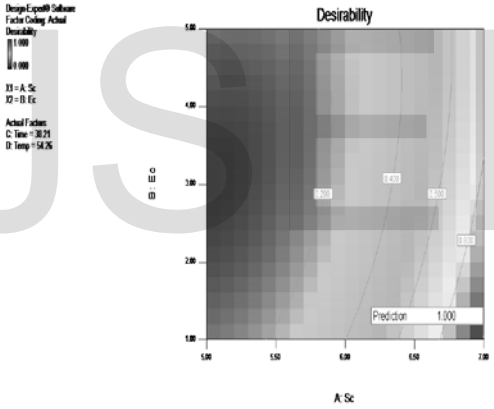

Fig 6 (m) Contour plot for production of enriched rice flour

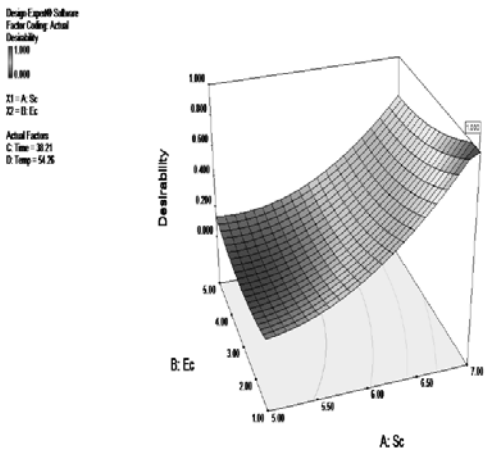

Fig 6(n) Surface plot for production of enriched rice flour

rice flour, increasing the resistant starch in about 5.7 folds in relation to the flour without treatment.

Final Equation in Terms of Coded Factors:

$$Pc=+13.80+3.00*A-0.51*B+0.51*C-0.15*D-0.67*A*B+0.48*A*C-0.15*A*D-0.025*B*C-0.18*B*D+0.18*C*D+1.68*A^2+0.67*B^2-0.33*C^2-0.071*D^2$$

Final Equation in Terms of Actual Factors:

$$Pc=+52.55185-16.47917*Sc+1.05417*Ec-0.11454*Time+0.076389*Temp-0.33750*Sc*Ec+0.031667*Sc*Time-0.0100*Sc*Temp-8.33333E-004*Ec*Temp-8.33333E-004*Ec*Time-5.83333E-003*Ec*temp+7.77778E-004*Time*Temp+1.67917*Sc^2+0.16667*Ec^2-1.48148E-003*Time^2-3.14815E-004*Temp^2$$

3.4. Effect of enzymatic treatment on proximate composition of rice flour

The effect of enzymatic treatment on comparison of proximate composition of treated and untreated rice flour has been shown in Table 5. Moisture content of treated rice flour decreased from 12.40% to 11.85%. The decrease in moisture content increases the shelf life and storage characters. This decrease may be attributed to the action of hydrolytic enzymes amylase and glucoamylase as these enzymes require water for their activity and hence reduce the moisture of the treated rice flour. Similar trend of moisture content was reported as a result of enzymatic pretreatment using commercial cellulase [19] in which there was a decrease in moisture content from 10% to 7.05%.

Table 5: Proximate composition of treated and untreated rice flour

S.No	Proximate composition	Untreated rice flour	Treated rice flour	CD (at 5 %)

1.	Moisture content (%)	12.40	11.85	.321636
2.	Protein (%)	8.08	20.79	.920351E-01
3.	Fat (%)	0.67	0.70	NS
4.	Crude fiber (%)	2.50	0.42	.323418
5.	Ash (%)	0.69	1.40	.647956E-01
6.	Carbohydrate (%)*	75.66	64.84	.436740

Protein content of treated rice flour was enhanced 3 fold as compared to untreated rice flour. This may be due to the hydrolysis of starch and release of protein fractions bound with the starch molecules. Similarly, a partially purified alpha amylase enzyme was used to study the effect of different cultural conditions such as concentration of alpha amylase, alpha amylase digestion time and effect of calcium on alpha amylase digestion. Optimum conditions used to hydrolyse rice starch in rice flour slurry were enzyme concentration of 0.8 ml/100 ml of slurry, digestion time of 90 min at a temperature of 60 °C with 2.5 ml/100 ml slurry of 2% calcium chloride/ 100 ml which resulted in production of rice flour with two-fold enhanced crude and true protein. Crude protein increased from 7.5 to 15.2 % and true protein increased from 5.9 to 12 % [20].

Fat content of treated and untreated rice flour differed insignificantly but crude fibre decreased from 2.50% to 0.42%. Ash content increased from 0.69% to 1.40% in treated rice flour as compared to untreated rice flour. Enzymatic hydrolysis of rice flour had no effect on the fat content but increase in ash content was observed. This may be due to release of inorganic components of rice flour which were trapped with the starch on its hydrolysis.

Carbohydrate concentration in the treated rice flour decreased significantly as compared to the untreated rice flour. It has decreased from 75.66 to 64.84 %. Similar results were found when rice starch was subjected to enzymatic treatment with pullulanase and the physicochemical properties of the modified rice starch were investigated as a function of the degree of hydrolysis which ranged from 0.9 to 10.2%. The enzymatic hydrolysis of rice starch caused a decrease in the fraction of rapidly digestible starch (78.31→44.65%), whereas the levels of slowly digestible starch (0.8→22.18%) and resistant starch (20.79→34.43%) increased, consequently reducing *in vitro* starch digestibility [21].

3.5. Effect of enzymatic treatment on mineral content of rice flour

Minerals are essential requirement of our body and they can be classified on the basis of their concentration in the body and dietary requirement. They can be classified as:

Major elements- Calcium, phosphorus, sodium, potassium, chloride and magnesium.

Elements needed in small amounts - iron and zinc.

Trace elements needed in tiny amounts - includes iodine, copper and selenium.

Mineral composition of both the treated and untreated rice flour has been studied. Enzymatic hydrolysis of rice flour has significantly increased few of the essential minerals shown in Table 6. Calcium and phosphorus are the minerals that are required by humans in

Table 6: Mineral composition of untreated and treated rice flour

S. No.	Element	Untreated rice flour (mg/kg)	Treated rice flour (mg/kg)
1	Arsenic	0.215	0.045
2	Boron	94.4	44.25
3	**Calcium**	**1069**	**2170**
4	Chromium	3.60	3.38

5	Copper	5.12	6.0
6	Iron	1979	2046
7	Potassium	5695	2457
8	**Magnesium**	**1017**	**1560**
9	Manganese	29.02	39.26
10	**Sodium**	**346**	**619**
11	Nickel	9.56	9.34
12	**Phosphorus**	**4806**	**10615**
13	Lead	4.39	4.72
14	Sulphur	3036	3286
15	Zinc	118	139

great amounts. The concentration of calcium and phosphorus has increased in treated rice flour from 1069 to 2170 mg/kg and from 4806 to 10165 mg/kg respectively. Magnesium is required for normal metabolism of calcium and phosphorus, it has also increased from 1017 to 1560 mg/kg in the treated rice flour. The increase in the mineral content can be attributed to the fact that the bound minerals with the starch molecules got released on its hydrolysis.

3.6. Effect of enzymatic treatment on functional properties of rice flour

Effect of enzymatic treatment on functional property of rice flour is shown in Table 7. In vitro protein digestibility was studied and found that the digestibility coefficient of treated rice flour was 1351.67 as compared to 644.63 of untreated rice flour. It may be attributed to the fact that due to enzymatic hydrolysis the protein in the flour got broken down to smaller peptides and was easily available.

Table 7: Functional Quality Analysis (In vitro Protein Digestibility)

Parameter	Untreated rice flour	Treated rice flour	CD (at 5%)
Digestibility Coefficient	644.63	1351.67	5.13249

P (<0.05)

3.7. Effect of enzymatic treatment on microbiological quality of rice flour

Microbiological quality of the treated and untreated rice flour has been evaluated and shown in Table 8. Microbiological limits are the criteria that indicate the microbiological conditions of the food concerned so as to reflect its safety and quality. These limits have been set by international and national authorities for a number of food commodities. The total plate count of the treated rice flour decreased significantly as compared to untreated rice flour from $85x10^3$ to $35.67x10^3$ cfu/gm which satisfy the standards given by Food and Drug Administration (FDA). Yeast and mould count also decreased in the similar manner from $12.33x10^2$ to $3.67x10^2$ cfu/gm which satisfies the standards given by FDA for cereal

flours. The contaminating micro-organisms such as coliforms and salmonella were not detected in both untreated and treated rice flour. The decrease in total plate count and yeast and mould count was may be due to the heat treatment given during the enzymatic treatment of rice flour.

Table 8: Microbiological Quality Analysis

S. No.	Parameter	Untreated rice flour (cfu/gm)	Treated rice flour (cfu/gm)	CD (at 5%)	Standards for cereal flours (cfu/gm)
1.	Total Plate Count (x 10^3)	85.00	35.67	8.06882	10^2 - 10^6
2.	Yeast and Mould Count (x 10^2)	12.33	3.67	4.13924	10^2 - 10^4
3.	Coliforms	Nil	Nil	---	Negative
4.	Salmonella	Nil	Nil	---	Negative

P (<0.05)

3.8. Toxic residue analysis of treated and untreated rice flour

Both the treated and untreated rice flour were subjected to toxic residue analysis. They were analyzed for the presence of aflatoxin content in them. Aflatoxin residues in both the flours were not detected.

4. Conclusion

The present study was conducted to produce nutritionally and functionally improved rice flour by application of microbial enzymes. Fungal enzymes amylase and glucoamylase were produced using a specific culture *Aspergilus oryzae*. Utilization of various agro-based residues for solid state fermentation by *Aspergillus oryzae* is an economical method of enzyme production. The optimized conditions for the production of enriched rice flour were slurry concentration (7% i.e. 7g/100ml), Enzyme concentration (1 ml), temperature (54.26 °C) and time (38.21 min).All the independent variables viz. slurry concentration, enzyme concentration, temperature and time were found to have statistically significant (P <0.05) effect on the production of enriched rice flour. The interactions between different variables were found to contribute to the response at a significant level. Enzymatically treated rice flour was compared with the raw untreated rice flour for various parameters such as physico-chemical properties, functional properties, toxic residue analysis and microbiological quality. Enzymatically treated rice flour was found to have more protein content and ash content as compared to untreated rice flour. Mineral content of enzymatically treated rice flour was enhanced as compared to untreated rice flour. In vitro protein digestibility increased in comparison to raw rice flour.Both the treated and untreated rice flour was devoid of any harmful toxins such as aflatoxin. Both the samples were found to be microbiologically safe for consumption.

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