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COVERED TRAY SSF AND HYDROPONICS LIKE SSF - NOVEL ALTERNATIVE METHODS TO THE CONVENTIONAL SOLID STATE FERMENTATION

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ABSTRACT
The work was focused at developing new methods, the Covered-Tray and Hydroponics-like solid state fermentation (CT-SSF and HP-SSF) and comparing them with conventional solid state fermentation in trays (T-SSF) for the extracellular protease production by *Aspergillus flavus* (NCIM 535) using wheat bran. The Revolving Drum SSF method, an available alternative to T-SSF, was used as a positive control. The protease production using CT-SSF was equivalent to that using the T-SSF whereas the HP-SSF presented a highly significantly more production ($p \leq 0.002$) and hence was selected for further optimization. Univariate optimization of the selected process parameters of HP-SSF and T-SSF followed the Plackett-Burman method for the determination of the three most significant process parameters. Using the central composite design contour plots for their interaction in pairs was obtained to determine the optimized yield. A novel stepwise supplementation method for nitrogen during the incubation enhanced the protease production by 82 % against the 31 % increase in a single dose manner traditionally practiced. Altering the moisture level on different days of incubation revealed a distinct phase wise requirement of it. Scale up of the method was done to 10 and 100 fold levels and the outcome was at par the pilot scale level.

KEY WORDS: Protease, *Aspergillus flavus*, hydroponics-like SSF, optimization.

INTRODUCTION

Several ways of production of industrially important enzymes and numerous optimization strategies to enhance the production are recorded in the history of fermentation biology (Omemu *et al*., 2008; Rauf *et al*., 2010; Sindhu *et al*., 2009a*)*. Solid state fermentation (SSF, for an instance, is the most widely accepted method for fungal inoculants. The method exhibits several advantages such as lower production cost, inexpensive raw materials, less water requirement, lower chance of bacterial contamination, lesser effluent volume, easier recovery and enhanced production rate (Diaz-Godinez *et al*., 2001; Divakar *et al*., 2006; Guneet Kaur and Satyanarayana, 2004; Paranthaman *et al*., 2009; Sindhu *et al*., 2009b). However, the SSF has several methodic difficulties, the most important being the problem of aeration in a three-phase system of solid (substrate), liquid (film over the substrate particles) and air (interstitial spaces between the particles) (Bashir *et al*., 2011; Rajagopalan and Modak, 1995; Sindhu *et al*., 2009b). Often the three cannot be simultaneously obtained with moisture been the most instrumental factor among them. Secondly, one cannot uniformly and effectively blend the additives or nutrition supplements with the fermentation medium during the incubation period as this leads to the mycelial damage of the fungal inoculants. Revolving drum method offers only a limited success in overcoming this difficulty and the method has its own set of limitations (Stuart and Mitchell, 2003; Wang *et al*., 2010). Yet another limitation of SSF is the difficulty in pH control because of unavailability of free flowing aqueous phase (Bashir *et al*., 2011). To develop new methods without these drawbacks was therefore considered to be a rational solution to enhance the food enzyme production in a cost effective manner (Omemu *et al*., 2008; Rauf *et al*., 2010).The present investigation was directed at developing novel methods for the production of extracellular fungal protease using *Aspergillus flavus* (NCIM 535). Further investigated aspects include the univariate and statistical optimization of the key process parameters and characterization and scaling up of the best of the methods.

MATERIALS & METHODS

Microorganisms

Aspergillus flavus (NCIM 535) with protease producing potential was obtained from the National Collection of Industrial Microorganisms (NCIM) facility of National Chemical Laboratory (NCL), India. The culture was grown on potato dextrose agar (PDA) slants at 28 ^{o}C and maintained thereafter at 4 ^{o}C with regular sub culturing every three weeks (Falony *et al*., 2006; Sindhu *et al*., 2009b).

Preparation of Inoculum

A potato dextrose agar (PDA) slant with fully sporulated mycelial growth was overlaid with sterile 0.01 % aqueous solution of Tween-80, gently tapped with a sterile loop and the spore suspension was collected in a sterile tube (Sindhu *et al*., 2009b). The tube was subjected to a gentle

vortex for uniform dispersal of the harvested spores. Spore count was determined and the suspension was diluted with sterilized distilled water to attain an approximate final count of $2x10^7$ spore mL^{-1} (Guneet Kaur and Satyanarayana, 2004). Aliquots from the same inoculum were used to inoculate in all the experiments.

Preparation of Substrate

Raw wheat bran was initially sieved (80 mesh) and washed with tap water and then dried at 80 ^oC (Guneet Kaur and Satyanarayana, 2004). This pre treatment was meant for the removal of impurities and making the bran more suitable for microbial attack because of partial soaking. The 1000 mL aqueous mineral solution was composed of $ZnSO_4.7H_2O$ (0.07 g), $FeSO_4.7H_2O$ (0.07 g) and H_2SO_4 (0.07 mL). This concentration was considered to be 10 X (meaning 10 fold) and was diluted with distilled water to 1 X and 0.1 X during the optimization experiments.

Methods of protease production

1. Conventional SSF method – Tray SSF

In the tray SSF (T-SSF), wheat bran (400 g) was blended with 240 mL 1 X mineral solution (60 mL for every 100 g dry weight of the substrate), spread on metal trays in a thin layer (5 mm thick) and sterilized at 121 ^oC for 30 min (Sekar and Balaraman, 1998). The substrate was inoculated with $2x10^7$ spores of *Aspergillus flavus* per gram of dry substrate, incubated at 28 ^oC for 5 days and harvested.

2. Advanced SSF method – Revolving Drum SSF

In the Revolving Drum SSF (RD-SSF), wheat bran (400 g) was blended with 240 mL of 1 X mineral solution (60 mL for every 100 g dry weight of the substrate) and sterilized at 121 ^oC for 30 min. The above blend was aseptically loaded into the chemically sterilized (using alcohol swabs) drum fermentor (radius = 25 cm, length = 100 cm). The substrate was aseptically inoculated with spores of *Aspergillus flavus* ($2x10^7$ per gram of dry substrate) and incubated at 28 ^oC. The drum was rotated at the rate of 3 revolutions per day for 5 days.

3. Novel SSF method – Covered tray SSF

In covered tray SSF (CT-SSF) method the wheat bran (400 g) was blended with 120 mL of 2 X (double strength) mineral solution (30 mL instead of 60 mL for every 100 g dry weight of the substrate) and spread on metal trays in 5 mm thick layers. The deficit moisture of 30 mL for every 100 g dry weight of the substrate was made up by covering the tray with a Whatman no. 1 filter paper soaked in the exact volume of distilled water. Covered trays were sterilized (121 ^oC for 30 min), inoculated with the spores of *Aspergillus flavus* ($2x10^7$ per gram of dry substrate) and incubated at 28 ^oC for 5 days.

To support more growth, extra moisture in the form of a new filter paper soaked in 60 mL distilled water was provided daily till the day 3 of incubation by replacing the previous filter paper.

4. Novel SSF method – Hydroponics like SSF (pilot scale)

In a pilot scale Hydroponics like SSF (HP-SSF), a circular filter paper (diameter 14 cm) was punched in the center, a 5 thread wick of freshly pulled cotton fiber (the cotton fiber was folded five times to obtain a wick with 5 threads) was passed through it and the paper was kept in a Petri plate over a support of 6 marble hemispheres (7mm diameter) stuck to the base. The above system, the substrate and the mineral solution were separately sterilized (121 ^oC for 30 min). A Petri plate was opened under laminar air flow, 2.4 mL mineral solution (60 mL for every 100 g dry weight of the substrate) was added to the base and the filter paper-wick system was adjusted over the support such that the moisture rose from the lower end of the wick to the surface of the filter paper and spread in a radial manner. Filter paper was loaded with spores of *Aspergillus flavus* ($2x10^7$ per gram of dry substrate) and left for 30 min to allow the spores to absorb moisture. Wheat bran (4g dry weight) was then aseptically layered over the filter paper and incubated at 28 ^oC for 5 days. Extra moisture (2 mL distilled water) was provided in a Petri plate on day1, day 2 and day 3 of incubation. Harvesting was done on the fifth day.

Extraction and Assay of Protease

Extraction of protease was done by soaking the moldy bran in chilled distilled water (volume approximately 5 times that of the moldy bran) for half an hour with intermittent stirring followed by filtration through muslin cloth (twice), centrifugation and then making up the filtrate to a fixed volume of 1000 mL for T-SSF, CT-SSF and RD-SSF each and 10 mL for HP-SSF (Paranthaman et al., 2009; Sindhu et al., 2009b). It is important to note that a large amount of water gets absorbed in the moldy bran and therefore does not contribute to an equal volume of the filtrate. Assay of protease was done with colorimetric estimation of hydrolysis of casein using Folin and Ciocalteu's Phenol Reagent, on the tyrosine standard curve (Divakar et al., 2006). One unit of protease accounted for 1 µg of tyrosine liberated per minute, under the standard assay conditions. Protease activity was expressed per gram of the substrate. All the experimentation was carried out in triplicate.

Optimization studies on T-SSF and HP-SSF

Seven process parameters of T-SSF mentioned in Table 1 were optimized within the selected range using univariate strategy (Anto et al., 2006; Sudhakar and Nagarajan, 2011). The Plackett-Burman statistical design, which allows the investigation of n-1 variables with n experiments, was used to assign an importance ranking to the parameters and to select the three most significant parameters of the seven. The method assumes no interaction among the selected factors. A high level (+) and a low level (−) of the selected factors around the optimum were considered.

After analyzing the performance of the four methods, the HP-SSF was selected for further optimization and the seven parameters optimized in T-SSF were optimized in HP-SSF as well and the results were compared.

Response surface methodology (RSM) and statistical analysis

The three most significant process parameters identified by the Plackett-Burman design were selected for further studies. Interactions among these three factors in HP-SSF were studied using a central composite design at three levels (-1, 0, +1) as indicated in Table 3 (Lima et al., 2007).

Novel applications of HP-SSF

1. Method of nutrient supplementation

Two methodologies of nutrient addition were evaluated in HP-SSF.

Single Dose nitrogen supplementation method: In this method the addition of NH_4Cl (0.1% w/v of mineral solution) on day 0 in trial 1, day 1 in trial 2, day 2 in trial 3, day 3 in trial 4 and day 4 in trial 5 was practiced. For the fourth day supplementation, an additional 2 mL distilled water had to be provided.

***Stepwise nitrogen supplementation method*:** In this method the addition of NH_4Cl (0.1% w/v of mineral solution) on day 0 in trial 1 (in a single dose), on day 0 and day 1 in trial 2 (dose split into two aliquots), on day 0, day 1 and day 2 in trial 3 (dose split into three aliquots), on day 0, day 1, day 2 and day 3 in trial 4 (dose split into four aliquots), on day 0, day 1, day 2, day 3 and day 4 in trial 5 (dose split into five aliquots) was practiced. Total NH_4Cl added in all the trials remained the same.

A control trial was the one with no NH_4Cl.

The methods were repeated using the same concentration of dextrose as a carbon supplement.

1. Analysis of moisture requirement in various phases of incubation in HP-SSF

The incubation period of HP-SSF was divided into three phases, namely the phase of spore germination (Day 0 – 1), the phase of growth (Day 2 – 3) and the phase of enzyme production (Day 4 – 5). A higher level of moisture of 80 mL mineral solution for every 100 g dry weight of the substrate was provided during only one of the three stages at a time as indicated in Table 5. The moisture level was brought down to a lower level of 40 mL of mineral solution for every 100 g dry weight of the substrate by blowing sterile air over the surface in laminar air flow to promote evaporation, when needed. Finally, the protease activity was determined after completion of incubation period of 5 days. HP-SSF with a lower level of moisture maintained in all the three phases acted as a control.

Characterization of the T-SSF and HP-SSF

The samples of moldy bran (substrate) in T-SSF and HP-SSF method were drawn and analyzed daily for various physico-chemical properties.

The liquid obtained by squeezing the moldy bran by cold pressing was checked daily for its pH. The amount of reducing sugar was estimated per gram of substrate using di-nitro salicylic acid (DNSA) method. Bulb of the thermometer was buried in the moldy bran to measure the daily temperature of the substrate bed. Daily moisture content of the moldy bran was determined by drying 1 g of it at 80 ^oC till no further weight loss was seen. The total weight loss accounted for the total moisture content. Moldy bran in 1 cm^2 area was suspended in 10 mL of distilled water and spore count mL^{-1} was determined.

Scale up of HP-SSF

In the first step, a 10 fold scale up of HP-SSF method was done by incorporating following changes in the methodology previously described. The process was performed in a tray instead of a Petri dish. The shape of the Whatman filter paper no.1 was rectangular instead of circular and its size was approximately 10 times more than that previously used. Amount of wheat bran used was 40 g and volumes of the inoculum, mineral solution and distilled water (to be supplemented in the form of daily feeds) were increased by 10 fold. Single wick of pilot trial was replaced by 10 wicks arranged uniformly over the surface.

In the second step a 100 fold scale up was done by incorporating following changes in the methodology previously described. The process was performed on a rectangular Whatman filter paper no.1 placed on wire gauze instead of marbles and its size was approximately 50 times more than that previously used. This resulted in a thicker than 5 mm layer (as in pilot scale) of the substrate. Amount of wheat bran used was 400 g and volumes of the inoculum, mineral solution and distilled water (to be supplemented in the form of daily feeds) were increased by 100 fold. Hundred wicks were used at a regular distance and they were clustered in a group of 10 to collect the mineral solution or distilled water from 10 beakers.

RESULTS & DISCUSSION

T-SSF was optimized with respect to seven process parameters by varying only one of them at a time (univariate procedure). Using this method, the optimum and hence the high (+) and low (-) levels of these variables were obtained. The optimization results obtained were similar to those reported in the literature (Divakar et al., 2006; Sindhu et al., 2009b). Table 1 indicates the selected range of seven parameters, the curve pattern for the 'parameter-protease production' correlations, the optimum values and the high (+) and the low (-) level values of these parameters.

TABLE 1. Univariate optimization, curve pattern and high/low levels of seven process parameters of T-SSF

Process parameters	Range selected (Interval)	Observed optimum	Observed curve pattern	High level(+)	Low level(-)
Inducer (de-oiled cotton seed cake **)	5% to 45% (5%)	25%	Normal Curve	40%	10%
Incorporated inert support (80 mesh particles of glass **)	10% to 50% (10%)	20%	Normal Curve	30%	10%
Carbon (w/v % of dextrose in mineral solution)	0.05% to 0.25% (0.05%)	0.05%	Skewed Curve*	0.5%	0.005%
Nitrogen (w/v % of ammonium chloride in mineral solution)	0.05% to 0.25% (0.05%)	0.1%	Insignificant increase after 0.01%	1%	0.01%
Initial Moisture content (mL for every 100 g dry weight of the substrate)	40 to 90 (10)	60	Normal Curve	90	30
Mineral concentration (strength)	0.1X, 1X and 10X (10 fold)	1X	Insignificant increase after 1X	10X	0.1X

pH of mineral solution	4 to 8 (1)	5	Normal Curve	8	4

* The curve pattern wherein low level of carbon supplementation boosted the protease activity, higher levels showed negative impact.
** Weight per 100 g mixture of inducer/ glass powder and substrate.

The high and low values selected in the univariate experiment were used as the basis for the experiment carried out using the Plackett Burman matrix (Table 2). This experiment gave the following order of significance of the variables: carbon supplementation level > initial moisture > pH >inducer level > inert support > nitrogen supplementation level > strength of mineral solution.

TABLE 2. Plackett-Burman Matrix for seven parameters of T-SSF.

Trial	A Protease Inducer	B Inert Support	C Carbon %	D Nitrogen %	E Initial Moisture	F Strength of M.S.	G pH
1	+	+	+	-	+	-	+
2	-	+	+	+	-	+	-
3	-	-	+	+	+	-	+
4	+	-	-	+	+	+	-
5	-	+	-	-	+	+	+
6	+	-	+	-	-	+	-
7	+	+	-	+	-	-	+
8	-	-	-	-	-	-	-

\+ High level; – Low level

The significant factors identified by Plackett-Burman design were considered for the next stage (Dilipkumar et al., 2011).

After the optimization of T-SSF, it was compared with CT-SSF, HP-SSF and RD-SSF in the next step. The results indicated that the protease production increased only marginally in CT-SSF over T-SSF, whereas the 12 % increase in the protease production with respect to T-SSF in RD-SSF was significant ($p \leq 0.006$) and the 55 % increase over T-SSF in HP-SSF was highly significant ($p \leq 0.002$) (Fig. 1).

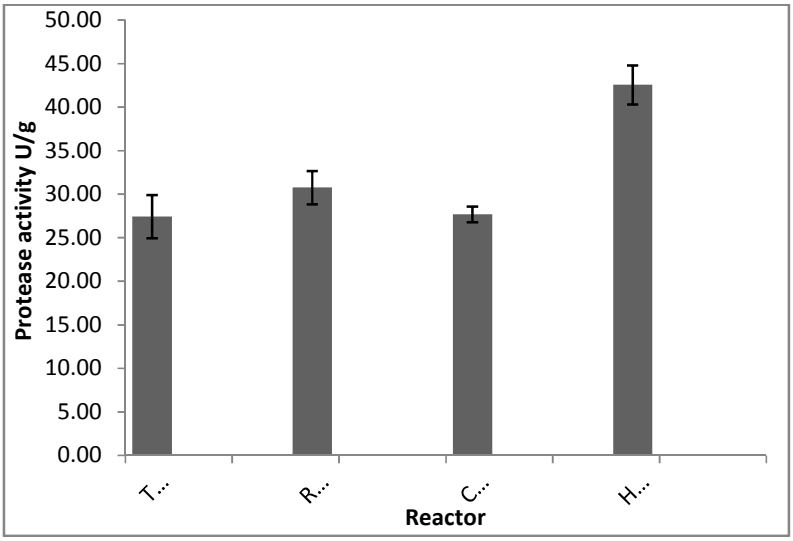


FIGURE 1. Comparison of bioreactors for their efficiency in protease production.

In view of significant improvement in protease production in HP-SSF, further studies were undertaken to optimize the process parameters in HP-SSF. The seven parameters studied in T-SSF were optimized in HP-SSF as well (Fig. 2).

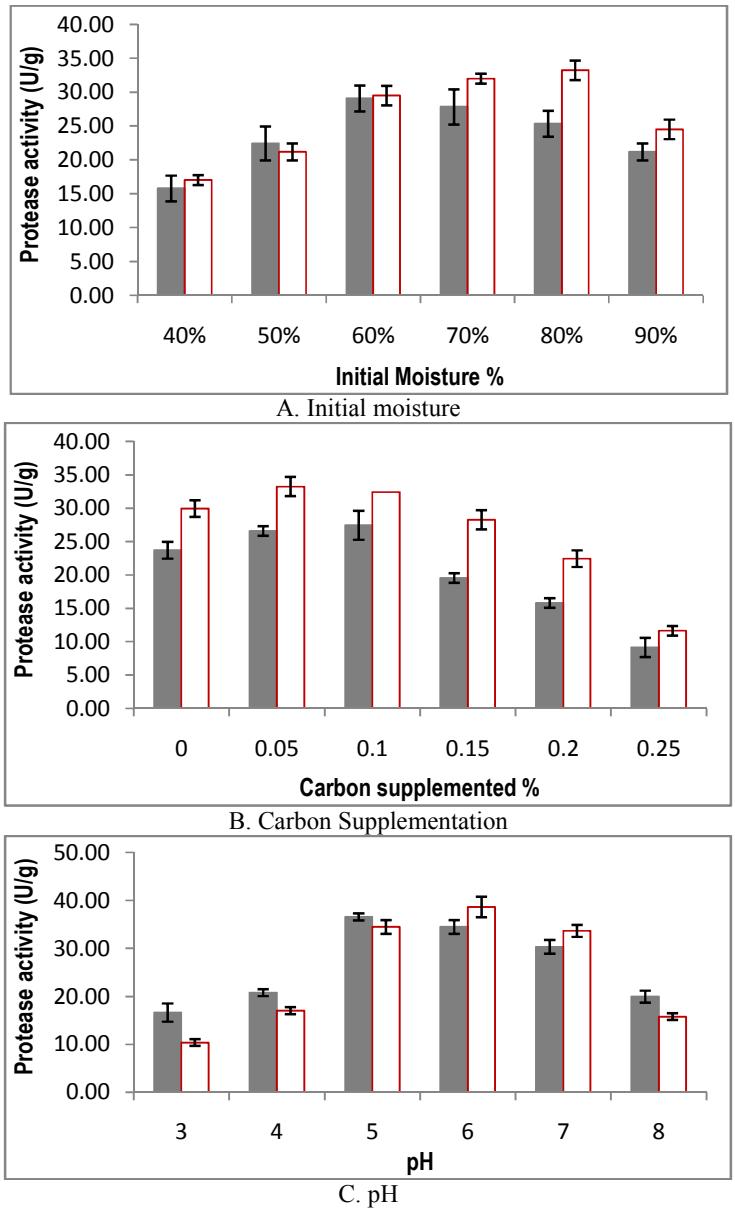


FIGURE 2. Comparison of results of optimization of the most significant process parameters in T-SSF■ and HP-SSF□.

The optimum initial moisture content requirement in HP-SSF was 80 mL for every 100 g dry weight of the substrate, against 60 mL for every 100 g dry weight of the substrate in T-SSF. This higher initial moisture requirement could probably be attributed to some moisture been absorbed by the paper and higher moisture loss due to evaporation through both the faces of the filter paper. Interestingly, even at the initial moisture level of 60 mL for every 100 g dry weight of the substrate, a higher protease production was seen in HP-SSF than in T-SSF at the same level. Thus, it is obvious that the moisture supplementation strategy is the key to the method's success.

An optimum carbon supplementation level of 0.1% (against 0.05% of T-SSF) can be attributed to a higher fungal growth (evident in terms of a greater spore count / cm^2) in the HP-SSF than in the T-SSF. In HP-SSF, this higher growth might have relatively starved more than that in T-SSF, forcing the system to shift its metabolism more towards the protease production.The reason for optimum pH requirement of 6.0 in HP-SSF (against 5.0 of T-SSF) can be cited in pH analysis of the medium during the incubation period of HP-SSF and T-SSF. A reduction in pH was observed in the course of both the methods. However it was more profound in the HP-SSF than the T-SSF. All other parameters along with the results of Plackett-Burman experiment showed trends similar to those observed in T-SSF. After knowing the three most significant process parameters (carbon supplementation level, initial moisture and pH) from the Plackett-Burman design, response surface analysis was carried out in three sets to study the interactive effect of two variables at a time on the protease production under HP-SSF (Dilipkumar et al., 2011). The three levels of the significant parameters are indicated in Table 3.

TABLE 3. Levels of the three most significant parameters required for the central composite design for statistical treatment of HP-SSF data.

Parameter Code	Parameter	Levels		
		-1	0	1
A	Carbon (g per 100 mL of mineral solution)	0.05	0.1	0.15
B	Moisture (mL of mineral solution for every 100g dry weight of substrate)	70	80	90
C	pH	4	6	8

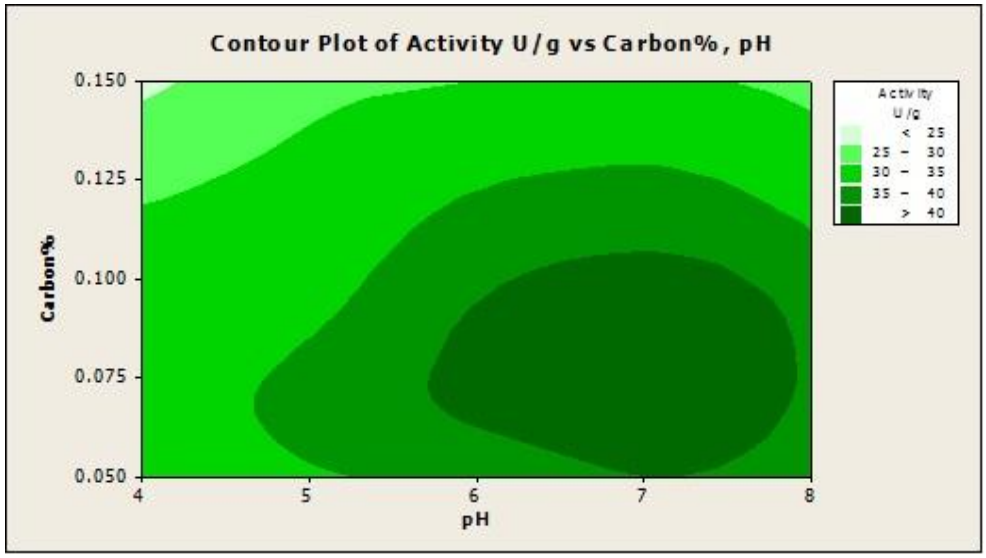
A central composite design having 13 experiments in each set was used for the purpose. Each set had 5 central points, 4 cube points and 4 axial points. The effects of interaction present in each set and the coefficient estimates of central composite design for each set are given in Table 4 (Lima et al., 2007).

TABLE 4. Coefficient estimates for the sets of central composite design

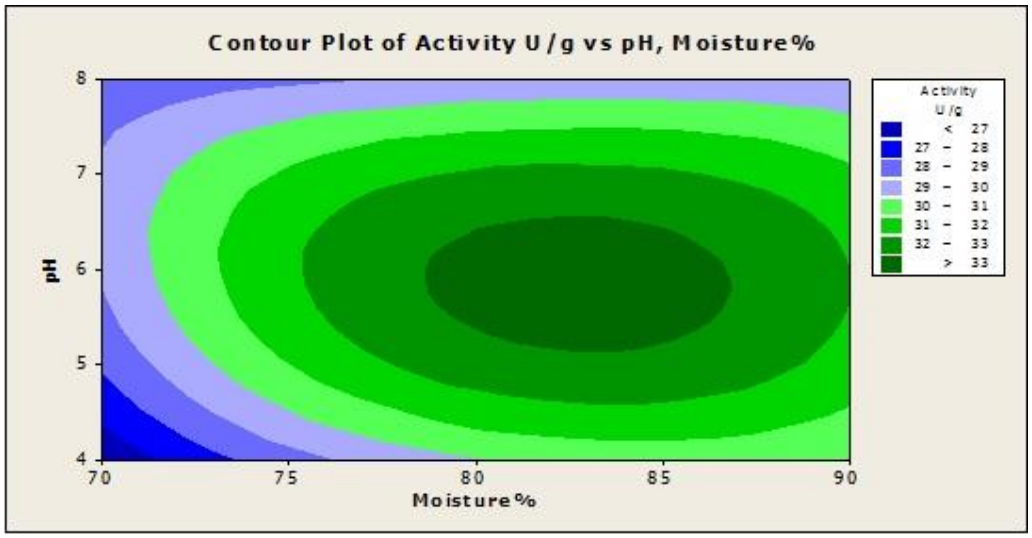
	Term	Coefficient	SE	P
Set I	Constant	38.83	0.28	0.000
	A	2.56	0.28	0.000
	B	- 3.32	0.28	0.000
	A*A	-3.97	0.41	0.000
	B*B	-5.43	0.41	0.000
	A*B	0.62	0.34	0.114
Set II	Constant	33.02	0.24	0.000
	A	1.24	0.24	0.001
	B	0.06	0.24	0.787
	A*A	-1.94	0.35	0.001
	B*B	-2.98	0.35	0.000
	A*B	-0.72	0.29	0.045
Set III	Constant	24.03	0.23	0.000
	A	-3.67	0.22	0.000
	B	-0.02	0.22	0.000
	A*A	-1.41	0.33	0.387
	B*B	-2.66	0.33	0.031
	A*B	0.73	0.27	0.000

Set I: A= Carbon%, B=pH; Set II: A= pH, B=Moisture%; Set III: A= Moisture%, B= Carbon%

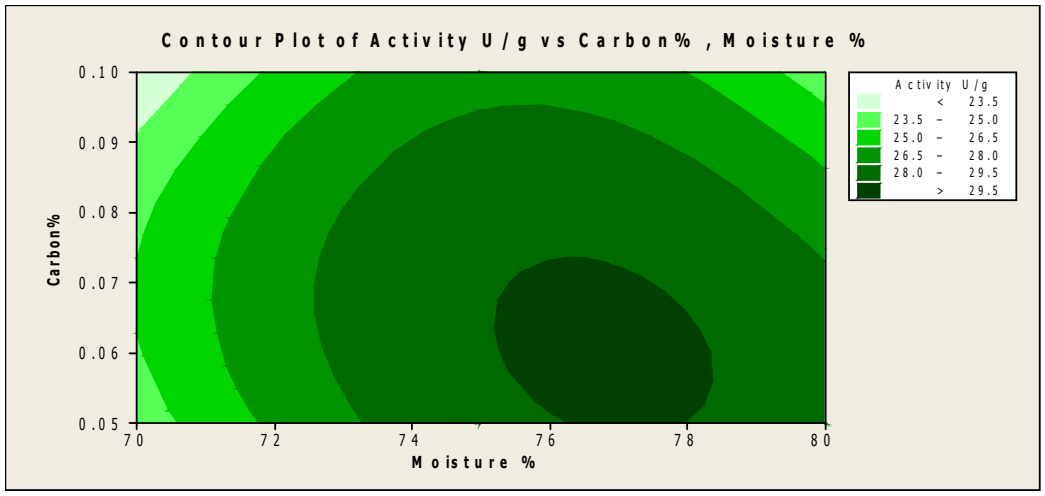
As can be seen, almost all the interactions among the important factors were statistically significant ($p < 0.05$) (Fig. 3). The results indicated presence of a curvature around the optimum points (Dilipkumar et al., 2011; Lima et al., 2007) and the maximum protease production was obtained at pH 6.0, carbon supplementation level approximately 0.06% and moisture level 75-80 mL for every 100 g dry weight of the substrate. The model was able to fit data satisfactorily.



A. Carbon – pH Interaction



B. pH – Moisture Interaction



c)

C. Carbon – Moisture Interaction

FIGURE 3. Contour maps of interaction of pH and carbon% (a), moisture % and pH (b) and moisture% and carbon % (c), against the protease activity (Ug^{-1}).

Nitrogen supplementation done in a single dose manner in HP-SSF increased the protease production by 15% to 32% against the control, with a maximum in case of the day 3 supplementation (Fig. 4). Protease production however got increased by 35% to 83% when the same dose of nitrogen was supplemented in a stepwise manner (Fig. 5). The step wise supplementation method thus proved to be more effective in bringing out the effect of the nutrient than that done by the single dose method

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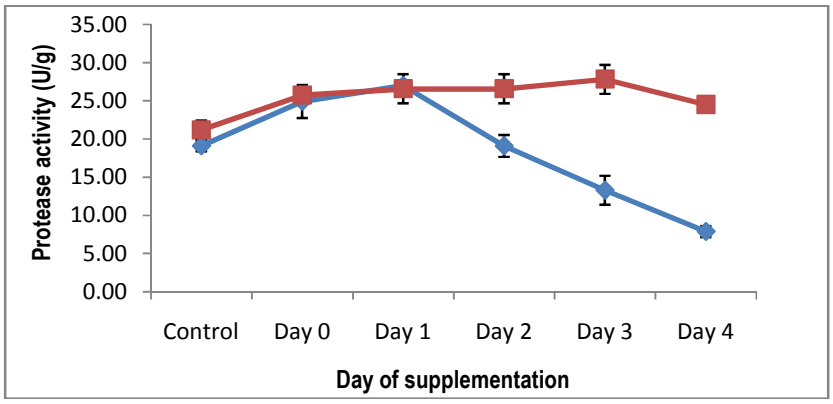

FIGURE 4. Effect of single dose supplementation of carbon ◆ and nitrogen ■ on protease activity.

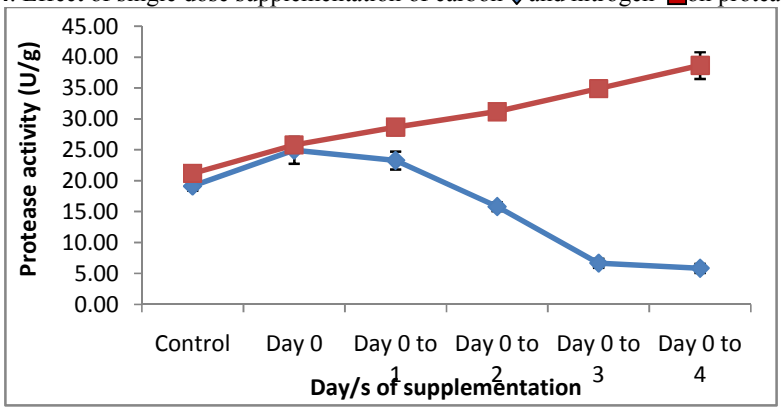

FIGURE 5. Effect of step wise supplementation of carbon ◆ and nitrogen ■ on protease activity.

Same conclusion was drawn about the two methods using supplementation of carbon. The step-wise supplementation caused an intense effect of catabolite repression than that in the single dose method. This may be attributed to the availability of dextrose in the enzyme production phase when step-wise supplementation method was used (Fig. 4, 5) (Sindhu et al., 2009b). To understand the moisture requirement of the fungus in various stages of its growth, a strategic study was done wherein a higher moisture level of 80 mL for every 100 g dry weight of the substrate was more critical during the spore germination phase, while a lower level of 40 mL for every 100 g dry weight of the substrate favored higher enzyme production (Table 5). Such kind of moisture requirement can be met easily using the HP-SSF by phase wise supplementation of the exact moisture.

TABLE 5. Effect of phase wise moisture levels on the protease production in HP-SSF.

Experiment Code	Moisture % during three phases			Protease Activity(U g^{-1})
	Germination Phase (I)	Growth Phase (II)	Enzyme production Phase (III)	
Control 1	40%	40%	40%	21.20 ± 1.25
M1	80%	40%	40%	24.94 ± 1.25
M2	40%	80%	40%	23.69 ± 1.25
M3	40%	40%	80%	19.12 ± 0.72

Results of the characterization of HP-SSF are summarized in Table 6.

TABLE 6. Characterization of HP-SSF with respect to selected physico- chemical parameters

Parameter	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
pH	6.0	5.5	5.2	4.9	5.0	5.5
Reducing Sugar (mg/g of moldy bran)	1.16	0.82	0.74	0.62	0.61	0.61
Temperature (^{o}C)	27	27.5	28.9	31.2	31.3	31.3
Moisture (mL for every 100 g of dry substrate)	62	59.5	57.3	54.8	53	52.8
Spore count (x 10^7)	NC	NC	NC	0.3	1.2	7.0

NC – Not countable

Regular drop in pH till day 3 followed by an increase indicated the presence of two distinct phases. The initial phase of acidogenesis coincided with the utilization of reducing sugars and with the stabilization of reducing sugar level after day 3, the pH showed an increasing trend. Temperature too appeared to obey this two phase pattern, where a rapid increase (because of generation of metabolic heat) was followed by stabilization (because of heat dissemination). Moisture loss too might have accounted for some cooling effect. It is interesting to note that the moisture content on day 0 was just 62 mL for every 100 g of dry weight of the substrate, though 80 mL was provided. This account of moisture is previously discussed. Significant spore count was seen only after day 3.These results are similar to those of T-SSF with two differences. Firstly, the moisture loss in T-SSF was not as prominent as in HP-SSF. The moisture level in T-SSF dropped to a minimum level of only 55.8 mL from initial 60 mL per 100 g substrate, against the 52.8 mL level of HP-SSF. As per the previous experiment, it can be said that lesser moisture content in the phase of production of enzyme favors the enzyme production. This fact can be correlated with the lesser productivity of T-SSF. Secondly, the spore count obtained in T-SSF was $5x10^7$ against that of $7x10^7$ of HP-SSF. This attribute refers to a lesser growth in T-SSF and hence a lower productivity.

The protease activity in pilot scale was 43.5 U/g of substrate whereas it was 43.8 U/g of substrate in first scale up and 40.9 U/g of substrate in the second scale up. Scale up in first step resulted in a more than10 fold increase in the protease production against the expected 10 fold. Whereas in the second step instead of the 100 fold

expected increase, the production went up by 94 fold. This can be attributed to a thicker substrate layer (8 mm) than in pilot scale experiments. However, this second step of scale up is very difficult to be set up and maintained in the laboratory and technical measures to make the method easy are required. Though tedious, a 94 fold increase can be viewed as a significant result in scaling up HP-SSF presented a significant status. HP-SSF has offered an approach to overcome the drawbacks of the currently used methods in the form of its newer methodic strategies and the methodology may be extended to more process parameters. As is evident from the results, with its scale up, the HP-SSF holds a potential for being used on a large scale. However, the engineering intervention would be the next step required for the development of a reactor suitable for the method.

CONCLUSION

A pilot scale HP-SSF has offered an approach to overcome the drawbacks of the currently used methods in the form of its newer methodic strategies. The methodologies can be extended to more process parameters and the further standardization may lead to a still better performance of the method. As is evident from the results, with its scale up, the HP-SSF holds a potential for being used on a large scale. However, the engineering intervention would be the next step required for the development of a reactor suitable for the method. Till then, even the pilot scale experiment performed in multiples, though would be very laborious, can be used in the capacity of scaled up HP-SSF.

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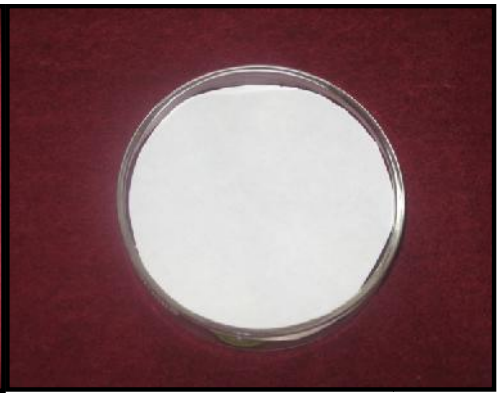
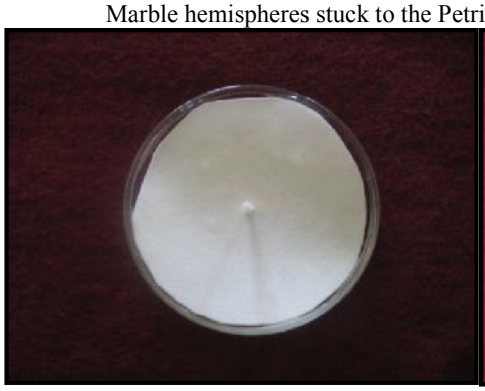
SUPPLEMENTARY MATERIAL

Photographs of pilot scale HP-SSF method:

Marble hemispheres stuck to the Petridish



Overlaid filter paper



Cotton wick inserted in the centre

Paper charged with the inoculum

Mycelial growth