3 Biotech (2019) 9:349
https://doi.org/10.1007/s13205-019-1883-5

ORIGINAL ARTICLE

L-Asparaginase production in rotating bed reactor from *Rhizopus microsporus* IBBL-2 using immobilized Ca-alginate beads

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Received: 27 June 2019 / Accepted: 24 August 2019

Abstract

The present work reports on the production of extracellular L-asparaginase from *Rhizopus microsporus* IBBL-2 using submerged fermentation (SmF) process free of glutaminase and urease activities. Primary studies done in shake flask showed that the highest L-asparaginase activity of 12.68 U mL^{-1} was produced at 72 h with optimized fermentation parameters such as pH 6.0, 4×10^6 fungal cells mL^{-1}, and agitation of 180 rpm at 30 °C using one-factor-at-a-time (OFAT). Different substrates, nitrogen sources, temperature, pH, the initial number of cells and metal ions were tested to determine the impact on enzyme production. L-Asparaginase activity of 17.68 U mL^{-1} was produced after 48 h using immobilized calcium-alginate (Ca-alginate) cells. 4×10^6 cells mL^{-1} was entrapped in 3% (W/V) of alginate bead of size 2 mm each at a temperature of 30 °C and pH of 6. The process was optimized using L9 (3^4) Taguchi Orthogonal Array (OA) technique with a regression coefficient (R^2) value of 0.9709, F value of 33.34 and p value of 0.0025. Scale-up studies involving 200-mL and 1-L rotating bed reactor (RBR) using immobilized beads were done and the results obtained are 20.21 U mL^{-1} and 19.13 U mL^{-1}, respectively, the increased activity with immobilization accounts for reduced shear on cells due to increased stability as compared to the free-flowing cells.

Keywords L-Asparaginase · *Rhizopus microsporus* IBBL-2 · Rotating bed reactor (RBR) · Submerged fermentation · Taguchi OA technique

Introduction

L-Asparaginase (EC 3.5.1.1, ASNase) is an amidohydrolase that catalyzes the deamination of asparagine to aspartic acid and ammonia. This key feature of the enzyme is utilized extensively in the treatment of acute lymphoblastic leukemia (ALL) in adults, children, and non-Hodgkin lymphoma in children. L-Asparaginase is also used in the food industry to prevent the formation of acrylamide during frying or baking of food items. Alternatively, it also finds an application as a biosensor to detect asparagine levels during chemotherapy of ALL (Kumar et al. 2013).

Acute lymphoblastic leukemia (ALL) is a common malignant disease found mostly in children and young adolescents (Batool et al. 2015; Kim et al. 2015). For normal cells, L-asparagine is a non-essential amino acid which can be synthesized in necessary amounts by asparagine synthetase. It is the enzyme that converts the glutamine present in the body to the asparagine required for growth of cells (Meena et al. 2015; Doriya and Kumar 2016; Doriya et al. 2016). In contrast, leukemic cells are deficient of enzyme asparagine synthetase, require a massive amount of L-asparagine for their malignant growth and hence are dependent upon an exogenous supply of L-asparagine from the plasma. L-Asparaginase is an enzyme that deprives tumor cells of this principle metabolite by hydrolyzing it, thereby starving and killing leukemic cells (Sajitha et al. 2015). The asparagine deficiency causes lack of protein synthesis and delay in DNA and RNA synthesis which impairs the cell functions resulting in the death of this cancerous cell; because of this selective action against leukemic cells, L-asparaginase is considered as a milestone for the treatment of ALL (Anderson and Cairney 2004; Kirk et al. 2004; Doriya and Kumar 2018).

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Published online: 31 August 2019

L-Asparaginase is synthesized using various microorganisms such as bacteria, fungi, yeast, and actinomycetes. In the current scenario, three L-asparaginase preparations are clinically available, asparaginase from *Escherichia coli*, *Erwinia chrysanthemi*, and PEGylated *E. coli* (Shrivastava et al. 2016). Clinically formulated L-asparaginase has an affinity towards glutamine, urea, and also obtained enzyme has reduced half-life; as a consequence, patients exhibit specific hypersensitive reactions. Several researchers have also reported that side effects obtained during the treatment hinder the complete treatment regimen (Badoei-Dalfard 2015). Hence, it is a specific challenge for the researchers to find the L-asparaginase with high specificity and lower allergic reactions. High-grade purification is necessary for the removal of these by-products; extensive research is being carried out to find effective alternates for the bacterial species and also in the production of L-asparaginase free of glutaminase and urease (Kumar et al. 2010; Doriya and Kumar 2016). Sajitha et al. (2015) has shown that the expression studies of *E. coli* asparaginase in a eukaryotic system such as *Pichia pastoris* would reduce the drawbacks when compared to expression in prokaryotic system. The current state-of-the-art technique of L-asparaginase production is using fungal sources as they are eukaryotic and have proven to be one of the antitumor agents (Baskar and Renganathan 2011a). *Rhizopus* strain previously isolated, which is free of glutaminase and urease, and characterized as *Rhizopus microsporus* IBBL-2 is employed in this study for the production of L-asparaginase (Doriya and Kumar 2016).

The primary concern for enzyme production in any industry is low stability and activity; therefore, the use of the immobilization technique helps in improving the productivity of any process (Mateo et al. 2007).

Usually, *Rhizopus* sp. can grow in a simple medium with glucose as a carbon source, but fermentation with filamentous fungi is complicated as mycelial growth causes an increase in medium viscosity. Immobilization of whole cells is a technique where the cells are confined to space without any loss in its biological activity. Cells can be supported on a solid matrix structure using different physical or chemical methods; the most commonly used technique is to entrap the whole cells, this technique is known as bioencapsulation or microencapsulation (Cirpan et al. 2003; Sheldon 2007; Rodrigues et al. 2013). Tay and Yang (2002) reported that immobilized cells of *Rhizopus oryzae* in rotating fibrous bed bioreactor for lactic acid production gave a virtually cell-free fermentation and provided higher yield in comparison to suspended fungal cells. The use of such a technique also decreases the time needed for the extraction and purification of the enzymes as the trapped whole cells have better reaction capability (Palmieri et al. 1994). The decision on the usage of any immobilization technique depends on the nature of the cells used, its reactivity with the solid support and the type of material being produced (Zhang 1998; Ashok and Kumar 2017). In this study, calcium-alginate (Ca-alginate) beads are used to entrap the whole-cell *Rhizopus microsporus* IBBL-2. Various factors, such as the concentration of the alginate solution and the size of the beads on the activity of the enzyme, were studied using the Taguchi OA statistical analysis method.

Rotating bed reactor (RBR) is a packed bed filled with a solid phase which in our case is the immobilized beads containing microorganism. The reactor works based on centrifugal acceleration, which helps to enhance the mixing and the mass transfer operations that are taking place in the reactor (Yang et al. 2005). Process intensification is being carried out using the reactor, which not only leads to a reduction in the economy of the process but also increases the overall output (Chen et al. 2010; Jiao et al. 2012). In the RBR, the media passes through the packing, and the fermentation process between the media and microorganism takes place within this packing, and the enzymes are released as output. Due to the compact nature of the beads in the rotor, the overall stress is reduced compared to a free-flowing liquid, which helps in increasing the activity of the enzyme.

The present study deals with the production of the L-asparaginase enzyme using *Rhizopus* sp. and the methods and parameters that can be varied for the enhanced production of the enzymes. Initially, individual parameters are varied to determine the effect of these parameters on the enzyme production, and later Taguchi technique is utilized to study the effects of immobilization on the production of the enzyme. A scale-up study using the 200-mL and 1-L RBR is also done to show the effectivity of the process.

Materials and methods

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1 ml (1×10^6 spores mL^{-1}) was used to inoculate 100 ml of sterilized modified Czapek–Dox (MCD) medium dispensed in 250-ml Erlenmeyer flasks. The composition of the medium is as follows (g/L): glucose, 2 (S D Fine-Chem Ltd., Mumbai, India); L-asparagine, 10 (Avra Synthesis Pvt. Ltd., Hyderabad, India); KH_2PO_4, 1.52 (Sisco Research Laboratories Pvt. Ltd., Mumbai, India); KCl, 0.52 (S D Fine-Chem Ltd., Mumbai, India); $MgSO_4.7H_2O$, 0.52 (S D Fine-Chem Ltd., Mumbai, India); $Cu(NO_3)_2.3H_2O$ 0.001 (S D Fine-Chem Ltd., Mumbai, India); $ZnSO_4.7H_20$ 0.001 (S D Fine-Chem Ltd., Mumbai, India); $FeSO_4.7H_2O$, 0.001 (Sisco Research Laboratories Pvt. Ltd., Mumbai, India).

F

The fungal species were isolated from Wheat Bran substrate using the conventional plate assay technique (Gulati et al. 1997; Mahajan et al. 2013) The isolate obtained was sub-cultured in potato dextrose agar (PDA) slants for 3 days at 30 °C and stored in a refrigerator at 4 °C. Confirmation of the genome species was done through gene sequencing and identified as *Rhizopus microsporus* IBBL2. The ITS rRNA sequences of *Rhizopus microsporus* IBBL-2 were deposited in NCBI GenBank under the accession number MH596007.

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The whole cell immobilization was done using Ca-alginate beads through the technique of microencapsulation. 1.5 g of sodium alginate (Na alginate, Finar Ltd., Ahmedabad, India) was dissolved in 50 ml distilled water and sterilized at 121 °C for 15 min. Alginate solution was then mixed with 3 mL of *Rhizopus microsporus* IBBL-2 suspension. The prepared solution was then added to 100 mL of pre-sterilized 2% $CaCl_2$ (S D Fine-Chem Ltd., Mumbai, India) solution using a sterilized syringe to obtain spherical beads entrapping *Rhizopus microsporus* IBBL-2. For further hardening, beads with cells were left in the solution for 80 min. Lastly, obtained beads were washed with distilled water to remove excess calcium ions and unentrapped spores. For optimization of the immobilization process, the Na alginate concentration is varied from 1 to 5% (w/v) and the size of the beads that are being produced is varied from 1 to 3 mm and the optimized parameters are determined.

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Immobilized *Rhizopus* sp. beads were then transferred to 250-mL Erlenmeyer flask containing 50 mL of the sterilized MCD medium. Fermentation experiments were carried out on a rotary shaker (180 rpm) at 30 °C. One-factor-at-a-time was used for optimization by varying the following process variables: pH of media ranging from 4 to 8, temperature ranging from 15 to 50 °C and initial cell number varying from 1×10^6 to 6×10^6 cells mL^{-1}. To investigate the effect of different carbon sources on L-asparaginase production, fructose, dextrose, sucrose, lactose, and starch were added at a concentration of 0.2% (w/v) to the MCD medium. Influence of nitrogen source on asparaginase production was obtained by substituting asparagine of MCD medium with glutamine, yeast extract, peptone, sodium nitrate and ammonium sulphate at a concentration of 1% (w/v). Effect of various metal ions on the production of L-asparaginase was determined by supplementing the growth with $CaCl_2$, $CuSO_4$, $MgSO_4$, $NaNO_3$, $FeSO_4$, and $ZnSO_4$. Optimized parameters obtained at shake flask level are taken for further study in the scale-up process.

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Subsequently, the experiments were carried out in a stirred SpinChem rotating bed reactor of the working volume of 200 mL and 1 L equipped with agitator driven by a variable speed motor and an internal thermocouple. The immobilized *Rhizopus microsporus* IBBL-2 cells were packed into the rotor of the rotating bed reactor between the two meshes. The media was prepared based on the optimized parameters that were obtained. The sterilized synthetic media was added to the column surrounding the rotor and agitated at 200 rpm. The packed immobilized bed is separated from the free-flowing media by the outer and inner filter meshes made of stainless steel (SS); as the rotor starts, the flow of the media is varied, and the media enters the packed bed chamber where the enzymatic reaction occurs within the encapsulated whole-cell beads. Porous nature of the beads allows the passage of the media components into and the enzyme out of the beads. The packed bead materials increase the stability of the cells and help in proper mass transfer across the material. Samples were collected at regular intervals of time to analyze the activity of the enzyme. A schematic of the reactor in use is shown in Fig. 1.

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Crude L-asparaginase enzyme was extracted by centrifugation. The collected supernatant was then subjected to spectrophotometric analysis by measuring the ammonia liberated using Nessler's reagent (Long et al. 2015). Enzyme assay mixture consisted of 900 µL of freshly prepared L-asparagine (10 mM) in Tris–HCl buffer (pH 8.6) (Sisco Research Laboratories Pvt. Ltd., Mumbai, India) and 100 µL of crude extract of the enzyme was prepared. The reaction mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 200 µL of 1.5 M trichloroacetic acid (TCA, Sisco Research Laboratories Pvt. Ltd., Mumbai, India). The obtained mixture was centrifuged at 10,000 rpm for 15 min at 4 °C to remove the precipitates. The quantity of ammonia released in the supernatant was calculated using the colorimetric technique by adding 100 µL Nessler's reagent into the sample containing 100 µL supernatant and 800 µL distilled water. The contents in the sample were centrifuged and then incubated at room temperature for 10 min, and the optical density (OD) was measured at 425 nm against the blanks to which TCA was added before crude enzyme. The ammonia released in the reaction was calculated based on the standard curve obtained with ammonium sulfate.

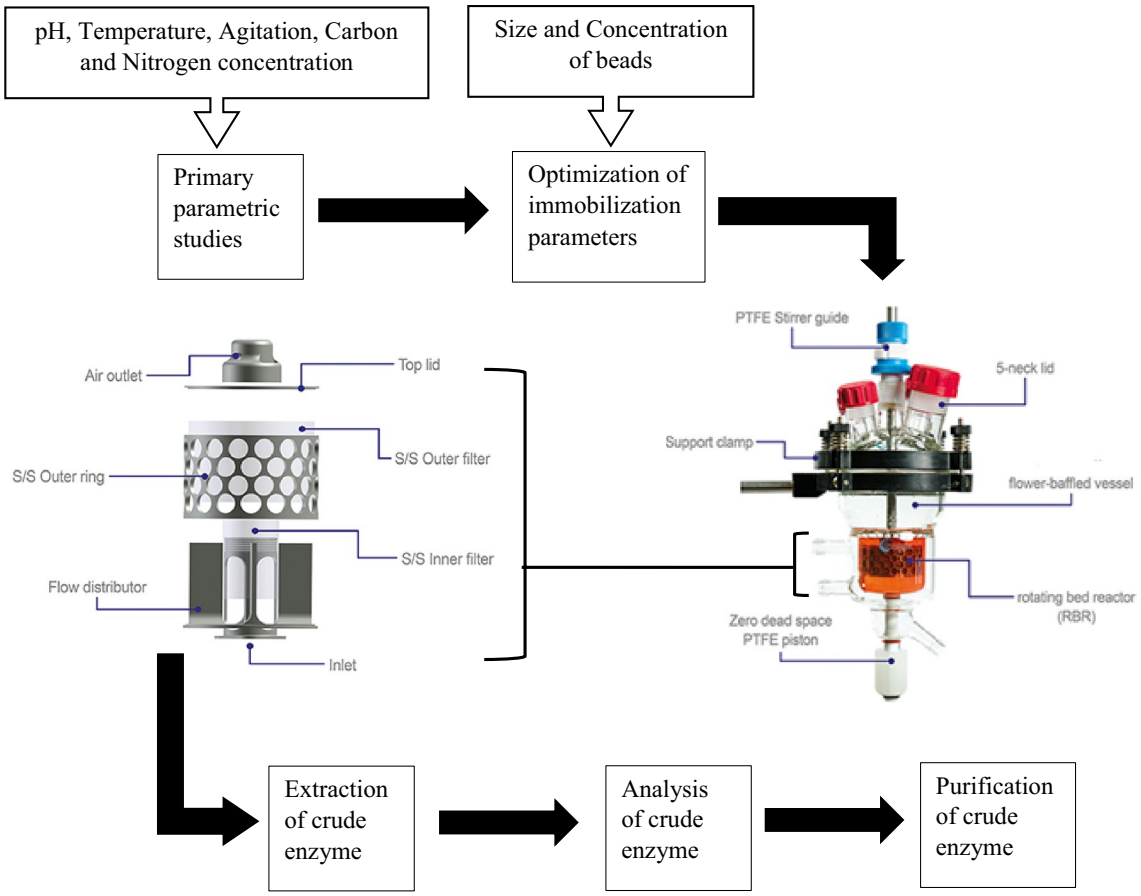

Fig. 1 Schematic representation of the working procedure: *S/S* stainless steel, *PTFE* polytetrafluoroethylene

One unit (U) of L-asparaginase activity was defined as the amount of the enzyme that liberates 1 mM of ammonia/min at 37 °C (Kumar et al. 2010).

O

The current study deals with the determination of the activity of crude enzyme and optimization of the basic process parameters using one-factor-at-a-time (OFAT) technique. Parameters such as temperature, pH, carbon and nitrogen source, and number of cells used initially were studied. The Taguchi OA technique (Design Expert 9, Stat-Ease Inc., Minneapolis, USA) was used to analyze the optimization of the immobilization technique using factors such as temperature, pH, % of the bead material (Na alginate) added and the size of the beads used. The analysis is done using the L9 (3^4) technique for which four parameters are tested at three different levels to obtain the results. The ranges of values that are used have been given in Table 1. The obtained results are later analyzed to determine which parameters have a significant effect on the optimization process.

Table 1 Range of variables used for Taguchi OA optimization method

Factor	Parameter	Units	Minimum	Maximum	Levels
A	Temperature	°C	25	35	3
B	pH		6	8	3
C	Bead material concentration		1	5	3
D	Size of bead	mm	1	3	3

P

The soluble protein content of the crude samples was determined using a spectrophotometer according to the method described by Lowry (Lowry et al. 1951; Peterson 1979). The protein liberated in the reaction was determined based on the standard curve obtained using bovine serum albumin (BSA). Specific activity is expressed as unit enzyme activity per mg of protein.

Results and discussions

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The one-factor-at-a-time technique was used to analyze the efficiency of the process for each factor. Every parameter has to be determined for the proper growth of the microorganism; any value below or the above the optimum value will have an adverse effect on the growth of the microorganism. L-Asparaginase production was performed at a temperature of 30 °C using 4×10^6 cells mL^{-1}. *R. microsporus* IBBL2 was grown on MCD medium at different pH values ranging from pH 4–8. The effect of pH on the L-asparaginase is shown in Fig. 2a, the *Rhizopus microsporus* IBBL-2 has shown a better activity at a slightly acidic pH, the activity curve suggests that the enzyme release or the microorganism growth has a rapid rise between the 2nd and 3rd day of the experiment with its maximum activity being reported on the 3rd day. The initial lag phase is responsible for the slower growth on the first 2 days. Results indicate that maximum enzyme activity was observed at pH 6.0 with an activity of 11.69 U mL^{-1}. However, asparaginase activity decreased at low pH (4 and 5) and high pH range (8–9). Maximum L-asparaginase activity for most microbial species was attained at pH ranging from 6 to 7 (Abbas Ahmed et al. 2015).

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Incubation temperature of the fermentation process has a profound impact on the growth of the microorganisms and production of enzymes. Effect of temperature on L-asparaginase activity was studied in the temperature range of 15–50 °C with pre-optimized pH. L-Asparaginase activity and specific activity for varied temperature are shown in Fig. 2b. The current study showed that the optimum temperature for the growth of the *Rhizopus microsporus* IBBL-2 was 30 °C, at which it showed maximum activity of 12.2 U mL^{-1} and specific activity of 19.29 U mg^{-1}. A decline in L-asparaginase activity was observed with lower and higher temperatures other than 35 °C. At higher temperatures, visually less growth was observed, indicating that the microorganism could not sustain at these temperatures. L-Asparaginase production from bacterial sources has shown very less activity (2.5 IU mL^{-1}) at high-temperature conditions (Sajitha et al. 2015).

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The initial number of cells that are added as inoculum for the growth of the microorganism is another essential factor to be studied. Lower initial number results in the abnormal growth of the microorganism while more significant number causes overcrowding, which leads to a lack of availability of the nutrient for the initial growth. Influence of initial spore concentration on L-asparaginase production by *R. microsporus* IBBL2 is shown in Fig. 3a. The current study indicated that at 4×10^6 cells mL^{-1} concentration, maximum L-asparaginase activity of 12.02 U mL^{-1} was obtained.

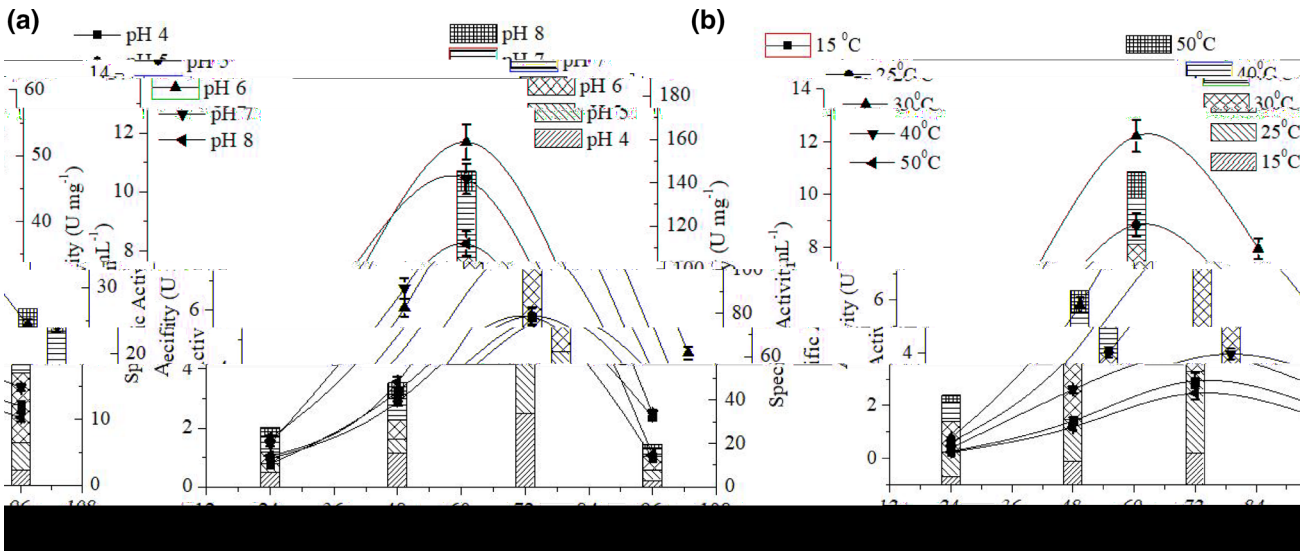

Fig. 2 L-Asparaginase activity and specific activity in submerged fermentation using *Rhizopus microsporus* IBBL-2 for varying pH (**a**) and temperature (**b**) values, respectively

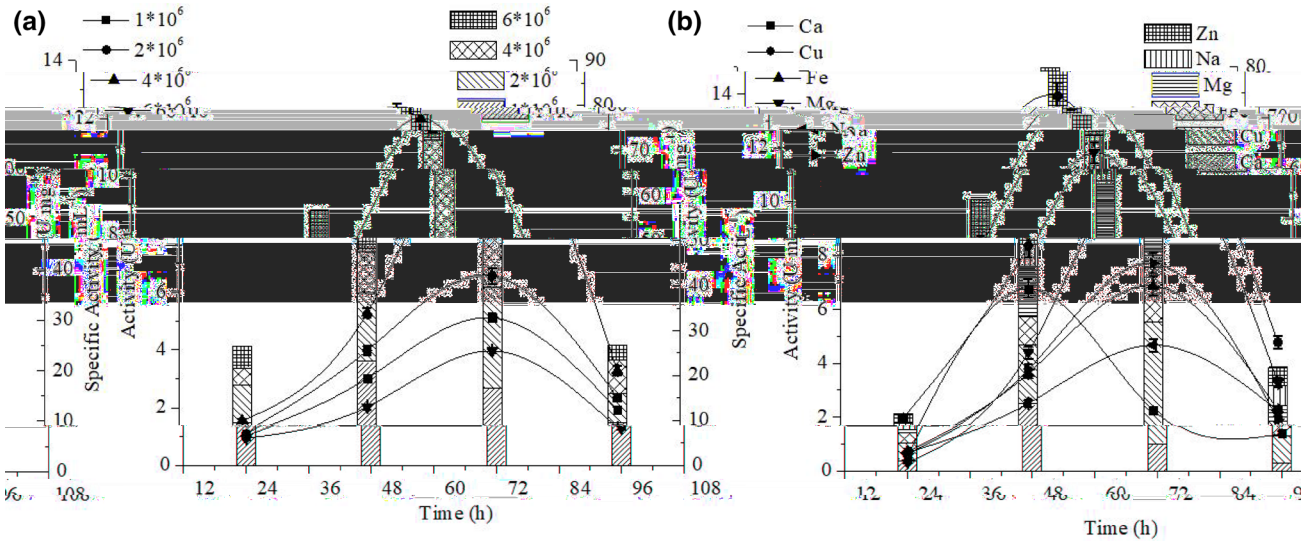

Fig. 3 L-Asparaginase activity and specific activity in submerged fermentation using *Rhizopus microsporus* IBBL-2 for varying the initial concentration of microbial cells (**a**) and varying metal ion types (**b**), respectively

E

Another factor that affects the growth of the microorganism is the presence of metal ions which enhances or reduces the growth based on its impact on the development of the microorganism and also acts as a co-factor for several biosynthetic enzymes (Hosamani and Kaliwal 2011). In the current instance, Cu^{2+} has shown a positive effect on the growth and has shown the maximum activity of 13.92 U mL^{-1}. While earlier studies involving other microbial species have demonstrated that the presence of Cu^{2+} has shown much lower enzyme activity as compared to the *Rhizopus microsporus* IBBL-2 (Mathew et al. 1994), the metal ions are varied in the prepared media, and its effects have been studied and reported as shown in Fig. 3b. Among the tested metal ions, the lowest enzyme activity was obtained when MCD media was amended with Na^{+} metal ions.

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The L-asparaginase production pattern using various carbon sources (dextrose, fructose, starch, lactose, and sucrose) is shown in Fig. 4a. The growth of the microorganism is governed by the nutrient source that is being supplied. The carbon source is the main factor responsible for the development of any microorganism. *Rhizopus microsporus* IBBL-2

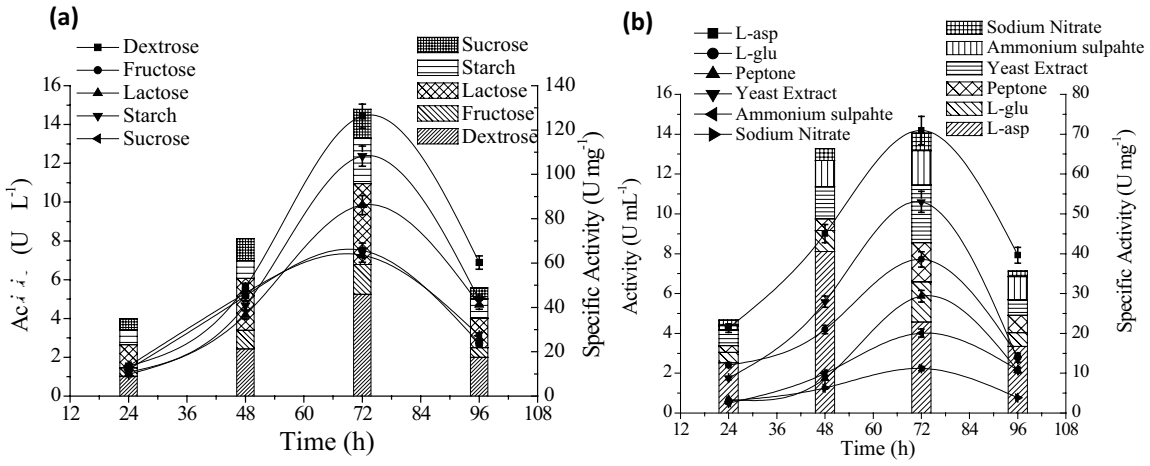

Fig. 4 L-Asparaginase activity and specific activity in submerged fermentation using *Rhizopus microsporus* IBBL-2 for varying carbon sources (**a**) and nitrogen sources (**b**) for enhanced production, respectively

has shown the maximum activity of 14.43 U mL^{-1} using dextrose as a carbon source. In comparison to the earlier studies where *Serratia marcescens* was used and not the considerable activity was obtained from the different sources with values ranging as low as 0.8 U mL^{-1} (Heinemann and Howard 1969). It has been ascertained from Fig. 4a that the *Rhizopus microsporus* IBBL-2 has the best compatibility with the dextrose and production of L-asparaginase was marginally favorable with lactose and starch. A decline in L-asparaginase activity was observed with sucrose and fructose as sole carbon sources. Similar results were obtained using *A. terreus* MTCC 1782 with glucose as a carbon source, which was found to be an important fermentation media component for L-asparaginase production (Baskar and Renganathan 2011b).

Effect of nitrogen compounds on L-asparaginase by *R. microsporus* IBBL2 was studied by supplementing nitrogen sources (asparagine, glutamine, peptone, yeast extract, sodium nitrate, and ammonium sulphate). The nitrogen source supplies the materials for the production of proteins required for growth. A comparison of the nitrogen factor with respect to *Escherichia coli* has shown that the reported fungal strain has a much better affinity to the L-asparagine than the bacterial species which suggests that the fungal species can be considered as a vital substitute for the bacterial source (Sarquis et al. 2004; Kenari et al. 2011). The strain *Rhizopus microsporus* IBBL-2, as seen in Fig. 4b, has a higher affinity towards L-asparagine (L-asparaginase activity 14.18 U mL^{-1}). Yeast extract also supported the production of L-asparaginase to a substantial quantity. Lowest L-asparaginase activity was obtained with sodium nitrate as the nitrogen source.

Table 2 Parameters set for the different runs in the Taguchi OA method

Run no.	Temperature (°C)	pH	% of bead material	Size of beads (mm)	Activity (U/mL)
1	30	8	1	3	15.5
2	25	6	1	1	10.75
3	25	8	5	3	8.35
4	35	7	1	3	7.15
5	25	7	3	2	10.5
6	35	6	5	2	9.26
7	30	6	3	2	17.63
8	30	7	5	1	14.36
9	35	8	3	1	6.04

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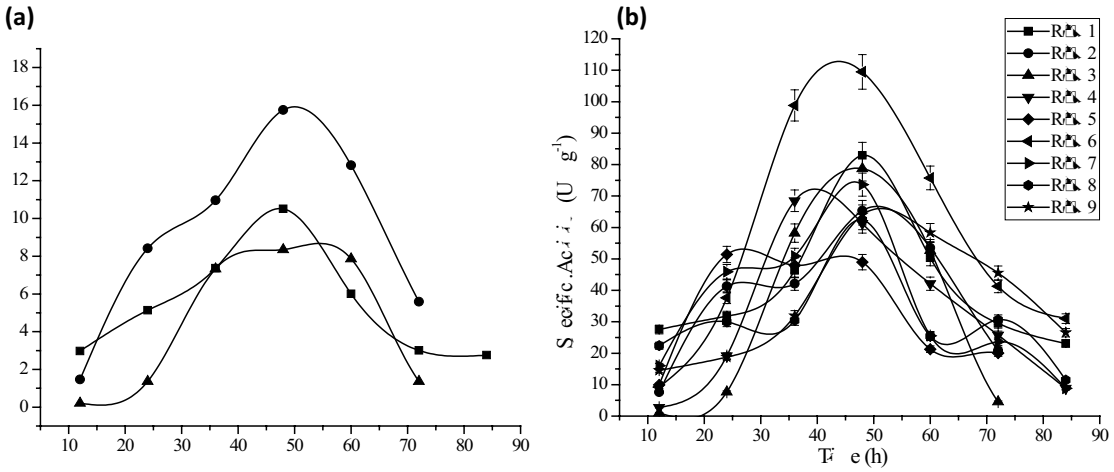
To achieve the best result, an optimized set of parameters was determined using the Taguchi OA method of optimization by varying four parameters over three levels. These parameters include temperature, pH, % of beads, and size of beads. A total of nine experiments were carried out to predict the correlation between variables and response, and results are given in Table 2. L-Asparaginase activity was examined at design points to determine the optimum level of variables. A linear regression model was fitted for variation in response with the coefficient of determination $R^2 > 0.90$. Enhanced activity has been obtained with immobilization with a value of 17.63 U mL^{-1} compared to 12.68 U mL^{-1} as reported in the individual experimentation given in Fig. 5a, b.

Statistical analysis of the obtained data shows the significant impact of factors on enzyme production. The accuracy of the model is demonstrated by the regression coefficient



(R^2) value of 0.9709 which indicates that there is only ~ 3% of variation in the response that cannot be explained using the model which is an acceptable value. The adjusted R^2 value of 0.9418 also shows that the model is appropriate for the obtained data. The significance of the Taguchi model is demonstrated by the F value and p value obtained in ANOVA analysis; the values obtained are 33.34 and 0.0025, respectively, and according to the analysis data, F value > 4.0 and p value < 0.05 give a clear-cut idea on how significant the model can explain the variations in data. Moreover, there is only a 0.25% chance that an F value this large could occur due to noise. A relation between the enzyme activity and the individual parameters is represented with the help of an equation developed based on the model as given below:

$$Y = 11.06 - 1.19 * x_1 + 4.77 * x_2 + 1.49 * y_1 - 0.39 * y_2, \quad (1)$$

where the 'x_1 and x_2' values correspond to the temperature data, and the 'y_1 and y_2' values correspond to the pH data in coded terms. The ANOVA analysis data are given in Table 3. The optimum parameters for current experimentation are at a temperature of 30 °C and a slightly acidic pH (6); although the effects of % of bead material and size of the beads are not relative, a concentration of 3% and a size of 2 mm, respectively, can be used to obtain the best results. L-Asparaginase activity variation was observed to be less when interactive level influential factors are in an optimized environment. The data were in line with the study conducted for L-asparaginase production by isolated *Staphylococcus* sp.—6A (Prakasham et al. 2007).

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The lab-scale production of the L-asparaginase enzyme was carried out in Erlenmeyer flask and in a solid-state tray bioreactor using the optimized culture composition and the optimal conditions (pH 6.0, 30 °C, inoculum volume 25 mL, % of beads 3 and size of beads 2 mm). In the present study, the synthetic media of 200 mL and 1 L capacity were prepared and reactions were carried out with the addition of the immobilized Ca-alginate beads using the RBR. From the obtained results, it was seen that the activity values are similar to that obtained in the flask-scale study. The activities obtained for laboratory-scale studies shown in Fig. 6a are 20.21 U mL^{-1} in 200-mL RBR and 19.13 U mL^{-1} in 1-L RBR, and the specific activities

Table 3 ANOVA analysis of the main effects with coefficients that help in determining the significance of each parameter and also the model efficiency

Source	Sum of squares	Degrees of freedom	Mean square	F value	p value Prob > F
Model	121.60	4	30.40	33.34	0.0025
A—Temperature	110.91	2	55.45	60.81	0.0010
B—pH	10.69	2	5.35	5.86	0.0647
Residual	3.65	4	0.91	–	–
Cor total	125.25	8	–	–	–

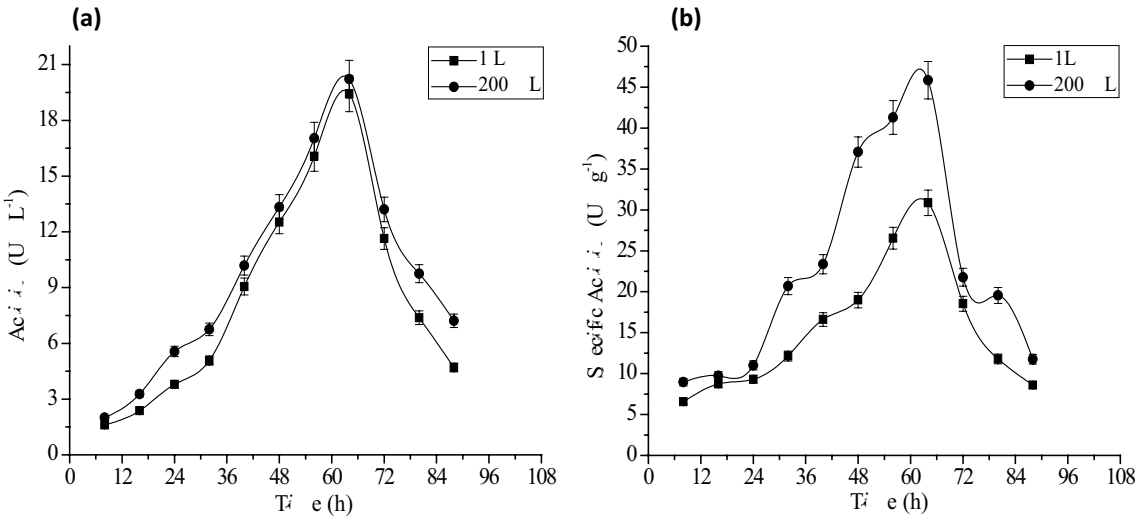

Fig. 6 Comparison of the enzyme activity (**a**) and specific activity (**b**) on the semi-pilot scale in RBR of 200 mL and 1 L capacity for enhanced enzyme production

are as shown in Fig. 6b. The increase in the activity of the enzyme due to immobilization is attributed to the fact that the stability of the cells is increased. It causes a steady input of the media and output of the products, i.e., there is efficient mass transfer in the process; also the cells are protected from any shear forces acting on it due to the flow of the media which are responsible for the damages that may occur in the cell.

Conclusion

The *Rhizopus microsporus* IBBL-2 fungal species has shown maximum activity of 17.68 U mL^{-1} at the optimum conditions as follows: temperature of 30 °C, pH of 6, initial microbial concentration of 4×10^6 cells mL^{-1} using dextrose as carbon source and L-asparagine as nitrogen source with Cu as an additional metal ion, using immobilized cells of size 2 mm prepared at a concentration of 3% (W/V). This accounts for a 1.4-fold increase in the activity. The activity without immobilization for similar parameters was around 12.68 U mL^{-1}. Optimization of the process was done using the Taguchi OA. The significance of the model is determined by F value (33.34), p value (0.0025), and regression coefficient ($R^2 = 0.9709$), which makes the Taguchi OA technique an optimum fit for the data that were obtained. The scale-up studies conducted have shown that the results are replicates of the flask-scale with activities of 20.21 U mL^{-1} in 200-mL RBR and 19.13 U mL^{-1} in 1-L RBR, and there is no loss in the efficiency during the scale-up process. From the current study, it can be said that the *Rhizopus microsporus* IBBL-2 can produce L-asparaginase free of glutaminase and urease, thereby replacing the existing production using bacterial species. It also has shown excellent stability in the use of immobilization techniques. The present study has been limited to the production of crude enzymes. The later stages of the work shall focus on the purification of the enzymes through various processes, which include ammonium sulphate process and/or aqueous two-phase process. The purified enzyme can then be used for trial tests after receiving approvals for the study at a later stage.

Acknowledgements This work is supported by a research grant from the Department of Science and Technology-Science and Engineering Research Board SERB (SB-EMEQ-048/2014). The authors would like to express their sincere gratitude to Dr. Kruthi Doriya, Vaibhav Lendekar, Venkatesh Mandari, and Ashutosh Nema for their support during the experimentation process.

Author contributions Anup Ashok has done the primary work including experimentation, analysis and writing the paper. Dr. Devarai Santhosh Kumar has supervised the entire process and supported in the experimentation, analyses and correction of the manuscript.

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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