J. Microbiol. Biotechnol. (2010), **20**(4), 737–748
doi: 10.4014/jmb.0903.03024
First published online 19 July 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Production of Glutaminase (E.C. 3.2.1.5) from *Zygosaccharomyces rouxii* in Solid-State Fermentation and Modeling the Growth of *Z. rouxii* Therein

Iyer, Padma* and Rekha S. Singhal

Food Engineering and Technology Department, Institute of Chemical Technology, University of Mumbai, Matunga, Mumbai-400 019, India

Received: March 25, 2009 / Revised: June 14, 2009 / Accepted: June 20, 2009

Glutaminase production in *Zygosaccharomyces rouxii* by solid-state fermentation (SSF) is detailed. Substrates screening showed best results with oatmeal (OM) and wheatbran (WB). Furthermore, a 1:1 combination of OM:WB gave 0.614 units/gds with artificial sea water as a moistening agent. Evaluation of additional carbon, nitrogen, amino acids, and minerals supplementation was done. A central composite design was employed to investigate the effects of four variables (*viz.*, moisture content, glucose, corn steep liquor, and glutamine) on production. A 4-fold increase in enzyme production was obtained. Studies were undertaken to analyze the time-course model, the microbial growth, and nutrient utilization during SSF. A logistic equation (R^2=0.8973), describing the growth model of *Z. rouxii,* was obtained with maximum values of μ_m and X_m at 0.326 h^{-1} and 7.35% of dry matter weight loss, respectively. A good-fit model to describe utilization of total carbohydrate (R^2=0.9906) and nitrogen concentration (R^2=0.9869) with time was obtained. The model was used successfully to predict enzyme production (R^2=0.7950).

Keywords: Glutaminase, solid-state fermentation, response surface methodology, *Zygosaccharomyces rouxii*, modeling, logistic model

Solid-state fermentation (SSF) is a fermentation process carried out on a solid medium with a low moisture content (A_w), typically 0.40–0.90, which occurs in a nonseptic and natural state [16]. SSF produces a high product concentration having a relatively low energy requirement and appears to be promising for the production of "low-volume and high cost" products such as biopharmaceuticals. Recent evidences indicate bacteria and fungi growing under SSF conditions to be capable of supplying the global demand for various metabolites [19]. The product titers produced in SSF are many-fold higher than that from submerged culture, although the reasons for this are not clear [18]. SSF also appears to possess several biotechnological advantages such as higher fermentation productivity, higher product stability, lower catabolic repression, cultivation of microorganisms specialized for water-insoluble substrates or mixed cultivation of various fungi, and lower demand on sterility owing to the low water activity used in SSF [7].

L-Glutamine amidohydrolase (E.C. 3.5.1.2), commonly referred to as L-glutaminase, has received much attention with respect to its therapeutic and industrial applications. It is a potent antileukemic drug and a flavor-enhancing additive in the production of fermented foods. Its commercial importance demands not only the search for new and better yielding microbial strains, but also economically viable bioprocesses for its large-scale production [21].

Statistical optimization not only allows quick screening of a large experimental domain, but also reflects the role of each of the components. Response surface methodology (RSM) provides important information regarding the optimum level of each variable along with its interactions with other variables and their effects on product yield [20]. The determination of an optimized level by standard univariate techniques is not always feasible for enzyme production. Multivariate experiments are designed to reduce the number of experiments necessary in the optimization process and to produce more precise results than those obtainable by univariate strategies [2]. This multivariate approach also improves statistical interpretation possibilities, and evaluates the relative significance of several factors even in the presence of complex interactions. Statistical design results in successful optimization even if the fundamental understanding of the mechanisms involved in the process under investigation is lacking.

**Corresponding author*
Phone: +91-22-24145616; Fax: +91-22-24145614;
E-mail: iyer_padma@rediffmail.com, rss24@udct.org

There is only one report on SSF from *Z. rouxii* [9] using wheat bran and sesamum oil cake as substrates. Glutaminase production by SSF from various organisms like *Rhizopus oligosporus* [6], *Vibrio costicola* [21], and *Beauveria* sp. [23] has also been reported. Reports on media optimization using any statistical tool for L-glutaminase production in SSF are also scant. We have reported earlier on the submerged fermentation of *Z. rouxii* for L-glutaminase production [8]. In the present study, we report on the production of extracellular L-glutaminase by halophilic yeast *Z. rouxii* under SSF using response surface methodology (RSM) on a substrate combination of oatmeal (OM) and wheat bran (WB), OMWB, which is as yet unreported. Screening of several substrates for optimal production of glutaminase using 100 g/l NaCl, 24 g/l NaCl, and artificial sea water (ASW) as the moistening medium has been discussed. Further, a study of the various supplements that can enhance production of glutaminase was done using one-factor-at-a-time approach, and finally a RSM was employed as a statistical approach to obtain the optimal medium for maximum glutaminase titers.

Modeling in a SSF system is another important aspect, which needs attention from researchers, as it plays an important role for optimum design and operation of bioreactors. Scaling up and optimizing the operation of a SSF bioreactor can be largely simplified if an accurate process model is available [12]. Not enough information is available on the kinetics of SSF systems. This is mainly because the heterogeneous nature of the structural and nutritional complex substrates causes difficulties in the measurements of growth parameters, analysis of cellular growth, and determination of substrate consumption [17]. To the best of our knowledge, there are no reports on modeling in SSF for *Z. rouxii*. Hence, an attempt has been made to model the SSF system for *Z. rouxii* using the RSM optimized medium for glutaminase production.

Materials and Methods

Media Components

Glucose, yeast extract, glutamine, glutamic acid, and other media components were procured from Hi-Media Limited, Mumbai, India. Buffer salts like Tris, dipotassium hydrogen phosphate, sodium chloride, hydrochloric acid, *etc*. were purchased from Merck (India) Limited, Mumbai, India. L-Glutamic dehydrogenase, nicotinamide adenine dinucleotide (NAD), and adenosine diphosphate (ADP) were purchased from Sigma-Aldrich Pvt. Ltd, Steinheim, Germany. UV transparent 96-well microplates were a gift sample from Corning, U.S.A. All the oil cakes were procured from the local market in Trivandrum, Kerala, India, whereas the agro-industrial residues were obtained from a local market in Mumbai, Maharashtra, India.

Microorganism and Culture Maintenance Conditions

Zygosaccharomyces rouxii NRRL-Y 2547, procured as a gift sample from Northern Regional Research Laboratories Type Culture Collection (NRRL), Peoria, U.S.A., was used in the study. The culture was maintained on GPYM agar medium slants containing (in g/l) glucose, 10; peptone, 5; yeast extract, 3; and malt extract, 3; supplemented with sodium chloride, 50 and agar, 20 (pH 7.0). Inoculated slants were grown in an incubator at 35°C for 2 days. The slants were kept at 4°C and subcultured every 20 days in the above-mentioned media. ASW used throughout the study contained (in g/l) NaCl, 24.7; KCl, 0.66; $MgCl_2 \cdot 6H_2O$, 4.7; $CaCl_2 \cdot 2H_2O$, 1.9; $MgSO_4 \cdot 7H_2O$, 6.3; and $NaHCO_3$, 0.18, with pH adjusted to 8.1 using 0.1 N NaOH filtered through #3 Whatman filter paper before use.

Inoculum Preparation

Inoculum was prepared in 250-ml Erlenmeyer flasks containing 50 ml of GPYM liquid media supplemented with 50 g/l NaCl (pH 7.0). The media so prepared was autoclaved at 121°C (15 lbs) for 20 min, and then inoculated with *Z. rouxii*. The inoculated flasks were kept on a shaker at 180 rpm for 48 h, and used as the inoculum. This inoculum (3.12×10^6 cells/ml) was used for all subsequent inoculations unless otherwise mentioned.

Fermentation

Five g of the substrate was taken separately into 250-ml Erlenmeyer flasks autoclaved at 121°C (15 lbs) for 20 min and cooled to room temperature (29±2°C) before inoculation. The sterilized solid substrate media were inoculated with 2 ml of the 48-h-old cell suspension of *Z. rouxii* under aseptic conditions and appropriate moistening medium. The contents of the inoculated flasks were mixed thoroughly and incubated at 30°C for 48 h.

Screening and Selection of Solid Substrates for SSF

A wide range of substrates were screened for glutaminase production, which included sesame oil cake (SOC), and traditional agro-industrial residues such as WB, rice bran (RB), green gram powder (GGP), OM, tea waste (TW), rice husk (RH), corn cob powder (CCP), cotton seed meal (CSM), orange peel powder (OPP), coconut waste (CW), soya flour (SF), wheat flour (WF), tamarind seed powder (TSP), and inert substrates like polyurethane foam (PF) and saw dust (SD). Further to study, the effect of mixed substrate fermentation OM (giving best response from initial screening) was mixed with above-mentioned substrates in 1:1% (w/w). A combination of OM and WB gave the best response. A study of different combinations of OM and WB (*viz*., 1:0, 1:0.25, 1:0.5,1:1, 0.5:1, 0.25:1, and 0:1) were studied, and 1:1 was found to be optimum (data not reported). Artificial sea water, 100 g/l NaCl, and 24 g/l NaCl were evaluated as moistening medium and were sterilized separately by autoclaving. The substrate OM:WB (1:1) combination supporting maximum production of glutaminase was selected for further study.

Optimization of Process Parameters

Optimization of various physicochemical parameters required for maximum glutaminase production by *Z. rouxii* under SSF was done. Various parameters studied included incubation temperature (25–50°C), initial moisture content of the substrate [37.5–75% (v/w)], and inoculum size (0.5 to 2.5 ml of 48-h-old culture containing 3.12×10^6 cells/ml). In order to determine the period of incubation required for optimum enzyme production, a time-course study was carried out under optimized conditions as above for a period of 60 h, with sampling at every 12 h for 60 h.

Effects of Supplements on Glutaminase Production: One-Factor-at-a-Time Approach

Further supplementations of solid substrate medium with additional carbon source at 10 g/l, additional organic nitrogen at 10 g/l, additional inorganic nitrogen sources at 0.05 M, and additional amino acids at 10 g/l were studied. These supplements were included in the moistening medium and added to the substrate after autoclaving. The procedure adopted for optimization of various parameters influencing glutaminase production was to evaluate the effect of independent parameters, keeping others constant, and to incorporate it at the optimized level in the next experiment while optimizing the next parameter. All the experiments were carried out in at least triplicates and the mean values taken. All the assays were carried out after 48 h of incubation.

Statistical Experiment Design

After identifying the variables affecting glutaminase production by "one factor-at-a-time" approach, the four most important variables, (*viz*., initial moisture content, glucose, corn steep liquor, and glutamine) were selected. The basal medium contained 0.05 g of ammonium sulfate. Inoculum was kept constant at 1 ml (3.12×10^6 cells/ml) and this moisture was included in the moisture content. RSM using face-centered central composite design (FCCCD) was adopted for improving glutaminase production using the software Design-Expert Version 6.0.10 (Stat-Ease Inc., Minneapolis, MN, U.S.A.) to find the interactive effects of four variables.

Production Profile of L-Glutaminase

The optimized medium was used to study the production profile of glutaminase for 60 h with samples being withdrawn every 4 h to determine glutaminase activity and protein content. All fermentation experiments were done in triplicate.

Enzyme Extraction

Crude enzyme was extracted employing a simple contact method using 0.1 M phosphate buffer (pH 8). After mixing the fermented substrate with buffer (41 ml), the flasks were kept on a rotary shaker at 150 rpm for 60 min. The slurry was centrifuged at 10,000 ×*g* for 10 min at 4°C in a refrigerated centrifuge. Supernatant was collected and used for enzyme assay.

Analytical Determinations

Total biomass estimation. Dry weight determination for dry substrate was determined by drying it at 80°C for 24 h. All enzyme activity results are given in terms of units/g dry fermented matter (units/gds). For modeling studies, the dry matter weight loss was calculated as follows [26]:

$$\text{Dry matter weight loss (\%)} = \frac{[(\text{Dry weight of initial solid substrate}) - (\text{Dry weight of fermented substrate})]}{[\text{Dry weight of initial solid substrate}]} \times 100\%$$

All measurements were carried out in triplicate.

Total carbohydrate estimation. One g of the dried solid substrate was hydrolyzed using 3 ml of 6 N HCl for 20 min and neutralized with 6 N NaOH. The hydrolysate was used for reducing sugar estimation by the DNSA method [4, 11].

Total nitrogen estimation. Total nitrogen was estimated by the Kjeldahl method in order to correlate the nitrogen utilization with respect to biomass generation and enzyme production [3].

Glutaminase Activity and Specific Activity

For estimation of glutaminase activity, a modification of the method of Moriguchi *et al*. [15] was used. In the first step of the assay, the glutaminase produced in the fermentation broth converts glutamine to glutamate. The glutamate so formed is converted in the second step of the assay to α-ketoglutarate by L-glutamic dehydrogenase, and in the process, NAD is reduced to NADH. This is measured as a change in absorbance at 340 nm. The final reaction mixture contained 184 mM potassium phosphate, 44 mM Tris-HCl, 0.18 mM EDTA buffer, pH 8.6, 20 mM L-glutamine, and crude enzyme extract (0.1 ml) in a final volume of 1.0 ml. After reacting for 35 min at 37°C, the reaction was terminated by adding 0.1 ml of 3 M HCl, and kept at 4°C for 5 min. Later, 130 mM Tris-HCl buffer (pH 9.4), 20 mM β-NAD, 0.5 mM ADP, and 25 U/ml of L-glutamic dehydrogenase were added in a total volume of 0.295 ml to 10 µl of reaction mixture from the earlier step. The reaction was conducted in UV transparent 96-well plates, which were read on a µQuant Elisa plate reader (Bio-Tek Instruments Inc., U.S.A.). The absorbance at 340 nm was measured before and after incubating the mixture for 1 h at 37°C. One unit of glutaminase activity was defined as the enzyme required for deamination of 1.0 µmole of glutamine per minute per milliliter of enzyme solution at pH 8.6, 37°C [1]. The protein concentration was determined by the Lowry method using bovine serum albumin as the standard [10].

RESULTS AND DISCUSSION

Screening of Substrates and Moistening Medium

As seen from Table 1, OM, WB, RB, OP, CSM, GGP, and SBM were found to be better substrates compared with the others used in this study. This could be due to the reducing sugar content of these substrates being higher, which was identified in a parallel study (not reported). This study helped in studying the effect of the substrates in all the three moistening media wherein it can be seen that the same substrate gave drastically different behavior with the three different moistening media. This could be due to interaction of the substrate itself with the salts, thereby producing a beneficial/detrimental effect on growth of the organism, and thereby on the production of glutaminase. Thus, for further studies, a 1:1 combination of OM and WB (OMWB) with ASW (0.61±0.04 units/gds) was selected. Kashyap *et al.* [9] have earlier reported better efficiency of NaCl medium for wheat bran and seawater for sesamum oil cake, with approximately 2.2 units/gds from WB and 2.17 units/gds from SOC. However the definition of enzyme units in their report was in terms of moles of ammonia released, whereas in the present report, we have defined enzyme units in terms of glutamine deamination.

A comparative study was undertaken wherein enzyme yield using OMWB as substrate was estimated using the method employed by Kashyap *et al*. [9] and an activity of 2.9±0.2 units/gds was obtained. Thus, we can infer that the present work gives an improved yield of glutaminase over the reported one by Kashyap *et al*. [9].

Table 1. Screening of solid substrates for L-glutaminase production.

Substrate	Yield (units/gds)[a] in medium		
	100 g/l NaCl	24 g/l NaCl	ASW
Oat meal (OM)	0.47±0.006	0.48±0.006	0.58±0.009
Wheat bran (WB)	0.46±0.009	0.37±0.007	0.38±0.005
Tea waste (TW)	0.40±0.008	0.32±0.006	0.37±0.006
Sesame oil cake (SOC)	0.23±0.004	0.20±0.004	0.18±0.004
Rice husk (RH)	0.04±0.001	0.09±0.001	0.11±0.002
Corn cob powder (CCP)	0.16±0.003	0.19±0.004	0.24±0.004
Orange peel powder (OP)	0.45±0.009	0.36±0.007	0.37±0.007
Rice bran (RB)	0.36±0.007	0.36±0.007	0.35±0.007
Cotton seed meal (CSM)	0.40±0.007	0.31±0.003	0.35±0.007
Tamarind seed powder (TSP)	0.35±0.007	0.39±0.008	0.41±0.008
Green gram powder (GGP)	0.46±0.009	0.40±0.007	0.47±0.009
Saw dust (SD)	0.13±0.002	0.12±0.002	0.12±0.002
Polyurethane foam (PF)	0.39±0.007	0.28±0.006	0.29±0.006
Coconut waste (CW)	0.14±0.002	0.13±0.002	0.13±0.002
Soya bean meal (SBM)	0.44±0.007	0.42±0.008	0.56±0.009
Soya flour (SF)	0.40±0.006	0.35±0.006	0.36±0.007
Wheat flour (WF)	0.28±0.006	0.41±0.006	0.47±0.01
OM+WB	0.53±0.010	0.47±0.009	0.61±0.012
OM+OP	0.41±0.004	0.41±0.006	0.42±0.008
OM+CSM	0.40±0.008	0.43±0.006	0.43±0.007
OM+GGP	0.43±0.008	0.42±0.005	0.37±0.004
OM+SBM	0.39±0.005	0.38±0.007	0.43±0.006

[a]Results are mean±SD of three determinations.

Optimization of Screened Variables and Their Interaction Analysis

Initial optimization of fermentation parameters yielded results as shown in Table 2. Further one-factor-at-a-time approach was employed for screening of the best supplemental nutrients that can be added to achieve higher glutaminase yields. Figs. 1A–1D show the results obtained from the one-factor-at-a-time approach.

One-factor-at-a-time study showed the initial moisture content to have a profound influence on the glutaminase activity, with optimum moisture for the OMWB system being about 65.52%. Moisture content is known to be the most studied parameter in solid-state fermentation, because the growth and metabolism of microorganisms in SSF mostly occur in the liquid phase. The transfer of solute as well as dissolved gas occurs in an aqueous film surrounding the microorganism. In SSF, microorganisms are in the liquid film on the solid surface Thus replacement of the liquid phase by a gaseous phase increases the oxygen transfer, but prevents solute diffusion in most substrates. Free water indicates a saturated substrate. The moisture content at which free water is apparent depends on the nature of the solid. Optimal moisture content depends on the microorganism and the substrate. Lower moisture levels reduce the solubility of the nutrients in the solid substrate, lower the degree of substrate swelling, and increase the water tension. Similarly, higher moisture is reported to decrease porosity, alter the particle structure, develop stickiness, and decrease gaseous exchange [5, 17].

Carbon source represents the energy source that will be available for growth of the microorganism. In SSF, generally the solid substrate itself acts as a source of carbon. However, in some cases, addition of a small amount of external carbon may lead to an increase in metabolite production. In this work, we found glucose supplementation alone to give a glutaminase production of 1.30±0.10 units/gds.

Ammonium sulfate supplementation gave a marginal increase in glutaminase production, and hence was included in the basal supplementation medium. Corn steep liquor increased it to 1.53±0.07 units/gds. The earlier report of Kashyap *et al*. [9] for SSF on wheat bran and sesamum oil cake on *Z. rouxii* had shown no beneficial effect of nitrogen supplementation on glutaminase titers. In the case of *Beauveria* sp., grown on polystyrene beads, malt extract (10 g/l) gave maximal enzyme production. Further organic nitrogen sources were observed to have a more pronounced effect on glutaminase production compared with inorganic nitrogen sources. The presence of additional nitrogen sources may have promoted enhanced growth and consequently enzyme production [23].

L-Glutamine enhanced L-glutaminase synthesis marginally to 1.57±0.10 units/gds. Similar results were obtained in the case of *Beauveria* sp., wherein glutamine at 0.25% (w/v) showed a substantial increase in glutaminase titers [23]. L-

Table 2. Physical fermentation parameters for glutaminase production from *Z. rouxii* using the OMWB system.

Parameter	Range	Other conditions	Units/gds
Temperature (°C)	15 to 45	Inoculum age: 48 h Innoculum size: 1 ml Initial moisture: 58.5% pH: 8.1	Optimum: 30°C 0.72 U/gds
Innoculum size ($3–3.15\times10^6$ cells/ml)	0.25 to 2.0 ml	Inoculum age: 48 h Temperature: 30°C Initial moisture: 58.5% pH: 8.1	Optimum: 0.5 ml 0.79 U/gds
Innoculum age (h)	24–60	Temperature: 30°C Innoculum size: 0.5 ml Initial moisture: 58.5% pH: 8.1	Optimum: 48 h culture 0.77 U/gds
Initial moisture (% v/w)	35 to 75	Inoculum age: 48 h Innoculum size: 0.5 ml Temperature: 30°C pH: 8.1	Optimum: 65.51% (w/w) 0.81 U/gds
pH optimum	3–10	Inoculum age: 48 h Innoculum size: 0.5 ml Initial moisture: 58.5% Temperature: 30°C	Optimum: pH 8 0.80 U/gds

Glutaminase production occurred even in the absence of L-glutamine, suggesting that glutaminase in *Z. rouxii* was a non-inducible enzyme.

These selected components were further studied at different concentrations to determine the optimum range. Thus, from the one-factor-at-a-time approach, a 0.5-ml

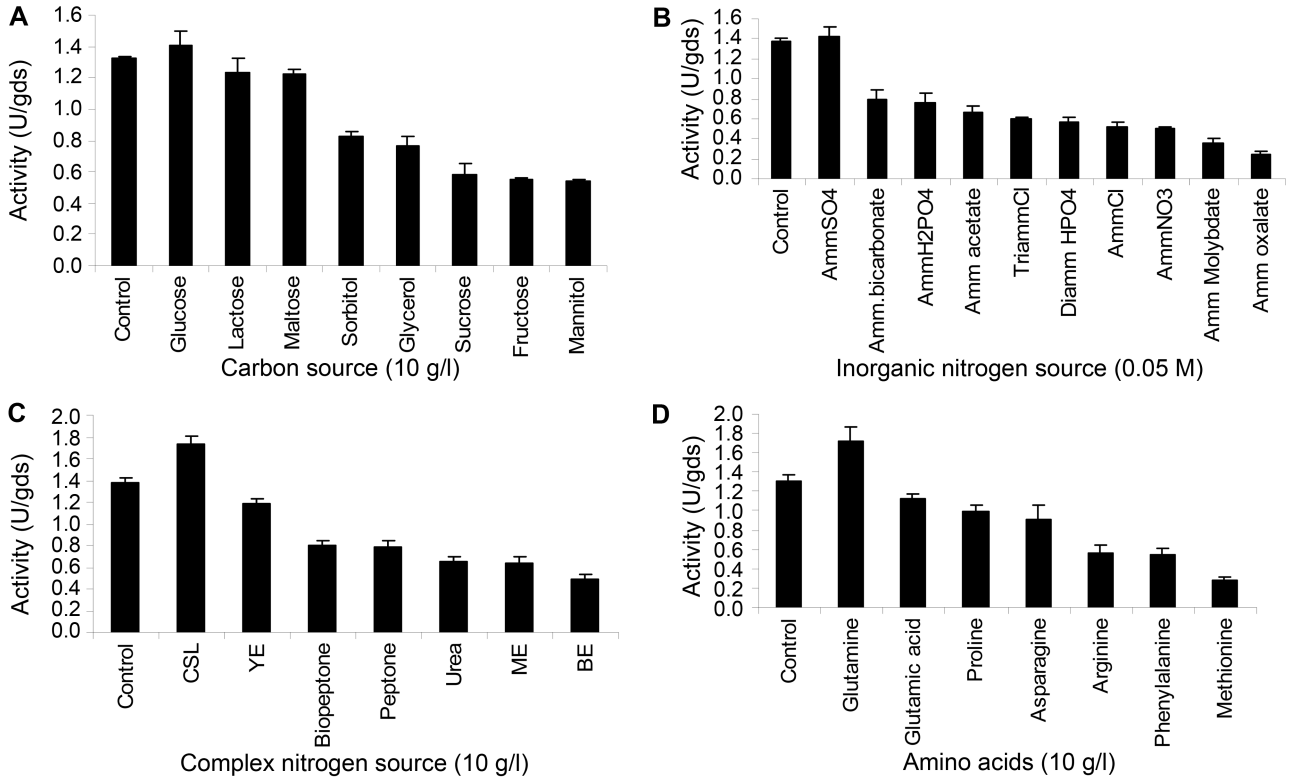

Fig. 1. Effect of addition of external (**A**) carbon, (**B**) inorganic nitrogen, (**C**) complex nitrogen, and (**D**) amino acids on glutaminase production by Z. rouxii in an OMWB system.

Table 3. Experimental range and levels of the independent variables used in RSM in terms of actual and coded factors employed in the FCCCD.

Media component	Range of levels					
	Actual (for 5 g substrate)	Coded	Actual (for 5 g substrate)	Coded	Actual (for 5 g substrate)	Coded
Initial moisture content (%)	62.96	−1	65.54	0	67.74	1
Glucose (g)	0.1	−1	0.2	0	0.3	1
Corn steep liquor (g)	0.05	−1	0.15	0	0.25	1
Glutamine (g)	0.03	−1	0.08	0	0.13	1

inoculum (3.12×10^6 cells/ml) in OMWB media with supplements containing (g) glucose 0.2, corn steep liquor 0.15, ammonium sulfate 0.05, glutamine 0.02, and moisture content of 65.52% led to a glutaminase production of 2.36±0.06 units/gds after 48 h at 30°C, compared with the initial production of 0.752±0.008 units/gds in media without additional supplements.

Optimum levels of these significant factors and the effect of their interactions on glutaminase production were determined by the face-centered central composite design (FCCCD) of RSM. The values obtained from the "one-factor-at-a-time" approach were employed in the RSM FCCCD design as center points. Table 3 gives the details of the actual and coded values employed in the FCCCD.

Table 4. Results of FCCCD using four independent variables and six center points showing observed and predicted response.

Std. order	Moisture (%)	Glucose[b]	Corn steep liquor[b]	Glutamine[b]	Enzyme activity (units/gds)	
					Predicted	Observed[a]
1	62.96	0.1	0.05	0.03	2.62	2.62±0.09
2	67.74	0.1	0.05	0.03	2.58	2.58±0.08
3	62.96	0.3	0.05	0.03	2.71	2.71±0.11
4	67.74	0.3	0.05	0.03	2.52	2.52±0.08
5	62.96	0.1	0.25	0.03	2.4	2.4±0.10
6	67.74	0.1	0.25	0.03	2.45	2.45±0.10
7	62.96	0.3	0.25	0.03	2.45	2.44±0.08
8	67.74	0.3	0.25	0.03	2.35	2.35±0.07
9	62.96	0.1	0.05	0.13	2.38	2.38±0.11
10	67.74	0.1	0.05	0.13	2.34	2.34±0.09
11	62.96	0.3	0.05	0.13	2.44	2.43±0.09
12	67.74	0.3	0.05	0.13	2.25	2.25±0.10
13	62.96	0.1	0.25	0.13	2.33	2.33±0.02
14	67.74	0.1	0.25	0.13	2.37	2.37±0.10
15	62.96	0.3	0.25	0.13	2.34	2.34±0.02
16	67.74	0.3	0.25	0.13	2.25	2.24±0.10
17	62.96	0.2	0.15	0.08	2.57	2.6±0.05
18	67.74	0.2	0.15	0.08	2.5	2.51±0.06
19	65.52	0.1	0.15	0.08	2.51	2.51±0.06
20	65.52	0.3	0.15	0.08	2.49	2.52±0.04
21	65.52	0.2	0.05	0.08	2.32	2.33±0.08
22	65.52	0.2	0.25	0.08	2.21	2.24±0.02
23	65.52	0.2	0.15	0.03	2.5	2.52±0.07
24	65.52	0.2	0.15	0.13	2.33	2.35±0.07
25	65.52	0.2	0.15	0.08	2.43	2.42±0.09
26	65.52	0.2	0.15	0.08	2.43	2.43±0.07
27	65.52	0.2	0.15	0.08	2.43	2.39±0.11
28	65.52	0.2	0.15	0.08	2.43	2.41±0.10
29	65.52	0.2	0.15	0.08	2.43	2.41±0.09
30	65.52	0.2	0.15	0.08	2.43	2.42±0.09

[a]Results are mean±SD of three determinations.
[b]Per 5 g of substrate.

Table 5. Model coefficients estimated by multiple linear regression and significance of regression coefficient for glutaminase yield and specific activity.

Factor	Coefficient estimate	Standard error	Prob>F
Intercept	2.431	0.007	
A	−0.036	0.005	<0.0001
B	−0.010	0.005	0.061
C	−0.056	0.005	<0.0001
D	−0.087	0.005	<0.0001
A2	0.106	0.013	<0.0001
B2	0.066	0.013	0.000
C2	−0.164	0.013	<0.0001
D2	−0.014	0.013	0.289
AB	−0.036	0.005	<0.0001
AC	0.022	0.005	0.001
AD	−0.001	0.005	0.907
BC	−0.011	0.005	0.061
BD	−0.008	0.005	0.142
CD	0.042	0.005	<0.0001

The results obtained by FCCCD were analyzed by standard analysis of variance (ANOVA), and the mean predicted and observed responses are presented in Table 4. The second-order regression equation provided the levels of glutaminase production as a function of initial values of moisture, glucose, glutamine, and corn steep liquor, which can be predicted by the following equation:

$$\text{Activity } Y_1 = 2.43 - 0.036\times A - 0.010\times B - 0.056\times C - 0.087\times D + 0.11\times A2 + 0.066\times B2 - 0.16\times C2 - 0.014\times D2 - 0.036\times AB + 0.022\times AC - 6.250E{-}004\times AD - 0.011\times BC - 8.125E{-}003\times BD + 0.042\times CD$$

A: Moisture; B: Glucose; C: Corn steep liquor; D: glutamine

Table 6. ANOVA for the experiments.

Term	Response Y_1 Glutaminase activity (units/gds)
F-value	58.54
P>F	<0.0001
Mean	2.43
R^2	0.98
Adjusted R^2	0.97
Coefficient of variance	0.86
Adequate precision	33.61

According to the present model, A, C, D, A2, B2, C2, AB, AC, and CD were significant model terms (Table 5). Table 6 gives the ANOVA values for enzyme activity. ANOVA for glutaminase production (activity Y_1 units/gds) indicated the F value to be 58.54, which implied the model to be significant. Model terms having values of Prob>F less than 0.05 are considered significant, whereas those greater than 0.10 are insignificant. The Lack-of-Fit F-value of 3.03 implies the lack of fit is not significant relative to the pure error and that the model fits.

ANOVA indicated the R^2 value of 0.9820 for response Y_1. This again ensured a satisfactory adjustment of the quadratic model to the experimental data, and indicated that the model could explain 90–95% of the variability in the response. The adequate precision, which measures the signal to noise ratio, was 33.60 (Table 6), which indicates an adequate signal. A ratio of >4 is desirable. This model can be used to navigate the design space. The predicted R^2 of 0.9486 is in reasonable agreement with the adjusted R^2 of 0.9652. A good correlation between observed and predicted results reflected the accuracy and applicability of the central composite design for process optimization.

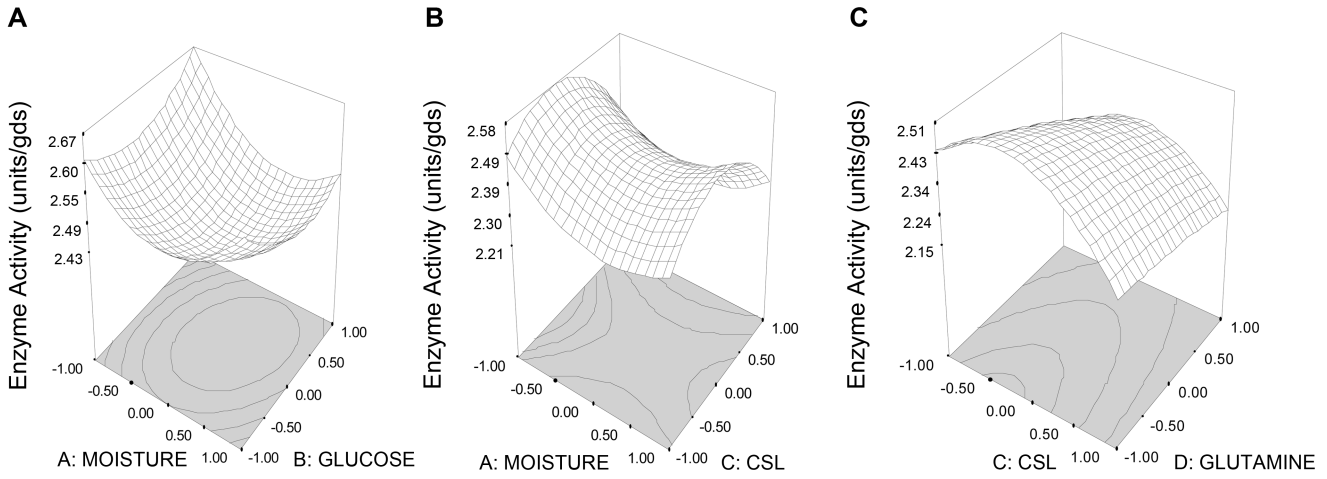

Fig. 2. 3D response surface.
Interactive effects of (**A**) varied initial moisture and glucose at 0.08 g glutamine and 0.15 g CSL, (**B**) varied initial moisture and CSL at 0.2 g glucose and 0.08 g glutamine, and (**C**) varied CSL and glutamine at 0.2 g glucose and 65.54% initial moisture, for 5 g of OMWB substrate, keeping ammonium sulfate constant at 0.05 g throughout the RSM.

Glutaminase yield for different levels of variables was predicted from the respective contour plots (Figs. 2A–2C). Each contour curve represents an infinite number of combinations of two test variables with the other two maintained at their respective zero levels. The elliptical nature of the contour in the 3D-response surface graphs (Figs. 2A–2C) depicted the mutual interactions of all the variables. There was a relatively significant interaction between every two variables, and there was a maximum predicted yield as indicated by the surface confined in the smallest ellipse in the contour diagrams.

Maximum glutaminase production was 2.4±0.03 units/gds when all the variables were kept at their central values. The model was used for optimization by point optimization. The model predicted a maximum glutaminase production of 2.77 units/gds in a medium containing (g) glucose 0.3, corn steep liquor 0.11, and glutamine 0.03, with an initial moisture content of 62.96%. Thus, an overall 1.13-fold increase in glutaminase production was being predicted after validation of RSM. A production of 2.93±0.089 units/gds at the end of 48 h was experimentally obtained using the predicted media. Glutaminase production in the unsupplemented OMWB media was 0.752±0.008 units/gds, which increased to 2.4±0.03 units/gds after supplementation of media using components selected by one-factor-at-a-time approach.

Production Profile of Glutaminase

In order to confirm the optimization results, the production profile of the culture was studied using predicted media composition and culture conditions. Maximum glutaminase production was observed during the exponential and stationary phases, where it attained a peak of 2.92±0.066 units/gds after 48 h incubation (Fig. 3).

Modeling of the Solid-State Fermentation System

Various kinetic profiles have been reported in SSF systems, including linear, exponential, logistic, and fast-acceleration/slow-deceleration. Mathematical models of SSF bioreactors most commonly use the logistic equation, including several of the most recently proposed models [12, 14, 24]. This is done on the basis of mathematical simplicity, because often the logistic equation in a single equation can give an adequate approximation of the whole growth curve including the lag phase and the cessation of growth in the latter stages of the fermentation.

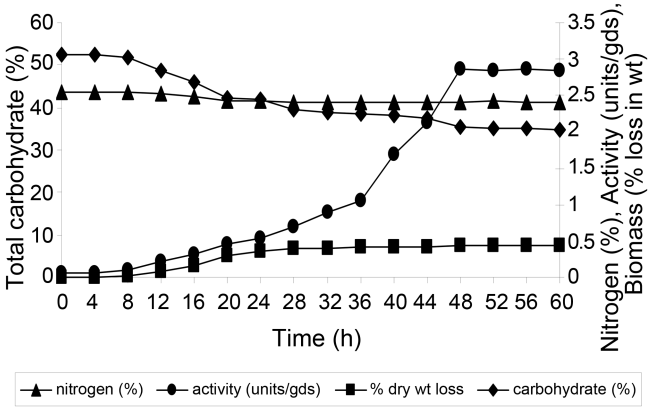

Fig. 3. Time course of SSF on OMWB for *Z. rouxii*, using an RSM optimized medium.

Direct determination of biomass in a solid medium is very difficult because it is impossible to separate the organism from the substrate. Terebiznik and Pilosof [25] found that dry matter weight loss was highly correlated with the biomass, and the biomass in solid-state fermentation system can be estimated by determining the dry matter weight loss. Hence, for the modeling studies, dry matter weight loss was determined.

In the present case, it was attempted to model nutrient utilization, biomass generation, and enzyme production for *Z. rouxii* in SSF using the logistic equation. Table 7 gives the observed and predicted values of the fermentation for *Z. rouxii* in the RSM optimized supplemented OMWB medium

Since data on the growth kinetics and utilization of nutrients for *Zygosaccharomyces rouxii* were not available, we did preliminary experiments to determine the production of biomass, enzyme production, and utilization of carbon and nitrogen versus time using the RSM optimized medium supplemented with the nutrients identified in the study. Table 7 and Fig. 3 indicate that growth initiation started only after 8 h. Log phase continued from 8 h to 48 h, after which there was a lag phase up to 60 h. Enzyme production was visible from 12 h and continued exponentially throughout the log phase and slowed down after 48 h. Carbohydrates, an important nutritional factor and indicator of biomass generation, decreased up to 42 h, after which there was a slight decrease in consumption. With respect to nitrogen utilization, there was a significant relative utilization of nitrogen during the log phase (and the production phase). However, it was not completely consumed, indicating that there was no nitrogen or carbon limitation to growth.

Modeling of Biomass Generation During SSF

Fig. 4 gives a correlation between biomass generation and time (log phase 8–48 h). A straight line with a linear fit equation (y=0.2045 x) and an R^2 value of 0.8973 was obtained, suggesting that the logistic equation can be used for the current data. Mitchell *et al*. [13] proposed an empirical model for *Rhizopus oligosporus*. Based on similar results, the growth pattern for *Z. rouxii* can be best described by Eq. (1)

$$\frac{dX}{dt}=\mu X\left(1-\frac{X}{X_m}\right) \quad (1)$$

Integrating Eq. (1), we get Eq. (2) that describes the growth pattern of *Z. rouxii*.

Table 7. An overview of the enzyme production, growth, and nutrient utilization patterns against time for SSF of *Z. rouxii*.

Time (h)	Activity (units/gds)	Biomass (% loss wt)		Carbohydrate (%)		Nitrogen (%)		Enzyme yield (U/gds)	
		Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
0	0.07	0.03±0.001	0.03	52.41±2.61	52.41	2.55±0.12	2.55	0.00±0.01	0.00
4	0.07	0.06±0.003	0.09	52.37±2.55	52.25	2.55±0.13	2.55	0.00±0.004	0.00
8	0.11	0.32±0.02	0.34	51.91±2.56	51.67	2.55±0.13	2.54	0.077±0.004	0.00
12	0.23	1.29±0.05	1.10	48.66±2.33	49.83	2.52±0.12	2.53	0.12±0.01	0.01
16	0.31	2.70±0.13	2.89	45.89±2.19	45.53	2.49±0.12	2.50	0.26±0.01	0.03
20	0.45	4.96±0.24	5.18	42.12±2.10	40.04	2.43±0.12	2.45	0.35±0.02	0.12
24	0.53	6.12±0.28	6.60	40.8±2.04	36.63	2.42±0.12	2.43	0.47±0.02	0.39
28	0.69	6.81±0.34	7.13	39.48±1.96	35.36	2.41±0.12	2.42	0.79±0.04	1.02
32	0.89	6.98±0.34	7.29	39.01±1.95	34.98	2.4±0.12	2.41	1.23±0.06	1.83
36	1.06	7.03±0.35	7.33	38.66±1.92	34.87	2.4±0.12	2.41	1.78±0.08	2.33
40	1.69	7.13±0.35	7.35	38.32±1.87	34.84	2.41±0.12	2.41	2.23±0.11	2.51
44	2.13	7.28±0.33	7.35	37.45±1.83	34.84	2.41±0.11	2.41	2.47±0.12	2.57
48	2.86	7.35±0.36	7.35	35.6±1.72	34.83	2.41±0.12	2.41	2.51±0.13	2.58
52	2.85	7.35±0.35	7.35	35.1±1.69	34.83	2.42±0.12	2.41	2.56±0.13	2.59
56	2.86	7.35±0.36	7.35	35.1±1.73	34.83	2.41±0.12	2.41	2.58±0.11	2.59
60	2.84	7.36±0.33	7.35	34.8±1.74	34.83	2.41±0.12	2.41	2.59±0.11	2.59

Predicted values indicate the values obtained from the model.

$$X_{(t)}=\frac{X_m}{(1+((X_m/X_0)-1)\exp(-\mu_m t))} \quad (2)$$

where, X is the microbial biomass, X_0 is the initial biomass, and X_m is the maximum possible microbial biomass, t is time, and μ is the specific growth rate constant.

When our data were put in this model, we found that there was a good correlation with the model obtained as follows:

$$X(t)=7.35/(1+283.99\ \exp(-0.326t))$$

where X(t) is the predicted value of dry matter weight loss at a given incubation time t(h). A good correlation between the calculated and measured biomass versus time (Fig. 4) indicated that the said model could be used to model the growth of *Z. rouxii*.

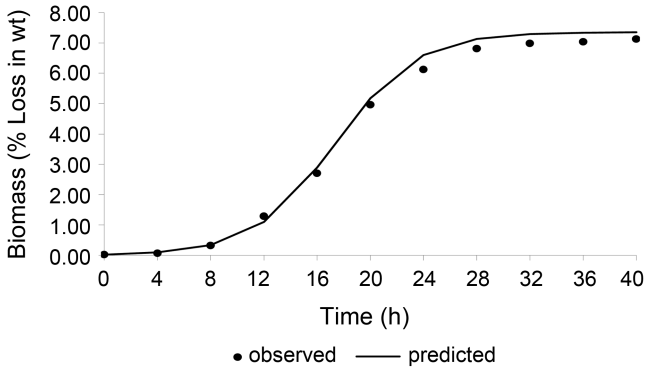

Fig. 4. Plot of measured and predicted values of dry matter weight loss.
A linear fit of y=0.7394x and R^2=0.8557 obtained for predicted values is in good agreement to the linear fit of y=0.7083x and R^2=0.8671 obtained for observed values. *Note*: Predicted values calculated from the equation expressed as follows: X(t)=7.35/(1+283.99 exp(−0.326t)), where X(t) is the predicted value of dry matter weight loss at a given incubation time t(h).

Modeling of Total Carbohydrate Concentration During SSF

From Fig. 5a, it can be seen that the carbohydrate content is inversely proportional to biomass generation. The carbohydrate consumption started just before the log phase and continued well beyond the log phase until 36 h, after which there was little, if any, change in carbohydrate content. The decrease in carbohydrate can be explained by the dry matter loss, which indicates a conversion of organic carbon to carbon dioxide released during the normal metabolism of the organism. However, as there was residual carbohydrate even at the end of the 48 h, we can infer that there was no carbon limitation to growth. Further carbohydrate utilization was positively correlated with biomass generation. Fig. 5b gives the relation between

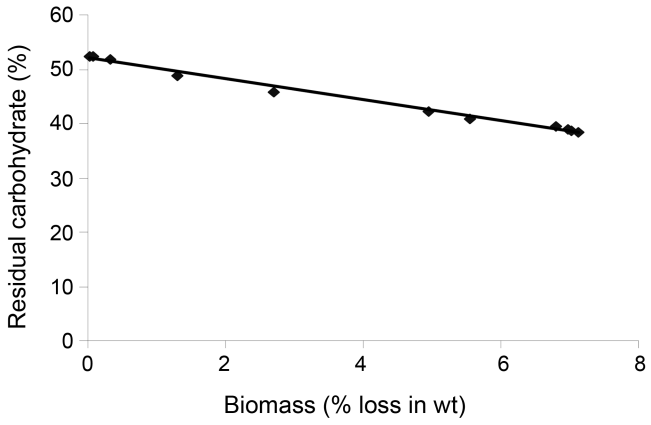

Fig. 5a. Correlation between biomass generation and residual carbohydrate for the incubation period 0–40 h.
A linear fit of y=−1.9204x+51.997 and R^2=0.9903 was obtained.

residual carbohydrate content and biomass generation; a negatively correlated linear equation (y=−1.9204x+51.997) was obtained with R^2=0.9903, suggesting that the carbohydrates supplied were utilized for biomass generation.

Generally, Eq. (3) given below can be used to model carbohydrate content in SSF

$$-\frac{dS}{dt}=\alpha\frac{dX}{dt} \quad (3)$$

where S (%) denotes total carbohydrate content in solid substrate, X (%) is the dry matter weight loss, and α represents the constant of correlation of maximum of total carbohydrate concentration and dry matter weight loss.

Equation (3) can be rewritten as follows by combining it with Eq. (1) and integrating.

$$S_{(t)}=S_0-\alpha(X_{(t)}-X_0) \quad (4)$$

where, S(t) (%) is the total carbohydrate concentration at t (h), corresponding to t=0, S=S_0, and X=X_0.

Equation (5) given below is the model of total carbohydrate concentration obtained by combining Eqs. (1) and (4). In this experiment, based on measured data, Eq. (5) (R^2=0.9241) describes the utilization pattern of total carbohydrate concentration, confirmed by comparing the measured values with predicted ones at different incubation times (Fig. 5a).

$$S(t)=52.41-2.4[(7.35/(1+283.99\ \exp(-0.326t))-0.026] \quad (5)$$

This equation can be used to predict carbohydrate utilization for the study.

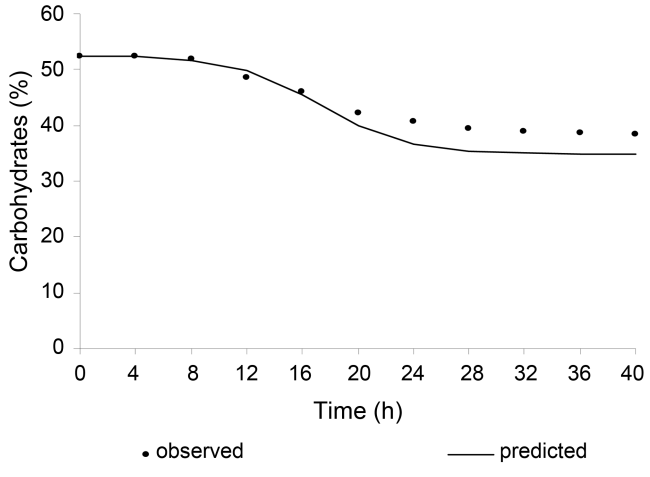

Fig. 5b. Plot of measured and predicted values of total carbohydrate content.
A linear fit of y=−2.2295x+55.96 and R^2=0.9038 obtained for predicted values is in good agreement to the linear fit of y=−1.704x+54.736 and R^2=0.9241 obtained for observed values. *Note*: Predicted values calculated from the equation expressed as follows: S(t)=52.41−2.4 [(7.35/(1+283.99 exp (−0.326t)) 0.026], where S(t) is the predicted value of residual carbohydrate at a given incubation time t(h).

Modeling of Total Nitrogen Concentration During SSF

The nitrogen consumption after the initiation of log phase continued up to 40 h, after which there was no significant change, indicating that nitrogen was used for biomass generation as well as for product generation. It is a well-known fact that glutaminase is a nitrogen regulated product like most enzymes in the nitrogen and urea metabolic cycles, and nitrogen may be utilized for biomass generation and product generation. However, total nitrogen in this case showed a slight decrease, indicating a possible loss of nitrogen as metabolic ammonia released. This observation is corroborated by the dry matter weight loss data indicating biomass generation during the same period. Fig. 6a gives the relation between residual nitrogen content and biomass generation and a negatively correlated linear equation, y=−0.0214x+2.5497, was obtained with an R^2 of 0.9869

Generally, Eq. (6) given below is derived in the same manner as for carbohydrate utilization, and the final equation can be used to model nitrogen utilization.

$$S(t)=2.55-0.019[(7.35/(1+283.99\ \exp(-0.326t))-0.026] \quad (6)$$

This equation was used to predict nitrogen utilization in the study.

The experimentally obtained values from Eq. (6) were used to describe the nitrogen utilization pattern, and Fig. 6b gives a comparison of the measured and predicted values of nitrogen content at different times.

Modeling of Glutaminase Production During SSF

It was seen on observing experimental data that glutaminase production follows the growth pattern with a small initial lag of 12 h (the time probably required for assimilation of nutrients, generation of the enzyme, and its excretion). When this is taken into consideration, the enzyme curve is

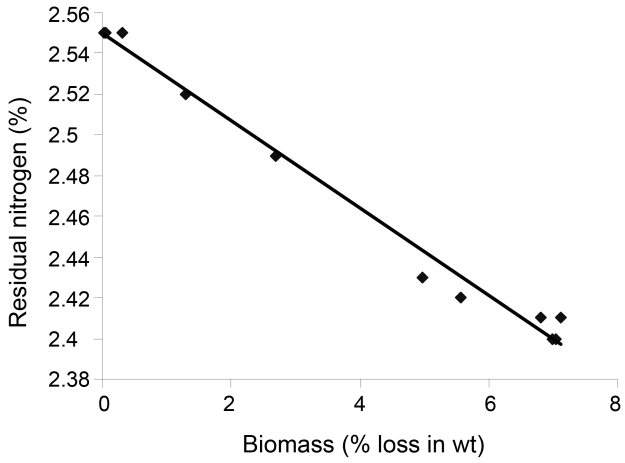

Fig. 6a. Correlation between biomass generation and residual nitrogen for the incubation period 0−40 h.
A linear fit of y=−0.0214x+2.5497 and R^2=0.9869 was obtained.

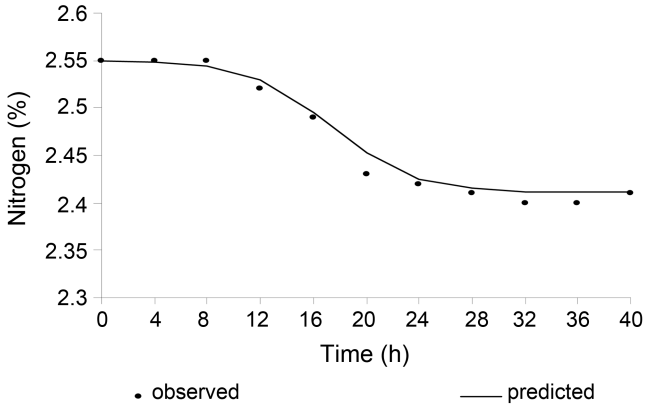

Fig. 6b. Plot of measured and predicted values of total nitrogen content.
A linear fit of y=−0.0177x+2.5781 and R^2=0.9038 obtained for predicted values is in good agreement to the linear fit of y=−0.0185x+2.5776 and R^2=0.8787 obtained for observed values. *Note*: Predicted values calculated from the equation expressed as follows: S(t)=2.55−0.019 [(7.35/(1+283.99 exp(−0.326t))−0.026], where S(t) is the predicted value of residual nitrogen at a given incubation time t(h).

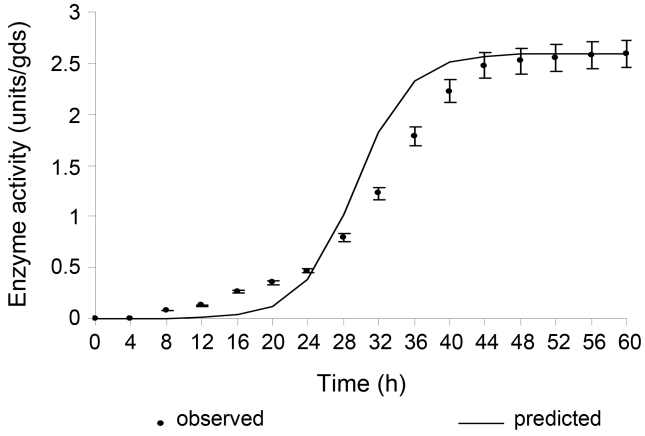

Fig. 7. Plot of measured and predicted values of enzyme titers.
A linear fit of y=0.1738x and R^2=0.795 obtained for predicted values is in good agreement to the linear fit of y=0.162x and R^2=0.8287 obtained for observed values. *Note*: Predicted values calculated from the equation expressed as follows: e[t]=2.59/(1+283.99exp(−0.326 (t-τ_e))), where e[t] is the predicted value of enzyme production at a given incubation time t(h) and a τ_e of 12 h considered from experimental values.

shifted in time compared with the biomass curve. The enzyme concentration, [e], at any time can thus be given as

$$[e(t)]=Y_{e/x}x(t-\tau_e) \quad (7)$$

where $Y_{e/x}$ is the mean yield coefficient for enzyme on total biomass and τ_e is the time delay between the enzyme and biomass production curves. It follows that biomass production correlates directly with enzyme production, and mean yield coefficient of enzyme production $Y_{e/x}$ will be given as

$$Y_{e/x}=\frac{[e_m]}{x_m} \quad (8)$$

where $[e_m]$ is the total enzyme produced.

Further combining Eqs. (2) and (8) we get

$$[e(t)]=\frac{[e_m]}{1+((x_m/x_0)-1)e-\mu_m(t-\tau_e)} \quad (9)$$

When this equation was used for modeling the enzyme production (Fig. 7), it was found that enzyme production was overpredicted by the model in the exponential phase. This deviation can be explained by the sudden increase in biomass at about 24 h, which increases the carbohydrate and nitrogen consumption but does not give a corresponding increase in enzyme production as nutritional requirements are directed towards biomass generation. Further studies on this aspect are in progress.

Overall, the modeling has contributed towards a better understanding of the physiological behaviour of *Z. rouxii* as a glutaminase producer in solid-state fermentation. These results have shown that *Z. rouxii* growth in SSF can be modeled using the widely used logistic model. Using this model, the growth of *Z. rouxii* and substrate utilization can also be predicted for other solid-state fermentations, as *Z. rouxii* is used widely in production of glycerol, polyhydric alcohols, and ethanol-based products.

Thus, in the present study, SSF for production of glutaminase from *Z. rouxii* has been detailed. Statistical optimization of media composition and culture conditions substantially increased glutaminase titers. *Zygosaccharomyces rouxii* (NRRL Y 2547) could be a promising source for the production of glutaminases. Moreover, the growth model of *Z. rouxii* could be described by a fairly good-fit logistic equation. A curvilinear regression model was obtained to show the kinetic pattern of total carbohydrate and total nitrogen concentrations. Furthermore, the model gives a reasonable prediction for glutaminase production at the end of fermentation.

Acknowledgments

We are indebted to Northern Regional Research Laboratories (NRRL) Type Culture Collection, U.S.A. for the supply of strains. The authors gratefully acknowledge the Department of Biotechnology (DBT), India, for financial support to the first author.

References

1. Curthoys, N. P. and R. F. Weiss. 1974. Regulation of renal ammoniagenesis – subcellular localization of rat kidney glutaminase isoenzymes. *J. Biol. Chem*. **249:** 3261.
2. Dahiya, N., R. Tewari, R. P. Tiwari, and G. S. Hoondal. 2005. Chitinase production in solid-state fermentation by *Enterobacter*

sp. NRG4 using statistical experimental design *Curr. Microb.* **51:** 222.
3. Erkan, H., S. Celik, B. Bilgi, and H. Koksel. 2006. A new approach for the utilization of barley in food products: Barley tarhana. *Food Chem.* **97:** 12.
4. Ghose, T. K. 1987. Measurement of cellulose activities. *Pure Appl. Chem*. **59:** 257.
5. Han, B. and R. J. Nout. 2000. Effects of temperature, water activity and gas atmosphere on mycelial growth of tempe fungi *Rhizopus microsporus* var. *microsporus* and *R. microsporus* var. *oligosporus. World J. Microb. Biotech*. **16:** 853.
6. Han, B., M. Yong, M. F. Rombouts, and M. J. Nout. 2003. Effects of temperature and relative humidity on growth and enzyme production by *Actinomucor elegans* and *Rhizopus oligosporus* during sufu pehtze preparation. *Food Chem.* **81:** 27.
7. Holker, U., M. Hofer, and J. Lenz. 2004. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Appl. Microbiol. Biotechnol.* **64:** 175.
8. Iyer, P. and R. S. Singhal. 2008. Production of glutaminase (E.C.3.2.1.5) from *Zygosaccharomyces rouxii*: Statistical optimization using response surface methodology. *Bioresour. Technol.* **99:** 4300.
9. Kashyap, P., A. Sabu, A. Pandey, G. Szakacs, and C. R. Soccol. 2002. Extracellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation. *Process Biochem*. **38:** 307.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193:** 265.
11. Miller, G. L. 1972. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*. **31:** 426.
12. Mitchell, D. A. and O. F. von Meien. 2000. Mathematical modeling as a tool to investigate the design and operation of the Zymotis packed-bed bioreactor for solid-state fermentation. *Biotechnol. Bioeng*. **68:** 127.
13. Mitchell, D. A., P. F. Greenfield, and H. W. Doelle. 1991. An empirical model of growth of *Rhizopus oligosporus* in solid-state fermentation. *J. Ferment. Bioeng.* **72:** 224.
14. Mitchell, D. A., A. Pandey, P. Sangsurasak, and N. Krieger. 1999. Scale-up strategies for packed-bed bioreactors for solid-state fermentation. *Process Biochem.* **35:** 167.
15. Moriguchi, M., K. Sakai, R. Tateyama, Y. Furuta, and M. Wakayama. 1994. Isolation and characterization of salt-tolerant glutaminases from marine *Micrococcus luteus* K-3. *J. Ferment. Bioeng.* **77:** 621.
16. Nigam, P. and T. Robinson. 2003. Bioreactor design for protein enrichment of agricultural residues by solid-state fermentation. *Biochem. Eng. J.* **13:** 197.
17. Pandey, A. 2003. Solid-state fermentation. *Biochem. Eng. J.* **13:** 81.
18. Pandey, A., C. R. Soccol, and D. Mitchell. 2000. New developments in solid-state fermentation: I - bioprocesses and products. *Process Biochem*. **35:** 1153.
19. Pandey, A., G. Szakacs, C. R. Soccol, A. Jose, Rodriguez-Leon, and V. T. Soccol. 2001. Production, purification and properties of microbial phytases. *Bioresource Technol*. **77:** 203.
20. Park, Y., S. Kang, J. Lee, I. Hong, and W. Kim. 2002. Xylanase production in solid state fermentation by *Aspergillus niger* mutant using statistical experimental designs. *Appl. Microbiol. Biotechnol.* **58:** 761.
21. Prabhu, N. and M. Chandrasekaran. 1997. Impact of process parameters on L-glutaminase production by marine *Vibrio costicola* in solid state fermentation using polystyrene as an inert support. *Process Biochem.* **32:** 285–289.
22. Roberts, J. 1976. Purification and properties of a highly antitumor glutaminase–aspariginase from *Pseudomonas* 7A. *J. Biol. Chem*. **251:** 2119.
23. Sabu, A., T. R. Keerthi, S. Rajeev Kumar, and M. Chandrasekaran. 2000. L-Glutaminase production by marine *Beauveria* sp. under solid state fermentation. *Process Biochem*. **35:** 705.
24. Sangsurasak, P. and D. A. Mitchell. 1998. Validation of a model describing 2-dimensional heat transfer during solid-state fermentation in packed bed bioreactors. *Biotechnol. Bioeng*. **60:** 739.
25. Terebiznik, M. R. and A. M. R. Pilosof. 1999. Biomass estimation in solid state fermentation by modeling dry matter weight loss. *Biotechnol. Tech*. **13:** 215.
26. Weng, X. and J. Sun. 2006. Kinetics of biodegradation of free gossypol by *Candida tropicalis* in solid-state fermentation. *Biochem. Eng. J.* **32:** 226.