GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

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AUGMENTATION OF EFFICIENCY OF SOLID STATE FERMENTATION BY MODIFYING THE CONVENTIONAL REACTOR DESIGN

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ABSTRACT
Considering the limitations of the conventional solid state fermentation in trays (SSF-T), a process modification in the form of solid state fermentation in stacked layers (SSF-SL) was constituted and compared with the former. The later was furnished with a newly devised drying treatment and a replacement of trays by metal gauzes which probably were responsible for an increase of 38.9 % in the production of fungal pectinase under optimized conditions. Univariate optimization of important process parameters of SSF-SL was done to determine their optimum levels which were then used to design the experiments in central composite design (CCD). The fit summary analysis suggested the use of quadratic model and the F-test showed that the model was highly significant at p=0.0001 with high regression coefficient of 0.933. Curvature in the response surfaces demonstrated the nature of interactions between independent variables. The interactions involving the duration of drying treatment suggested the sensitivity of the method to the parameter and its significance in the enhanced productivity.

KEY WORDS: SSF, fungal pectinase, stacked layers, drying treatment, CCD, RSM

INTRODUCTION

Numerous industrially important substances such as extracellular enzymes, organic acids, ethanol, bio-fuels and antibiotics are produced on a large scale using solid state fermentation (SSF) (Couto and Sanroman, 2006). The usefulness of SSF to the industry may be attributed to a relatively higher productivity, lower energy requirement, simpler production media, easy recovery of the product, less or no effluent, lesser chance of contamination and easy waste management (Paranthaman *et al*., 2009). Besides these benefits, certain limitations of SSF are reported in the literature (Couto and Sanroman, 2006). Only few restricted groups of micro-organisms can effectively grow under the SSF conditions, difficulty in regulation and control of process parameters like temperature, pH, humidity (Falony *et al*., 2006), intra particle oxygen diffusion (Oostra *et al*., 2001) and heat and mass transfer limitation (Rajagopalan and Modak 1995) are some of the drawbacks.

Attempts to understand and hence minimize the shortcomings by means mathematical modeling, redesigning the bioreactor and study of process kinetics have been made with some success (Mitchell *et al*., 2003). Research in modification of reactor design is expected to provide feasible alternatives. The objective of this study was to incorporate alterations in the conventional process of SSF. The research deals with comparison of the conventional SSF in tray with the modified SSF in stacked layers for the production of extracellular fungal pectinase using *Aspergillus foetidus* (NCIM 505), followed by the statistical optimization of key process parameters of the altered method using central composite design and response surface methodology.

MATERIALS AND METHODS

Microorganisms

The organism used in the current study was *Aspergillus foetidus* (NCIM 505). The strain was grown on potato dextrose agar (PDA) slants at 28 °C for 5 days and was maintained at 4 °C (Rauf et al., 2010). Inoculum was prepared by suspending the spores from a PDA slant by adding sterile distilled water. A final spore count of 1×10^6 spores/ml was obtained.

Substrate and Inducer

Sieved wheat bran (80-mesh) was washed with tap water to remove the impurities. It was then pretreated to make it more suitable for fungal penetration by partially soaking it in tap water for an hour followed by drying at 80°C (Guneet Kaur and Satyanarayana T, 2004).Orange peel used as an inducer in the optimization experiments too was washed, dried, powdered and sieved (80 mesh) on the above line.

Moistening agent

Mineral solution used as a moistening agent was constituted of 0.1% (w/v) of $MgSO_4$ and $CaCl_2$ and 0.05% of $FeSO_4$ and $ZnSO_4$. The pH of the solution was adjusted to 5.0 with components of phosphate buffer *viz*. NaH_2PO_4 and KH_2PO_4.

Methods used for the production of pectinase

Following two methods were compared for the pectinase production

Method 1: *Solid State Fermentation in Tray (SSF-T) – a conventional method*

A 3 mm thick layer of the fermentation medium, prepared by mixing 100 g pretreated wheat bran with 70 ml mineral solution to get a final substrate: moisture ratio of 1.0: 0.7 w/v (70%) was laid in a metal tray. The medium was then sterilized by autoclaving at 121^{o}C for 30 min, inoculated with 5ml of spore suspension and incubated at 28 ^{o}C for 5 days. A pile of 4 such trays was prepared. The assembly was not artificially aerated at any point of incubation. The moisture content and spore count of the inoculated bran was estimated at intervals of 24 hours, during the incubation period.

Method 2*: Solid State Fermentation in Stacked Layers (SSF-SL) – a modified method:*

A 3 mm thick layer of the fermentation medium, prepared as mentioned above was laid on square shape wire gauze: 10 cm x 10 cm in size. The medium was sterilized by autoclaving at 121^{o}C for 30 min, inoculated with 5ml of spore suspension and incubated at 28 ^{o}C for 5 days. Four such gauzes were prepared and tied together at the corners using sterilized nylon threads to form a pile without any gap in between, under laminar air flow.

During the incubation period sterile air was blown over the assembly for 15 minutes, after every 24 hours of incubation and the reduction in moisture content was determined by drying it to the constant weight (Adinarayana *et al.,* 2004). The minimum moisture level of 35% as in the substrate: moisture ratio of 1.0: 0.35 w/v was ensured. The system was allowed to withstand the dry condition for four hours. After that the loss in moisture was compensated with spraying of suitable amount of sterilized distilled water over the system and thus the substrate: moisture ratio of 1.0: 0.7 w/v (70%) was restored. As an indicator of magnitude of fungal growth, spore count of the inoculated bran was estimated using Neubauer's chamber, at intervals of 24 hours during the incubation period, just prior to the drying treatment.

The two methods were compared with respect to the pectinase production and the spore count. The better of the two was considered for the further study.

Extraction and Assay of pectinase

Extraction of pectinase was done by shaking the moldy bran with 250 ml of 0.05 M citrate buffer (pH 5.0) for 30 min followed by filtration through muslin cloth. The filtrate was centrifuged and the supernatant was used as a crude source of the enzyme.

Assay of pectinase was done using viscometery in a manner similar to the assay of hydrolase activity of pectinase described by Bhat *et al.* (2012) The percent reduction in viscosity (R) of the substrate was determined using the formula:

$$R = [(Tc - Te) / (Tc - Tw) * 100$$

Tc and Te indicate the flow time of the substrate in the viscometer, for control and experimental sets respectively while Tw indicates that for water.

Enzyme activity (U) was expressed as the percent reduction in viscosity of the substrate after 10 min of its incubation with the enzyme and reported as U per ml of the crude enzyme.

Univariate optimization of the process parameters of SSF-SL

The process parameters optimized for the pectinase production by univariate strategy, were the amount of inducer, initial moisture content, amount of ammonium sulphate supplemented (nitrogen source), pH of mineral solution and duration of the drying treatment. In this approach, the magnitude of only one of the independent parameters was varied in a fixed range at a time.

Statistical optimization of the process parameters of SSF-SL

Optimization of the SSF-SL for the pectinase production was based on initial moisture content of the fermentation medium (X_1), amount of ammonium sulphate supplemented through the mineral solution (X_2), proportion of inducer (powdered orange peel) in dry fermentation medium (X_3) (Hande Demir *et al.,* 2012), the duration of the drying treatment (X_4) and their interactions.

A 4 variable – 5 level central composite design (CCD) with total 30 experiments (16 factorial points, eight star points and six replicas of the center point) was employed to fit the following polynomial model.

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4$$

Y – Response (Pectinase activity)
β – Regression coefficients

The five levels of the four variables (X_1 - X_4) in the coded and actual values are shown in Table 1.

TABLE 1. Experimental domain in terms of coded and actual values of the process parameters

Variables	Coded Levels				
	-2	-1	0	1	2
X_1	50	60	70	80	90
X_2	1	2	3	4	5
X_3	5	10	15	20	25
X_4	1	2	3	4	5

[X_1 is volume (ml) of mineral solution added per 100g dry fermentation medium (expressed as % initial moisture), X_2 is weight (g) of ammonium sulphate added through the mineral solution per 100g dry fermentation medium (expressed as % w/w), X_3 is weight of powdered orange peel (inducer) in 100g of dry fermentation medium (expressed as %) and X_4 is duration of drying treatment in hours]

The five level - four factors CCD consisting of 30 experiments with different combinations of these levels is expressed in Table 2.

TABLE 2. Central composite design (16 factorial points, eight star points and six center points)

Standard run order	Run number	X_1	X_2	X_3	X_4	Combination points
1	30	60	2	10	2	Factorial points
2	14	80	2	10	2	
3	12	60	4	10	2	
4	7	80	4	10	2	
5	17	60	2	20	2	
6	28	80	2	20	2	
7	1	60	4	20	2	
8	8	80	4	20	2	
9	3	60	2	10	4	
10	23	80	2	10	4	
11	24	60	4	10	4	
12	21	80	4	10	4	
13	16	60	2	20	4	
14	2	80	2	20	4	
15	26	60	4	20	4	
16	10	80	4	20	4	
17	9	50	3	15	3	Star
18	15	90	3	15	3	points
19	4	70	1	15	3	
20	19	70	5	15	3	
21	27	70	3	5	3	
22	5	70	3	25	3	
23	13	70	3	15	1	
24	6	70	3	15	5	
25	22	70	3	15	3	Center
26	29	70	3	15	3	points
27	25	70	3	15	3	
28	18	70	3	15	3	
29	20	70	3	15	3	
30	11	70	3	15	3	

Response surfaces and contour plots were generated to understand the interactions between the four process parameters.

SSF-SL was performed at the combination of levels of the process parameters that resulted in the best response from CCD experiments and the response was compared with that of non optimized SSF-SL.

RESULTS AND DISCUSSION

The results of pectinase production using SSF-T and SSF-SL (mean of triplicate with standard deviation) are shown in Figure 1.

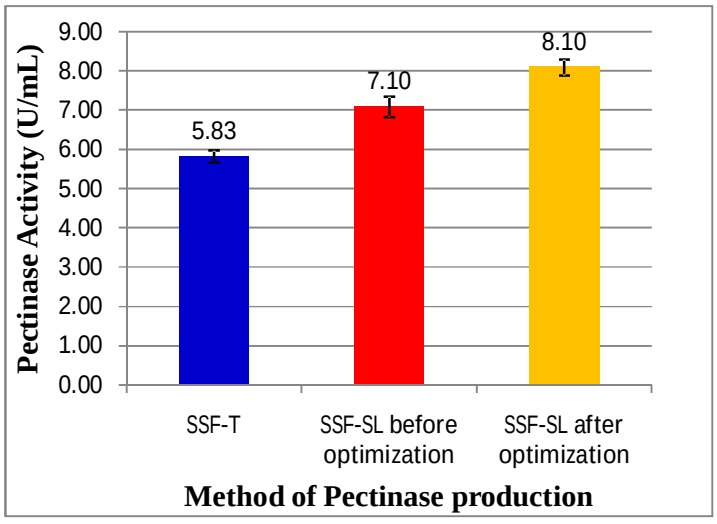


FIGURE 1. Production of pectinase in the conventional and modified methods
[SSF-T – Solid state fermentation in trays, SSF-SL – solid state fermentation in stacked layers]

Before being optimized the SSF-SL showed 21.78 % increase in pectinase production and slight decrease in the fungal growth (in terms of spore count) with respect to SSF-T (Fig. 2). The productivity of SSF-SL under optimized conditions was found to be more than that in SSF-SL before optimization by 14.08 %.

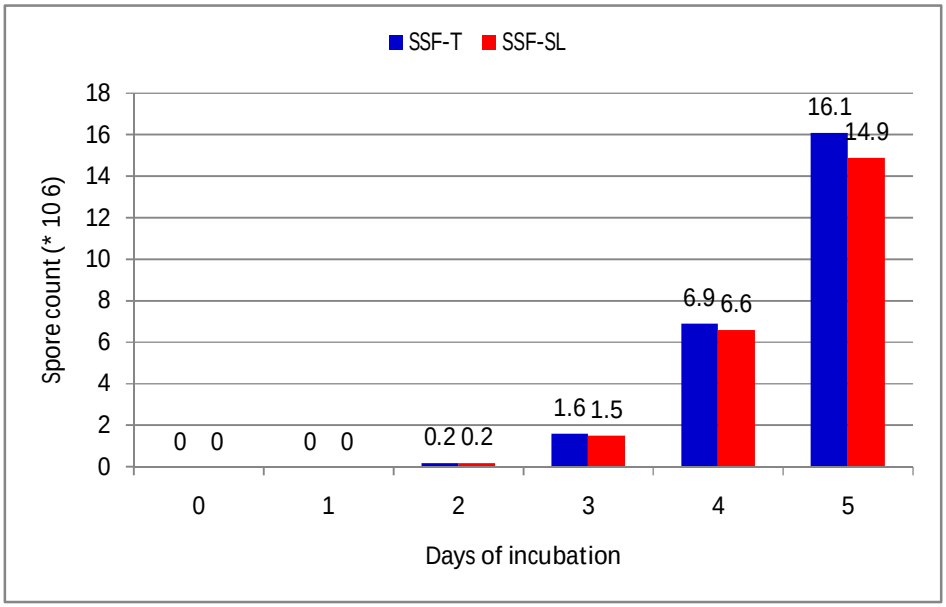


FIGURE 2. Spore count over the incubation period in the conventional and modified methods

The findings may be attributed to a better availability of oxygen to the aerobic microorganism due to the use of wire gauzes instead of trays and incorporation of the drying treatment in the method, as was also evident from the reduction of the compactness of the medium. Another aim of the drying treatment was to temporarily retard the fungal growth thereby enforcing the induction of pectinase production. Higher moisture levels are believed to interfere with the oxygen penetration**,** whereas lower levels retard the fungal growth (Mekala et al., 2008). Further the reduced moisture level for a short duration did not result in a permanent loss of viability of the mould as evident from the continuous increase in the spore count over the incubation period.

The slightly less spore count in SSF-SL with respect of SSF-T may be attributed to the system withstanding the drying treatment daily. Thus the SSF-SL achieved an increased productivity with minimum changes in the currently available design of SSF-T unlike most of the reactors available for SSF (Couto and Sanroman 2006). SSF-SL was hence selected for the further optimization. Results of univariate optimization of five process parameters are shown in Table 3.

TABLE 3. Univariate optimization of five process parameters of SSF-SL

Process parameters	Range selected (Interval between two levels)	Observed optimum
Inducer (weight of powdered orange peel in 100g of dry fermentation medium, expressed as %)	5% to 30% (5%)	15%
Initial Moisture content (volume in ml of mineral solution added per 100g dry fermentation medium, expressed as % initial moisture)	30% to 90% (10%)	70%
Nitrogen source (weight in gram of ammonium sulphate added through the mineral solution per 100g dry fermentation medium, expressed as % w/w)	0.5% to 5% (0.5%)	3%
pH of mineral solution	4.0 to 8.0 (1.0)	6.0
Duration of drying treatment (expressed in hours)	1 hr to 6 hr (1 hr)	3hr

The peakedness (kurtosis) of the normal curve for pH appeared to be very less and hence the remaining four parameters were selected for CCD experiments. CCD is a very useful statistical tool where the optimal level of the process parameters and their interaction can be determined.

The actual and predicted pectinase activity values (response) of the 30 experiment CCD are expressed in Table 4.

TABLE 4. Actual and predicted response values

Standard run order	Pectinase Activity (U/ml)	
	Actual	Predicted
1	3.5	3.94
2	4	4.26
3	5.6	5.70
4	6.1	6.27
5	6	5.83
6	6.9	6.65
7	7	6.79
8	8.2	7.86
9	3.9	3.95
10	4.2	4.57
11	5	5.40
12	6.4	6.28
13	5.6	5.59
14	7.1	6.71
15	6.8	6.25
16	7.9	7.62
17	6	5.92
18	7.4	7.62
19	5.1	4.88
20	7.2	7.55
21	5.5	4.60
22	6.8	7.83
23	4.4	4.33
24	3.9	4.10
25	8.1	7.97
26	7.3	7.97
27	7.8	7.97
28	8.4	7.97
29	8.3	7.97
30	7.9	7.97

It can be observed that most of the high pectinase activity responses were clustered at the center points (run number 22, 29, 25, 18, 20 and 11) with few exceptions. The maximum and the minimum response values were 8.4 U/ml and 3.5 U/ml respectively. The fit summary comprising of sequential model sum of squares and lack of fit tests suggested a quadratic model to be preferentially used over a linear, two factor interactions or cubic model. The statistical significance of the regression model was checked by the *F*-test. The analysis of variance for the response surface quadratic model is shown in Table 5.

TABLE 5. Analysis of Variance for the response

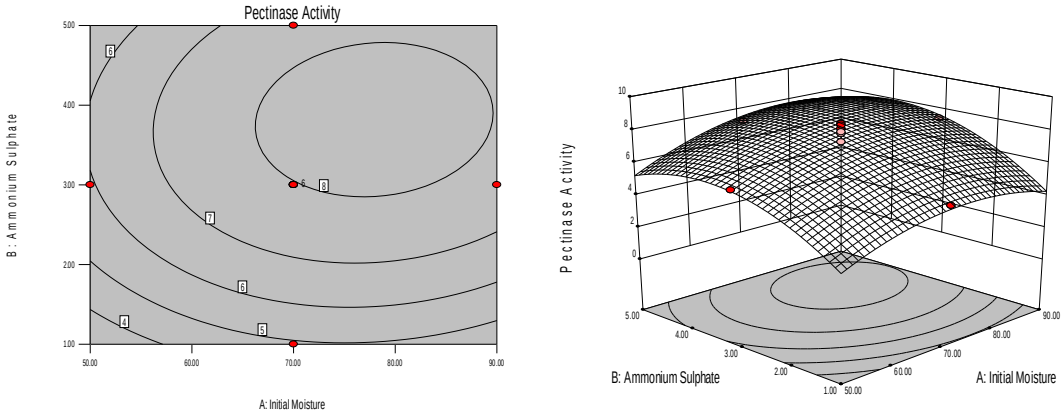
Source	Df	Sum of squares	F Value	P > F
Model	14	60.93	15.05	< 0.0001
X_1	1	4.34	14.99	0.0015
X_2	1	10.67	36.87	< 0.0001
X_3	1	15.68	54.21	< 0.0001
X_4	1	0.08	0.28	0.6030
X_1*X_2	1	0.06	0.22	0.6487
X_1*X_3	1	0.25	0.86	0.3673
X_1*X_4	1	0.09	0.31	0.5852
X_2*X_3	1	0.64	2.21	0.1576
X_2*X_4	1	0.09	0.31	0.5852
X_3*X_4	1	0.06	0.22	0.6487
X_1^2	1	2.47	8.53	0.0105
X_2^2	1	5.25	18.15	0.0007
X_3^2	1	5.25	18.15	0.0007
X_4^2	1	24.11	83.34	< 0.0001
Residual	15	4.34		

The F value = 15.05 and ($P_{model} > F$) < 0.0001 imply that the model was highly significant. High R^2 value of 0.933 marked the goodness of fit (Haaland, 1989). The adjusted R^2 of 0.87 reconfirmed the significance of the model.

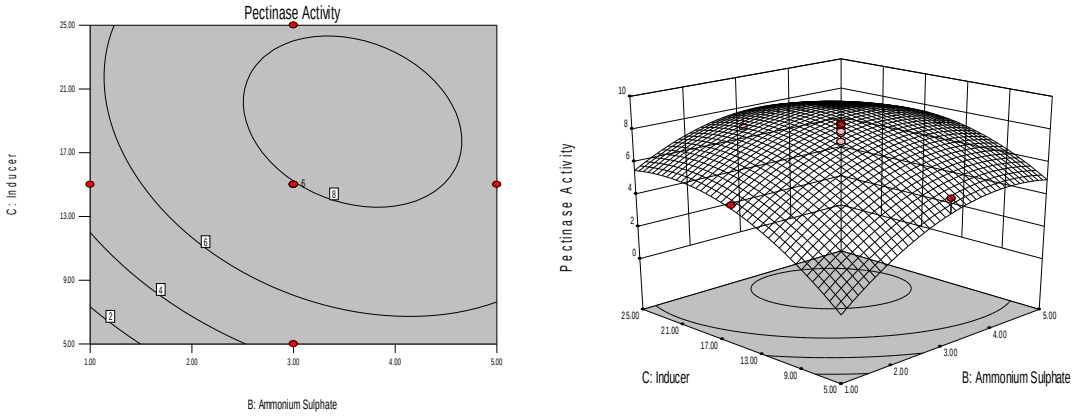
Final equation in terms of coded factors suggested by the model can be expressed as:

$$Y = 7.97 + 0.43*X_1 + 0.67*X_2 + 0.81*X_3 - 0.058*X_4 + 0.063*X_1*X_2 + 0.13*X_1*X_3 + 0.075*X_1*X_4 - 0.20*X_2*X_3\ 0.075*X_2*\ X_4 - 0.063*X_3*X_4 - 0.30*X_1^2 - 0.44*X_2^2 - 0.44*X_3^2 - 0.94*X_4^2$$

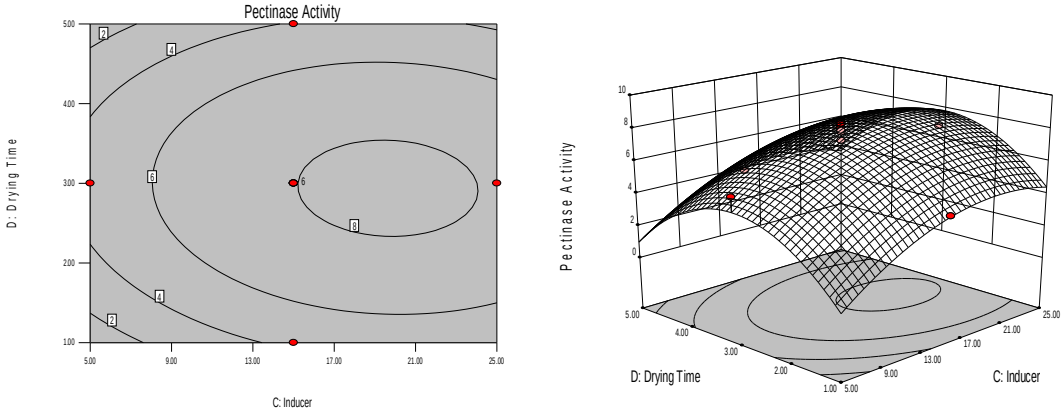
The contour and 3D response surface plots generated to determine the interactions between the process parameters that caused effect on pectinase production are shown in Figure 3A to 3E.



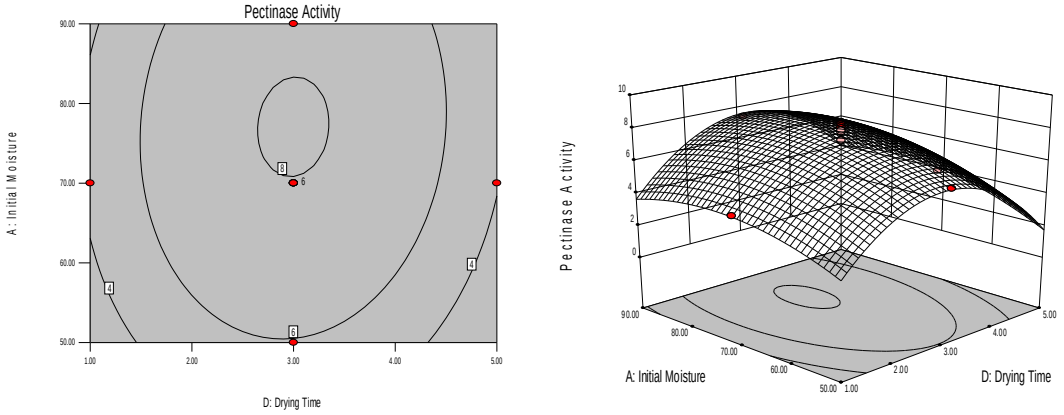
A. Initial moisture – Ammonium sulphate interaction



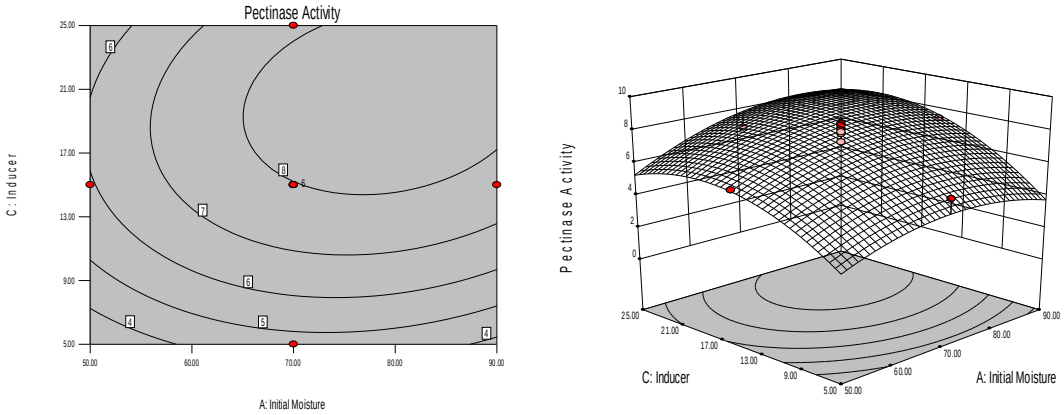
B. Ammonium sulphate – Inducer interaction



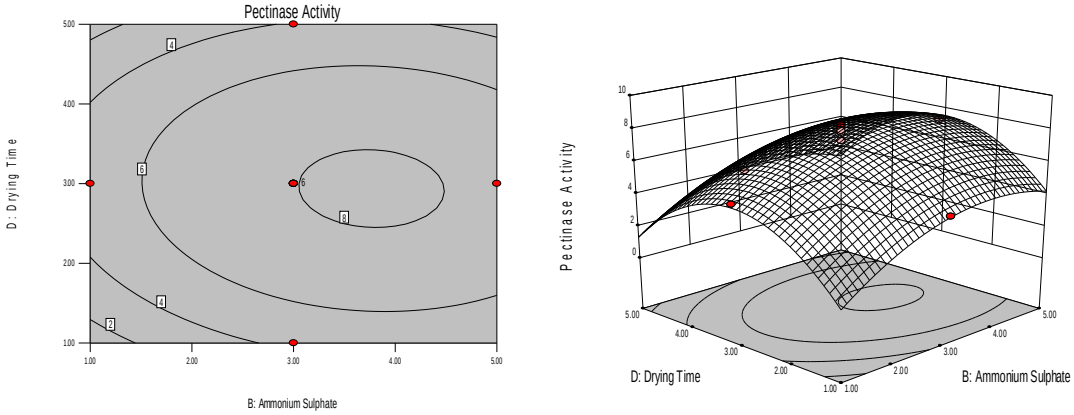
C. Inducer – Drying time interaction



D. Drying time – Initial moisture interaction



E. Initial moisture – Inducer interaction



F. Ammonium sulphate – Drying time interaction

FIGURE 3.Contour and Response surface plots for pectinase production

The plots describe the effect of interaction of two out of four parameters over a range of -2 to +2 at the middle levels (0) of remaining two parameters on the pectinase production. Findings indicated the presence of curvature with a maximum in the studied range (Dilipkumar *et al*., 2011).

The interactions between X_1 and X_2 (A), X_2 and X_3 (B) and X_1 and X_3 (E) appeared to be mutual with a shift in peak of the pectinase production towards their respective (+1, +1) positions. The X_3 and X_4 (C) and X_2 and X_4 (F) interactions appeared to shift the peak of the pectinase production near their respective (+1, 0) positions while that between X_4 and X_1 (D) shifted the peak near (0, +1)

position. These three interactions seemed to be under a total control of the drying time as any change in it adversely affected the response. Though beneficial at lower levels, a higher concentration of readily available nitrogen source adversely affects the production of inducible enzymes (Valeria *et al.*, 2003). The response peak near (+1, +1) position in figure 3A revealed that the negative impact of higher level (+1) of ammonium sulphate was not seen at a higher moisture level (+1). This may be attributed to the dilution effect. With lower moisture value the response got worsened even with decrease in the level of ammonium sulphate. A similar response was observed in moisture – inducer interaction (figure 3E). The orange peel has substantial pectin in it whose rheological aspect suggests its tendency to form gel with water (Danielle *et al*., 2009). A higher inducer level means more of pectin, which therefore like most of the other hetero polysaccharides requires more of water to be effectively utilized and thus attain the desired water activity (a_w).

The peak position in figure 3B suggested that the negative impact of higher level (+1) of one of the two parameters on the response is nullified by the higher levels (+1) of the other. The nutritional deficiency at higher inducer level, due to a corresponding reduction in the substrate, seemed to be taken care of by the higher level of ammonium sulphate. Alternatively, at higher ammonium sulphate level, a higher inducer level might be required for the maximum induction.

Other interactions involving X_4 (figure 3C, 3D and 3F) appeared to be one dimensional. The optimum level of drying time (0) appeared to produce more response at +1 level of other three parameters instead at their middle level (0). More significantly, any deviation in the duration of drying time resulted in a declined response at all the levels of the interacting parameters. This indicated the important role of the parameter in enhancing the pectinase production in SSF-SL over SSF-T.

CONCLUSION

A comparison of conventional method of solid state fermentation (SSF-T) with the modified method (SSF-SL) was done in the first place. The results exhibited that there was a significant ($p<0.005$) increase of 21.78 % in the pectinase production in the non optimized SSF-SL over the conventional method which further increased by 14.08 % over the non optimized SSF-SL in the case of SSF-SL under optimized conditions (a net increase of 38.9 % over SSF-T). This superior response observed in the SSF-SL can be attributed to the use of wire gauzes instead of trays and the drying treatment that was incorporated in the method. Statistical optimization of the modified method was done using central composite design experiments and response surface methodology. The pectinase activity was found to be between the highest and the lowest values of 8.4 U/ml and 3.5 U/ml respectively and the curvature in the response surfaces suggested the nature of interaction between the studied parameters. The F value of 15.05 suggested the significance of the model at $p<0.0001$ while the near one R^2 value of 0.933 revealed the goodness of fit of the model.

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