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ARTICLE

A permissive approach for optimization of L-glutaminase production using wheat bran as supporting substrate and assessment of its cytotoxic potentialities

Jyoti P. Soren[1], Suman K. Halder[1], Joy Mondal[1], Papan K. Hor[1], Pradeep K. D. Mohapatra[2], Keshab C. Mondal[1]*

[1]Department of Microbiology, Vidyasagar University, Midnapore-721 102, West Bengal, India
[2]Department of Microbiology, Raiganj University, Uttar Dinajpur, West Bengal

ABSTRACT Microbial L-glutaminase has considered as one of the most important therapeutic enzymes considering its anticancer or antitumor activity. In this study, one L-glutaminase producing potent fungus was isolated from the coastal soil and identified as *Fusarium nelsonii* KPJ-2. During parametric optimization, it was noted that wheat bran supported maximum L-glutaminase production than other agro-industrial wastes tested. Solid substrate fermentation was mechanized with optimum pH of 4.0, incubation temperature at 25 °C, inoculums concentration of 2.0% (v/v), substrate concentration of 7.0% (w/v) and moisture of the production media suits at 20.0% (w/v). Statistical optimization using Response Surface Methodology (RSM) was improved the L-glutaminase production by 14.5% (68.93 U/gds) than unoptimized state. The SEM-EDX analysis demonstrated the overgrowth of fungus on wheat bran and utilization of its associated minerals. A comparative cytotoxic effect of the partial purified glutaminase was examined on both cancerous HCT cell and normal Vero cell line. The result clearly demonstrated that L-glutaminase from *F. nelsonii* KPJ-2 is specifically cytotoxic against cancer cell line with IC_{50} of 203.95µg/ml, but, non-responsive against normal cell. The newly isolated fungal strain can produce a considerable amount of L-glutaminase utilizing very low-cost substrate and the enzyme have therapeutic value for real life application owing to its anticancer effectiveness. **Acta Biol Szeged 64(1):1-10 (2020)**

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***Corresponding author**
E-mail: mondalkc@gmail.com

Introduction

L-glutaminase (E.C.3.5.1.2) is an amidohydrolase that catalyzes the deamination of L-glutamine and produce L-glutamic acid and ammonia. This enzyme has significant applications in food, therapeutic and analytical industries. In food sector, addition of glutaminase leads to improvement of glutamate content, which enhances pleasant and delicious taste of the foodstuff (Kurihara 2009). The palatable taste of traditional food like soya sauce, miso and sufu is the involvement of L-glutaminase during course of fermentation and leads to accumulation of high content of L-glutamate (Lioe et al. 2010; Binod et al. 2017). In diagnostic and health monitoring, analysis of L-glutamine and glutamate level in the blood is very important (Madeira et al. 2018). Similarly, glutaminase based monitoring of glutamine and glutamate levels in mammalian cell culture media is effective for mass culturing (Cattaneo et al. 1992). Kikkoman Corporation, Japan first developed L-glutaminase based biosensor to determine the L-glutamine levels for diagnostic purpose and now it is worldwide applied. High rate of L-glutamine consumption is a characteristic of cancerous cells for biosynthesis of proteins and nucleic acids. Unlike normal cells, tumor cells have lack of L-glutamine biosynthetic machinery. As a consequence, growth of the tumor cells depends upon uptake of L-glutamine from serum. Administration of exogenous L-glutaminase in serum or at the site of tumor leads to depletion of glutamine level, therefore, tumor cells are starved for glutamine (Soren et al. 2019). Based on this principle, L-glutaminase is being extensively used as therapeutic agent to restrict growth of tumor or cancer cell. Recently for prolonged stability and better distribution of the enzyme, immobilized and *nano* forms of glutaminase are being developed.

Since the discovery of glutaminase, a vast number of microbes belongs to fungi and bacteria have been reported to be L-glutaminase producer (Soren et al. 2019). Among the fungal genera, *Aspergillus* and *Trichoderma* are the major

producer of L-glutaminase. For mass cultivation, both submerged and solid-state fermentation are employed by supplementing glutamine in the culture media. But report on raw or low-cost substrate is scanty in this regard. Due to existence of natural habitat and adequate oxygenation, the rate of productivity in solid state fermentation (SSF) of any metabolite is much higher than submerged fermentation (SmF) (Binod et al. 2017). Sabu et al. (2002) reported that salt stable enzyme from marine origin is effective in the treatment of acute lymphoblastic leukemia (ALL), as the isolated enzyme is more stable in salt-rich human plasma.

Considering these perspectives, the present study deals with the isolation of a potent L-glutaminase producing fungi from the soil of coastal area. The physicochemical environment of the organism has been optimized to recover maximum enzyme and under SSF to minimize the production cost. And finally, the anti-cancerous effectiveness of the isolated enzyme was explored under *in vitro* condition to highlight its therapeutic potentialities.

Materials and Methods

Isolation and identification of L-glutaminase producing fungi

Soil samples were collected from the coastal belt of Bay of Bengal from Bhitarkonika (20°45′N 87°0′E) and Digha (21°38′18″N 87°30′35″E). For exploring L-glutaminase producing fungi, soil samples were serially diluted, and 0.1 ml of each dilution was spread on glutamine supplemented (1.0%, w/v) potato dextrose agar (PDA) that containing 0.75% (w/v) phenol red and 100 mg/l ampicillin. The initial medium pH was maintained at 7.0. The plates were incubated at 28 °C for 7 days. Glutaminase producing fungi were selected by observing the pink zone around the colonies (Meghavarnam and Janakiraman 2017).

In secondary screening, primarily selected fungal isolates were cultured into the liquid medium (pH 7.0) having same composition as like isolation media (without agar) for 96 h. The activity of glutaminase in the culture supernatant was determined. The culture was maintained and sub-cultured on PDA slants frequently. The most potent fungal strain was identified on the basis 28S rDNA sequencing. Phylogenetic tree was constructed by the different nucleotide sequence which have maximum match with the query sequence. The sequences were aligned by ClustalW2 (Larkin et al. 2007) and the phylogenetic tree (dendrogram) was constructed by PHYLIP software (version 3.69) (Felsenstein 1981). Bootstrap value 500 was taken to build the tree by using Neighbour-joining method.

Inoculum preparation

Spore suspension of the potent fungal isolate was prepared by addition of 5 ml sterilized distilled water containing Tween 80 (2 drops/100 ml) onto the 3 days old fungal slants. The final concentration of the spore suspension was adjusted to about 2.0×10^2 spores/ml and preserved at 4 °C for further use.

Optimization of enzyme production through SSF

One variable at a time (OVAT) approach

Raw substrates were first treated with hot 1.0 % NaOH followed by repeated washing with cold and warm water to remove the dirt and other undesirable compounds. The washed substrates were then sun dried and cut into small pieces (2-3 mm). Solid state fermentation was carried out in Roux flasks containing 2.0 g of agro-industrial raw substrates moistened with 1.0 ml of glutamine (1.0%) solution (as inducer). Different raw substrates viz, sugarcane bagasse, wheat bran, rice straw, mustard oil cake and orange peel were used to examine their supporting effect on enzyme production. The effect of medium pH on enzyme production was checked by adjusting the pH (4.0-12.0) of moistening solution. The effect of temperature on enzyme production was studied by incubating the culture flaks at different incubation temperatures (20 - 50 °C). The optimization of important parameters like substrate concentration (1.0-10.0%, w/v), inoculum concentrations (0.5-3.0%, v/v) and moisture content (10-50%, w/v) were also done for enzyme production.

Response surface methodology (RSM) for optimization of L-glutaminase production

Five most influencing parameters (with their respective levels) were selected from OVAT experiments and further optimization was made using RSM. To explore the effects of these variables on the L-glutaminase production, a Box-Behnken factorial design was employed. An experimental design (5 factors with 3 levels) comprising multiple experiments were conducted for statistical optimization. Full experimental design is listed in Table 1. The relation between the coded value and actual values are described as in following.

$$X_i = \frac{x_i - x_o}{\Delta x_i}$$

Where, x_i is the independent variable coded value, x_o is the actual value of independent variable at their center point, and Δx_i is the step change value.

A model was generated by the regression analysis of the responses, and, its efficiency was tested by ANOVA and F-test. Three-dimensional (3D) response surface

Table 1. Experimental design used in RSM studies by using four independent variables each at three levels showing observed and predicted values of L-glutaminase production.

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**	**Factor 5**	**L-Glutaminase (U/gds)**	
Run	A: pH	B: Temperature	C: Inoculum	D: Substrate	E: Moisture	Observed value	Predicted value
1	4	27.5	2	8	30	64.38	54.77
2	4	30	2	7	10	55.87	55.92
3	3	30	2	7	20	55.8	56.29
4	4	27.5	1	6	20	57.81	65.58
5	5	27.5	3	7	20	64.67	60.86
6	4	25	3	7	20	60.79	62.23
7	/5	27.5	2	6	20	60.85	61.20
8	3	27.5	2	7	10	60.42	68.51
9	4	27.5	2	7	20	68.93	58.61
10	4	25	2	7	10	59.65	68.51
11	4	27.5	2	7	20	68.93	56.63
12	4	30	2	7	30	56.31	57.83
13	3	27.5	2	6	20	57.87	57.12
14	4	30	2	8	20	57.94	64.14
15	4	27.5	2	6	10	63.87	54.99
16	4	25	1	7	20	54.43	58.68
17	4	30	2	6	20	58.22	55.20
18	4	25	2	6	20	53.53	60.41
19	5	27.5	2	7	30	60.34	56.79
20	4	27.5	1	7	10	55.37	54.84
21	3	25	2	7	20	55.95	55.17
22	4	27.5	2	6	30	56.65	62.13
23	3	27.5	1	7	20	63.12	57.12
24	5	25	2	7	20	58.04	67.34
25	4	27.5	1	8	20	67.28	62.43
26	4	27.5	1	7	30	61.19	58.37
27	5	27.5	2	7	10	58.82	68.51
28	4	27.5	2	7	20	67.37	57.86
29	5	27.5	1	7	20	59.23	59.07
30	4	27.5	2	8	10	57.8	55.00
31	3	27.5	2	7	30	53.7	56.23
32	5	30	2	7	20	55.93	58.72
33	3	27.5	3	7	20	57.43	68.51
34	4	27.5	2	7	20	67.98	60.41
35	4	25	2	8	20	60.02	68.51
36	4	27.5	2	7	20	68.93	68.51
37	4	27.5	2	7	20	68.93	60.27
38	4	27.5	3	8	20	59.43	62.77
39	3	27.5	2	8	20	64.11	60.96
40	5	27.5	2	8	20	60.88	56.87
41	4	27.5	3	7	30	58.21	57.24
42	4	30	3	7	20	57.12	67.67
43	4	27.5	3	6	20	68.41	52.60
44	4	25	2	7	30	52.22	66.67
45	4	27.5	3	7	10	67.82	58.80
46	4	30	1	7	20	58.2	57.24

plots were generated to highlight the interactions among variables using Design-Expert 10.0 software (USA) (Das et al. 2013).

Enzyme extraction

After fermentation, sterile distilled water (5.0 ml) was added to each flask and shaked for 30 min at 100 rpm. The mixture was filtered through cheese cloth and centrifuged at 5000 rpm for 10 min. The crude enzyme was subjected to $(NH_4)_2SO_4$ precipitation (80%) and then dialyzed against the phosphate buffer (1M, pH 7.0). Finally, enzyme was collected by membrane filtration using 100 kDa molecular weight cut-off (Amicon, Merck). The partial purified glutaminase was stored for further use.

L-Glutaminase assay

The activity of L-glutaminase was determined by estimating the amount of ammonia liberates from the specific hydrolysis of substrate L-glutamine according to the method described in our previous publication (Soren et al. 2019). In brief, the reaction mixture consisting of 0.2 ml of crude enzyme, 0.5 ml of 0.04 M L-glutamine and 0.5 ml of 0.1 M phosphate buffer (pH 8.0). Then the mixture was incubated at 37 °C for 30 min. After that, 0.5 ml of 1.5 M Tri-chloroacetic acid (TCA) was added to stop the reaction. After dilution with distilled water (200 times), 100 µl of Nessler's reagent was added and absorbance was measured at 450 nm. A blank was prepared in the similar way, where, TCA was added before enzymatic reaction. One unit of L-glutaminase was defined as the amount of enzyme that liberates 1 µmol of ammonia under optimum conditions.

Field emission Scanning Electron Microscopy (FE-SEM) and Energy Dispersive X-ray (EDX) study

The surface morphology of solid substrate before and after fermentation was examined using FE-SEM (MERLIN, Zeiss). For this, dehydrated materials were put onto 1 cm ×1 cm glass slide and kept in an auto-sputter (Quorum-Q150R ES) under vacuum for gold coating. Images of surface morphology of the substrate were taken at various magnifications. Energy dispersive X-ray (EDX) spectrum was also taken for analysis of metal composition of solid substrate (Kar et al. 2013).

In vitro cytotoxicity assay

Vero cell line (kidney of an African *Cercopithecus aethiops*) and cancerous HCT (*Homo sapiens* colon colorectal carcinoma) cell line (IMGENEX, India) were maintained at 37 °C in Dulbecco's modified eagle medium (supplemented with Fetal Bovine Serum and 100 µL each of penicillin and streptomycin) (Himedia, India) in a 5.0% CO_2 incubator. Feasible influence of cell lines by addition of partial purified enzyme was determined through MTT assay. Cells were prepared at a concentration of 1.7 x 10^5 cells per well and incubated with 20 µl of L-glutaminase (30.93 U/ml) for 96 h and 20 µl phosphate buffer saline (PBS, pH 7.0) was used as control. Then 20 µl of MTT stock (Himedia) was added to each well and incubated for 4 h at 37 °C. Thereafter, MTT solubilizing agent was added into each well and color intensity measured at 570 nm. The concentration of enzyme needed for 50% (IC_{50}) inhibition of cell growth was determined from the dose response curves for each cell line. The percentage growth inhibition was calculated using the following formula:

$$Growth\ inhibition\ (\%) = 100 - (AE \times \frac{100}{AC})$$

Where, AE and AC are the mean absorbance measured for cell viability in culture medium containing enzyme and PBS, respectively.

Results

Isolation and primary screening of suitable fungi

After primary and secondary screening, the best extracellular L-glutaminase producing fungus designated as KPJ-2 was selected. The phenotypic identification was confirmed by phylogenetic analysis. The sequence homology of 28S rDNA amplicons revealed that KPJ-2 was most similar with *Fusarium nelsonii* CIB04 (GenBank accession no. MN117676.1) (Fig. 1).

Optimization of solid-state fermentation following OVAT approach

Physicochemical conditions are the key factor that moni-

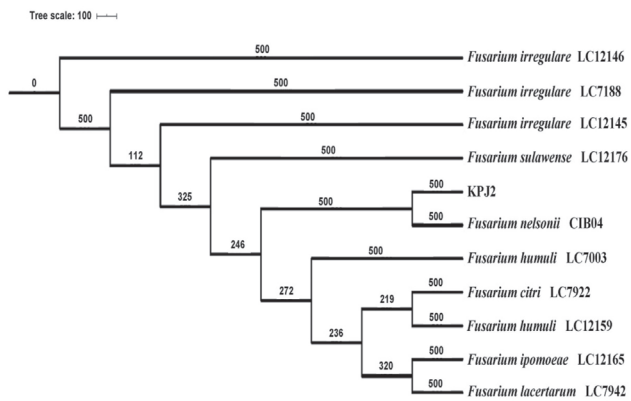

Figure 1. Dendrogram based on 28S rDNA sequence of the isolate KPJ-2 within the genus *Fusarium*. Sequence was aligned with ClustalW2 and tree was constructed by PHYLIP program.

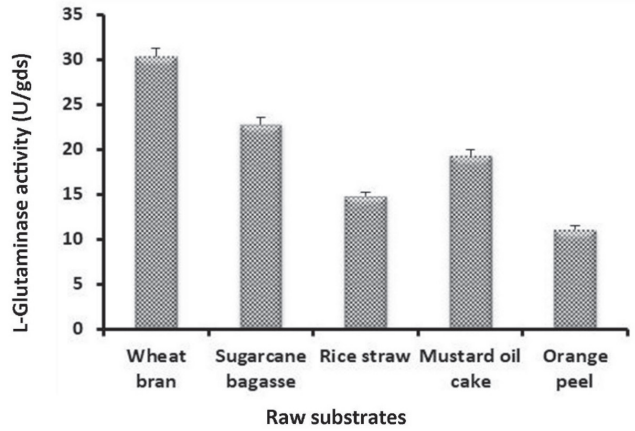

Figure 2. Optimization of different raw substrates for L-glutaminase production.

tored growth of microbes and its productivity (Halder et al. 2012; Elegbede and Lateef 2019). L-glutaminase production by *F. nelsonii* KPJ-2 was carried out under SSF in presence of various agro-industrial residues like sugarcane bagasse, wheat bran, rice straw, mustard oil cake and orange peel. The results revealed that wheat bran supported higher productivity of L-glutaminase (30.38 U/gds) by the isolate (Fig. 2). Among the various concentration of wheat bran, the highest enzyme production of about 47.82 U/gds was found at 7.0% (w/v) (Fig. 3a). Maximum L-glutaminase production by the fungal isolate (47.28 U/gds) was noted in presence of 20.0% (v/w) moistening agent (Fig. 3a).

The pH of the fermentation medium greatly influences the metabolic activities as well as growth of the organism. The highest enzyme activity of 35.53 U/gds was found at pH 4.0 (Fig. 3b). Production of enzyme was also studied in broad range of incubation temperatures (20-50 °C) (Fig. 3b) and optimum enzyme production was noted at 25 °C (41.29 U/gds). Inoculum concentration was found optimum for L-glutaminase production (47.37 U/gds) at 2.0% (v/w, 2×10^2 spore/ml) (Fig. 3b).

Optimization of enzyme production using RSM

Box-Behnken factorial design of RSM was employed for optimization of culture conditions for L-glutaminase production by newly isolated *F. nelsonii* KPJ-2. By taking three levels of five factors, a group of 46 experimental trails were performed. The levels of enzyme production have been represented in Table 1. A quadratic model was generated in response to L-glutaminase production by applying multiple regression analysis of the experimental data. The following second-order polynomial equation was derived:

$$\textit{L-glutaminase activity}\ (Y) = +68.51 + 0.5338 \times A + 0.1756 \times B + 0.9644 \times C + 0.9144 \times D - 1.17 \times E - 0.4900 \times A \times B + 2.33 \times A \times C - 1.55 \times A \times D + 2.06 \times A \times E - 1.86 \times B \times C - 1.69 \times B \times D + 1.46 \times B \times E - 4.61 \times C \times D - 3.86 \times C \times E + 3.45 \times D \times E - 4.85 \times A^2 - 7.62 \times B^2 - 2.90 \times C^2 - 2.87 \times D^2 - 4.91 \times E^2$$

Where, Y represents glutaminase production (U/gds), and A, B, C, D and E are initial pH, temperature (°C),

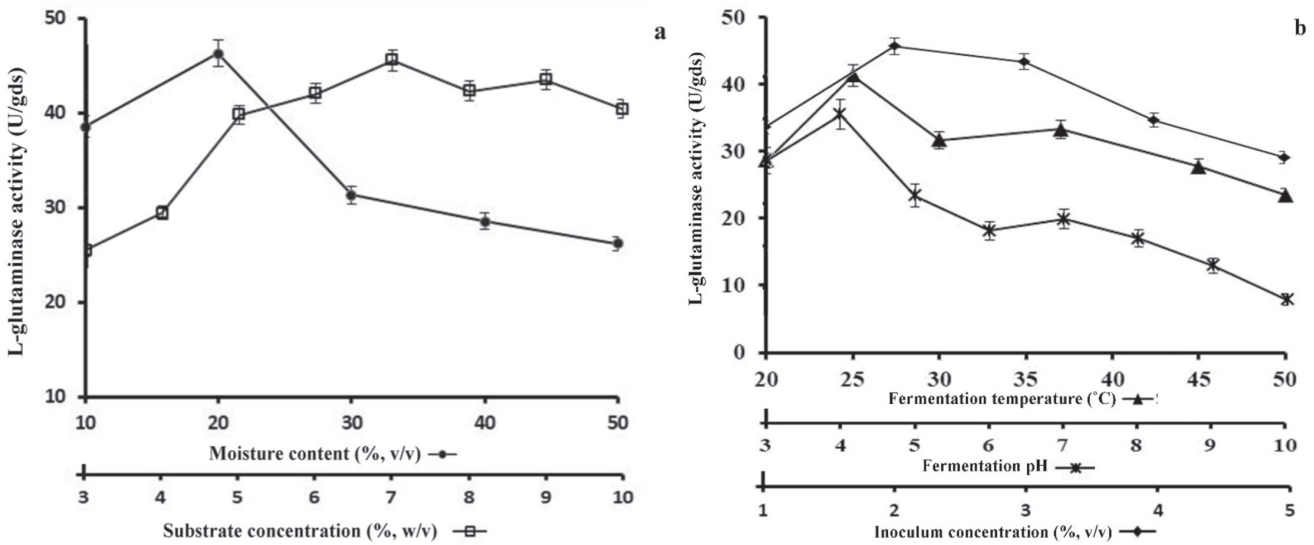

Figure 3. (a) Production of L-glutaminase in different substrate concentration (%, w/v) of wheat bran and moisture content (%, v/v), (b) Production of L-glutaminase in different initial fermentation pH, fermentation temperature (°C) and variable inoculum concentration (%) by strain KPJ-2.

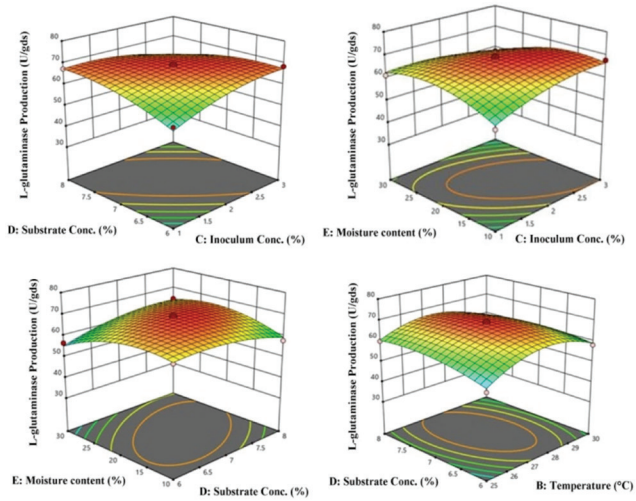

Figure 4. 3D response surface plots showing interactions between l-glutaminase production with substrate and inoculum concentrations, inoculum concentration and pH and temperature concentration and pH respectively using Box-Behnken response surface design.

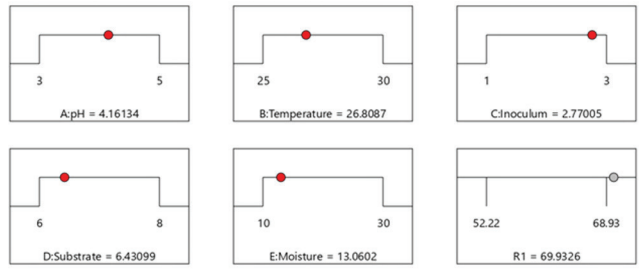

Figure 5. Desirability response surface plots showing the statistically actual concentration of L-glutaminase production using Box-Behnken response surface design.

inoculum concentration (%), substrate concentration (%) and moisture content (%), respectively.

The significant model terms were evaluated by analyzing variance (ANOVA) in the optimization study ($p < 0.01$) and represented in Table 2. The statistical analysis revealed that F-value of 24.61, and Prob > F was less than 0.0001. This result indicated that the model is significant for best utilization of optimized parameters for L-glutaminase production. The lack of fit value 5.08 also implies to be non-significant related to pure error; hence the model seems to be fit (Table 2). There is 3.99% chance of Lack of Fit F-value. The Predicted R^2 of

Table 2 ANOVA for quadratic model representing the F and P value to determine the most significant value.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	997.68	20	49.88	33.26	< 0.0001	Significant
A-pH	6.71	1	6.71	4.47	0.0446	
B-Temperature	0.0361	1	0.0361	0.0241	0.8780	
C-Inoculum	18.60	1	18.60	12.40	0.0017	
D-Substrate	13.38	1	13.38	8.92	0.0062	
E-Moisture	17.26	1	17.26	11.51	0.0023	
AB	0.9604	1	0.9604	0.6404	0.4311	
AC	30.97	1	30.97	20.65	0.0001	
AD	9.64	1	9.64	6.43	0.0179	
AE	16.97	1	16.97	11.32	0.0025	
BC	13.84	1	13.84	9.23	0.0055	
BD	11.46	1	11.46	7.64	0.0106	
BE	15.48	1	15.48	10.32	0.0036	
CD	85.10	1	85.10	56.74	< 0.0001	
CE	59.52	1	59.52	39.69	< 0.0001	
DE	47.61	1	47.61	31.75	< 0.0001	
A^2	192.31	1	192.31	128.23	< 0.0001	
B^2	529.49	1	529.49	353.06	< 0.0001	
C^2	65.92	1	65.92	43.95	< 0.0001	
D^2	71.89	1	71.89	47.93	< 0.0001	
E^2	224.55	1	224.55	149.73	< 0.0001	
Residual	37.49	25	1.50			
Lack of Fit	35.21	20	1.76	3.85	0.0702	not significant
Pure Error	2.29	5	0.4572			
Cor Total	1035.17	45				

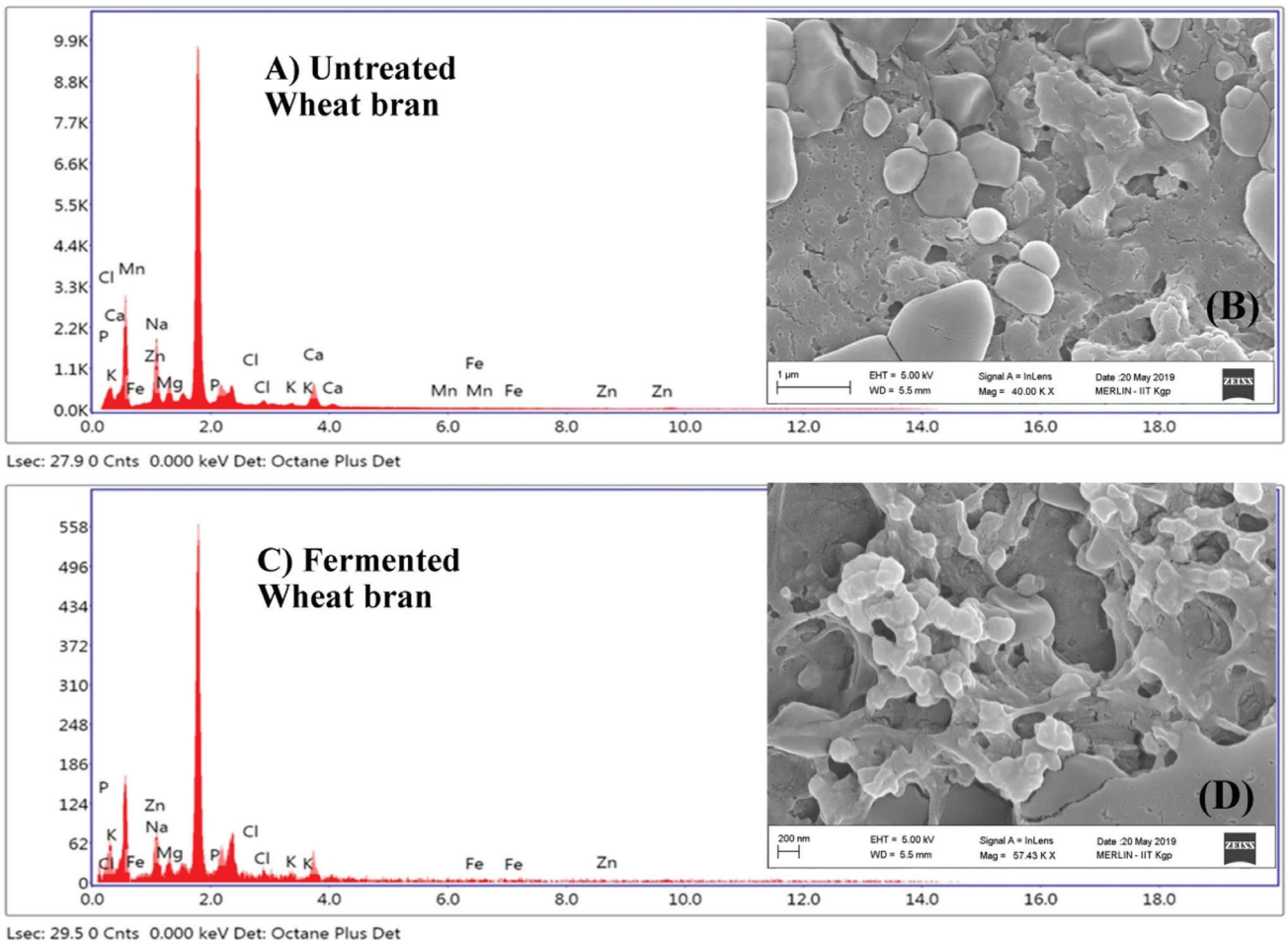

Figure 6. SEM-EDX spectrum of intact wheat bran incubated with strain KPJ-2. Energy dispersion analysis by X-ray (EDX) studies was conducted using the energy dispersive microanalysis of untreated and C) fermented wheat bran. SEM magnification bars show in 200 nm of B) untreated and D) fermented bran.

0.8124 is in reasonable agreement with the Adjusted R^2 of 0.9130 as the difference is less than 0.2. The adequate precision value, which measures the 'signal to noise ratio' was desirable to be > 4.0, and in the present case, the value of the said indices was 16.243. Thus, it can be stated that the model was statistically sound and can be used to navigate the design space (Das et al. 2013). The most significant mutual interaction was noticed among substrate concentration, temperature, moisture content and inoculum concentration for L-glutaminase production by *F. nelsonii* KPJ-2 (Fig. 4).

Confirmation experiment

Using Design Expert 10.0, numerical optimization design space was explored with a fitted quadratic model to arrive at an optimum fermentation condition. The desirability of optimized variables was found using independent function that assigns comparative importance to responses. Solutions with higher desirability for SSF gave an optimum pH of 4.1, fermentation temperature of 26.8 °C, inoculum percentage of 2.3%, moisture content of 13.6% and substrate amount of 6.4% (w/v), which could support L-glutaminase production of 68.93 U/gds (Fig. 5). Under these conditions, confirmation experiments were conducted in three replicates and the activity of 69.75 U/gds was determined, which suggested that experimental and predicted values were in good agreement.

Evaluation of pattern of wheat bran utilization and elemental analysis during glutaminase production

The SEM study revealed the gradual dislodgement of cortical layer and formation of mycelial mat on the surface of wheat bran after 72 h of fermentation. Numerous lateral pores on wheat bran surface were formed due to microbial interaction (Fig. 6). The peak analysis of EDX spectra revealed that the content of sodium, magnesium,

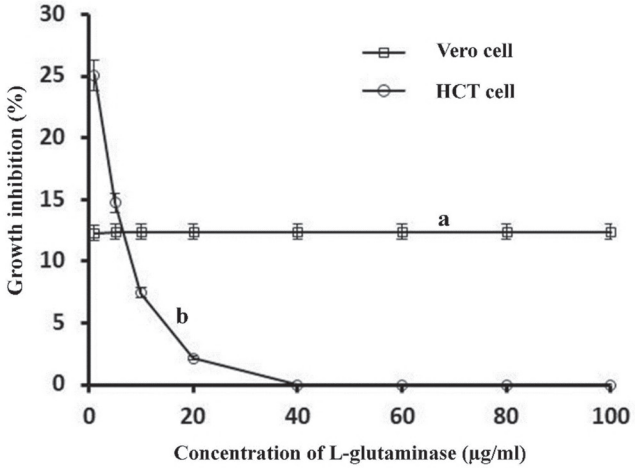

Figure 7. Growth inhibition (%) against different glutaminase concentration (µg/ml) of a) Vero and b) HCT cell line

phosphorous, potassium and calcium ions gradually decreased during course of fermentation and these may utilize for growth of fungus (Fig. 6). These data clearly demonstrated that wheat bran was became decomposed during fungus growth and the ingredients of the bran supported the biosynthesis of L-glutaminase.

In vitro cytotoxicity assay of L-glutaminase

The partial purified L-glutaminase from KPJ-2 has been subjected to cytotoxic study against the HCT and Vero cell line in different doses. The treatment of cancerous cell line (HCT) with increasing concentration of L-glutaminase resulted in appreciable inhibition of cell growth after 48 h in comparison to Vero cells (Fig. 7). This study indicates that this enzyme has potential cytotoxic effect against cancer cells. The concentration of IC_{50} value was found to be 203.95 µg/ml for HCT (Fig. 7). This *in vitro* screening model provides preliminary data on drug selection for clinical trials.

Discussion

In this study, an L-glutaminase producing fungal strain was isolated from the marine resources considering its natural habitat as a salt tolerant organism. Presently marine bioresources have tremendous therapeutic importance for development of anticancer, antibacterial and antiviral drugs (Malve 2016). Based on the morphological and phylogenetic analysis, the isolated organism was identified as *F. nelsonii* KPJ-2. The production of L-glutaminase from marine organism like *Aspergillus* sp. ALAA-2000, and *Beauveria* sp. was also reported by Ahmed et al. (2016) and Sabu et al. (2002), respectively. Selection of suitable substrate for solid state fermentation process is very crucial determinant of fermentation productivity (Nathiya et al. 2011). OVAT method is primarily employed to select the effective physicochemical condition on the growth of organism for production of L-glutaminase. In SSF, among the tested agricultural residues wheat bran supported maximum L-glutaminase production and this suggested the compatibility of this substrate for enzyme production as it may provide optimum carbon, nitrogen and mineral demand for growth of *F. nelsonii* KPJ-2 as well as enzyme synthesis. Nathiya et al. (2011) reported maximum production of L-glutaminase (42.37±0.47 U/g) by *Aspergillus flavus* KUGF009 utilizing tea dust followed by Bengal gram husk, groundnut oilcake, wheat bran and rice bran. Optimum inoculum concentration is essential because low inoculum density may produce insufficient biomass that leads to less product formation, whereas, a higher inoculum size may favor too much biomass formation that deplete the nutrients very fast or accumulates some self-limiting waste products (Kashyap et al. 2002). El-Sayed (2009) and Ye et al. (2013) reported that 2.0% inoculum concentration supports maximum glutaminase production by *Trichoderma koningii* and *Bacillus amyloliquefaciens*, respectively, and our finding is in good agreement with them. Temperature beyond the optimum zone exerted adverse effect on the enzyme production by any microbes as at both low and high temperature the growth of the organism is restricted (Binod et al. 2017). In this study the fungal isolate *F. nelsonii* KPJ-2 produced maximum L-glutaminase at 25 °C, which is in accordance with the report of Kashyap et al. (2002) on *Zygosaccharomyces rouxii.* Moisture level regulates the metabolic activity of the microorganism during SSF process (Pandey et al. 2000). Higher moisture levels may decrease porosity, alter particle structure, enhancement of bacterial growth or low oxygen transfer; whereas, the lower moisture level may lead to improve water tension, lower degree of swelling and reduced solubility of the nutrients of the solid substrate (Raimbault and Alazard 1980; Pandey 1992). For critical analysis of the most vibrant parameters, response surface methodology (RSM) based statistical analysis was carried out. The precise effect of five factors was examined by Box-Behnken factorial design. The relationship between the independent parameter were evaluated by examining the 3D response surfaces curves (Fig. 4) which showed the variation in the yields of L-glutaminase as a function of two variables when the other variables at their central values. Evaluation of response surface curves indicate the range of optimum conditions within the experimental area covered or show the way to confirmative experiments to achieve better results. The observed mean of L-glutaminase activity found to be 68.93 U/gds which is closed to predicted values and hence validates

the model. At optimized condition 14.50% increment in L-glutaminase production was achieved in comparison to un-optimized state.

The SEM-EDX studies show a thin lump of cell aggregates on the surface of wheat bran and distortion of surface layer as well as utilization of its mineral content after 72 h of fermentation. Wheat bran composed of fructans, xylans, cellulose, lignin, galactan in addition to other biomolecules including proteins and minerals (Onipe et al. 2015). The perforation of surface wall of wheat bran indicates KPJ-2 liberated an array hydrolytic enzyme including L-glutaminase which initiates hydrolysis from the surface layer. The enzymatic end products induced the fungus for liberation of reasonable amount of L-glutaminase. The elementary analysis revealed the quantity of iron and zinc ions decrease during fermentation while sodium, magnesium, phosphorous, potassium and calcium ions quantity gradually increase after fermentation. These ions may contribute to maintain the growth of the organism, physiological requirement and conformational stability of the enzyme molecule.

Cytotoxicity study revealed that L-glutaminase arrested cell proliferation of HCT cells with IC_{50} value of 203.95 μg/mL. On the contrary, the enzyme has no impact on Vero cell which implies that it become non-toxic to normal cells and, therefore, well-suited for anticancer or antitumor therapy. The literatures reflected that effectiveness and IC_{50} value of L-glutaminase varied depending upon the target cells. Reda (2015) reported IC_{50} of 64.70 μg/mL as growth inhibition doses for HCT cell line. Singh and Banik (2013) reported that in case of hepatocellular carcinoma (Hep-G2) cell lines IC_{50} dose was 82.27 μg/mL. Also, IC_{50} of 63.30 μg/mL was recorded by Elshafei et al. (2014) against hepatocellular carcinoma (Hep-G2) cell.

Conclusion

This is probably the first report that *F. nelsonii* KPJ-2, which is generally regarded as plant pathogen, is an excellent producer of extracellular L-glutaminase. Utilization of wheat bran as supporting substrate minimizes the production cost, therefore, will be economic and applicable for pharmaceutical and other industries. As the organism isolated from marine environment and the isolated L-glutaminase can restricted the growth of HCT cell line, therefore, this enzyme deserves for human wellness. Future work has been targeted towards the characterization the enzyme property and construction of nano-form structure to enhance its effectiveness.

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