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Optimization of solid substrate mixture and process parameters for the production of L-asparaginase and scale-up using tray bioreactor

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ABSTRACT

L-asparaginase is a key enzyme that degrades asparagine and this aspect of the enzyme has found a dominant role in chemotherapeutic and food processing industry. The aim of the present study is to sequentially optimize L-asparaginase production from newly isolated *Aspergillus* sp. using agro-industrial residues as solid substrate. In the first stage, through simplex centroid design maximum L-asparaginase activity was observed using a ternary mixture of cotton seed cake (2/3), wheat bran (1/6), and red gram husk (1/6). In the next step, cultivation parameters such as pH, temperature and moisture content were optimized using Box-Behnken design. After 6 days of fermentation using optimized ternary mixture, maximum activity of 12.57 U/mL was obtained at temperature 35 °C, pH-8 and moisture content 70% (v/w). Optimized bounds were further translated to lab-scale tray bioreactor and L-asparaginase activity was found to be 5.41 and 6.67 U/mL in tray bioreactor with 500 g and 1000 g of substrate mixture respectively. L-asparaginase production increased 5 fold through mixture design and 1.3 times increase in enzyme activity was observed with Box-Behnken design. Present work signifies the importance of optimization in the bioprocess industry for complete understanding and evaluation of enzyme production.

1. Introduction

L-asparaginase is an amido-hydrolytic enzyme that received tremendous attention ever since its inhibitory action on lymphoma 6C3HED cells were demonstrated (Broome, 1961). Subsequently, L-asparaginase is considered as anti-leukemic agent which can convert asparagine in tumour cells to aspartic acid and ammonia leading to death of cancer cells (Hill et al., 1967). Besides this, L-asparaginase also finds potential application in food industry to inhibit acrylamide development in fried and baked foods by depleting the precursor (Hendriksen et al., 2009). For the treatment of Acute Lymphoblastic Leukaemia (ALL), L-asparaginase from *Escherichia.coli* and *Erwinia chrysanthemi* are currently in use (Kumar and Sobha, 2012). Even with wide applications, L-asparaginase production has few challenges one being that the enzyme obtained from prokaryotes induces adverse reactions due to the presence of other contaminating enzymes such as L-glutaminase and urease. To lower these toxic effects associated with bacterial L-asparaginase, fungi is chosen as it is eukaryotic and evolutionarily closer to human. Numerous fungal species were isolated and tested for their ability to synthesize glutaminase free L-asparaginase in order to reduce immunological reactions in the course of ALL treatment (Doriya and Kumar, 2016).

On the other hand, existing industrial production of L-asparaginase is carried out using submerged fermentation (SmF) at elevated production cost (Cachumba et al., 2016). However, in the past few years solid state fermentation (SSF) with agro- industrial residue as substrate have received tremendous focus for production of enzyme. SSF is the process that enables cultivation of microorganism on non-soluble substrate with minimal water activity. Though, solid substrate must possess adequate water activity to support the growth and metabolism of micro-organism (Pandey, 2003). In comparison to SmF, SSF provides high enzyme titers and provides natural environment to microbes as similar to composting process (Couto and Sanromán, 2006). Among all the microflora investigated, filamentous fungi fit better for cultivation in SSF, as the hyphal mode of fungi contribute to consumption of nutrients from solid substrate (Raimbault, 1998). Various agro-industrial residues such as soy bean meal, leguminous crops, red gram husk, bengal gram husk, ground nut cake, coconut oil cake, rice bran, sesame oil cake and wheat bran have been used for the production of L-asparaginase (Abdel-fattah and Olama, 2002; Ghosh et al., 2013; Hosamani and Kaliwal, 2011; Hymavathi et al., 2009; Mishra, 2006; Venil and Lakshmanaperumalsamy, 2009).

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Optimization of fermentation medium involves selection of significant medium components and further optimizing concentration of each selected component (Rispoli and Shah, 2007). In mixture experiments, proportions of the individual components in the mixture are accountable for the final response (Cornell, 1973). Several optimization tools such as Box-Behnken design are frequently taken into account for lowering number of experiments. As a result, chemical consumption, laboratory work and time needed to perform can be reduced. In comparison to one variable at a time method, chemical consumption and laboratory work can be reduced using multi-variate techniques. For the development of any bioprocess, it is important to optimize physical parameters and their interactions that affect the process. Box- Behnken design is a three-level design, used to evaluate parameter coefficients with second degree polynomial (Box and Behnken, 1960).

To date, most of the experiments conducted using SSF were of single substrates. In the current study, simplex centroid design has been employed to evaluate solid substrates for their synergistic or antagonistic effect using *Aspergillus* sp. In the next phase Box-Behnken design was used to study the interactive effects of physical parameters (pH, temperature and moisture content). Further, optimized results using mixture design and Box-Behnken design were transferred to 50 g and 100 g in flask, 500 g and 1000 g in tray bioreactor.

2. Material and methods

2.1. Microorganism and culture maintenance

Fungal strain C_7 was isolated from solid substrate as reported earlier and was grown on potato dextrose slants at 30 °C for 5 days and were stored at 4 °C. Isolated fungi was identified as *Aspergillus* sp. The isolated strain has the ability that it is free of glutaminase and urease (Doriya and Kumar, 2016).

2.2. Simplex centroid design to screen solid substrates

Wheat bran, cotton seed oil cake and red gram husk were tested in various combinations using mixture design at a fixed total concentration of 10 g for L-asparaginase production. 10 g of solid mixture was collected in 250 mL Erlenmeyer flasks and supplemented with 0.1 M potassium phosphate buffer (pH 7.0) to maintain initial moisture content of 70%(v/w) and then sterilized at 121 °C for 15 min 1 mL (5×10^7 spores per mL) of homogenized inoculum was added to the flasks and incubated at 30 °C for 7 days. After 144 h of incubation, crude enzyme was extracted and L-asparaginase activity was determined. A total of 14 experiments (Table 1) were conducted and analysed using Design expert version 9.0 (Stat-Ease Inc., Minneapolis, USA).

Table 1
Simplex centroid design layout for L-asparaginase production using varied proportions of solid substrates; cotton seed cake, wheat bran and red gram husk.

Run	Component 1 Cotton seed cake	Component 2 Wheat bran	Component 3 Red gram husk	L-asparaginase activity (U/mL)	Specific activity (U/mg)
1	0.00	0.00	10.00	2.65	1.09
2	5.00	0.00	5.00	7.65	2.65
3	5.00	5.00	0.00	6.39	3.12
4	0.00	0.00	10.00	2.62	0.90
5	6.67	1.67	1.67	9.43	4.60
6	10.00	0.00	0.00	4.43	2.12
7	5.00	5.00	0.00	6.6	3.03
8	3.33	3.33	3.33	7.05	3.04
9	1.67	6.67	1.67	5.45	2.36
10	1.67	1.67	6.67	4.33	1.72
11	0.00	5.00	5.00	8.38	2.56
12	10.00	0.00	0.00	4.23	2.05
13	0.00	10.00	0.00	1.78	0.72
14	0.00	10.00	0.00	1.78	0.72

Table 2
Experimental ranges and the levels of the independent variables involved in optimization using Box-Behnken design.

Factor		Coded values		
		−1	0	+1
A	Temperature (°C)	25	30	35
B	pH	6	7	8
C	Moisture Content (% v/w)	60	70	80

$$Y = \sum \beta i xi + \sum \beta ij xixj$$

Where Y is the response function, βi is the linear coefficient, βij is the interaction terms, xi,xj are independent variables.

2.3. Box Behnken design to optimize culture conditions

Box-Behnken design was used as a following step of optimization with pH, moisture content and fermentation temperature as process variables using optimized tri-substrate composition. For statistical calculations parameters were coded as shown in Table 2. Cultivation parameters were varied at three levels as −1, 0 and +1 and experiments were conducted according to the design matrix shown in Table 3. A total of 17 experiments with 4 replicates were carried out to predict the correlation between variables and response. L-asparaginase activity was examined at design points to determine the optimum level of variables. Design-Expert was used to design and analyse the experiments. ANOVA was performed and results were used to fit a quadratic equation by multiple regressions and the interactions were assessed using response surface plots.

2.4. Scale-up of L-asparaginase production

50 g and 100 g of optimized solid mixture containing cotton seed cake: wheat bran: red gram husk are taken in the ratio of 6.5:1.5:2 in 500 mL and 1LErlenmeyer flask respectively. Mixture was moistened with 0.1 M phosphate buffer at pH-8 and was sterilized at 121 °C for 15 min. After cooling the substrates to room temperature, inoculum was added and incubated at 35 °C for 6 days. Similarly, further scale-up in a tray bioreactor was performed using a steel tray of dimensions' length: 45.2 cm, breadth: 42.7 cm and height: 2.5 cm. 500 g and 1000 g solid tri-substrate mixture was moistened with phosphate buffer and autoclaved at 121 °C for 15 min. Solid mixture was distributed on trays

Table 3
Evaluation of L-Asparaginase activity using Box–Behnken design.

Run no.	Temperature (°C)	pH	Moisture Content (% v/w)	L-asparaginase activity (U/mL)	Specific activity (U/mg)
1	25	8	70	7.09	3.19
2	35	7	80	9.08	4.21
3	35	8	70	12.57	5.69
4	25	7	60	6.74	3.17
5	25	6	70	6.46	3.36
6	30	7	70	9.29	4.65
7	35	7	60	5.06	2.27
8	30	6	80	4.82	2.69
9	35	6	70	6.91	3.31
10	30	7	70	9.29	4.58
11	30	8	60	5.17	2.13
12	30	7	70	9.32	4.62
13	30	7	70	9.36	4.80
14	30	8	80	7.89	4.11
15	30	6	60	4.71	2.42
16	30	7	70	9.32	4.36
17	25	7	80	7.72	4.06