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Optimization of Process Parameters for High Biomass, α- amylase and Protease enzyme by *Piriformospora indica* using Mathematical Model.

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ABSTRACT

Piriformospora indica is an endophytic fungus which exerts plant growth promotional effects on broad host spectrum. The present study was undertaken to optimize the process parameters to achieve high α- amylase, protease activity and high biomass of *Piriformospora indica* using response surface methodology. Glycerol, peptone and pH played a significant role in achieving high biomass. Increase in concentration of glycerol and pH gave maximum biomass of 7.6 gl^{-1}. A maximum amylase activity of 0.08U/ml was observed at mid concentration of the variables. Increase in pH and decrease in glycerol concentration depicted a maximum protease activity of 0.25 U/ml.

Keywords: *Piriformospora indica*, Response Surface Methodology, Glycerol, α- amylase activity, Protease activity.

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INTRODUCTION

Piriformospora indica (*P. indica*) is an axenically cultivable arbuscular mycorrhiza like fungus. It was isolated from the *Glomus mosseae* spore in the rhizosphere of the shrubs *Zyzyphus nummularia* (*Rhamnaceae*) and *Prosopis juliflora* (*Fabaceae*) of the Indian Thar desert [1]. *P. indica* belongs to Basidiomycota and possess pear shaped chlamydospores at hyaline Hyphae with dolipore septa. *P.indica* colonizes various mono and dicot plants. They are known to enhance the growth and yield of host plants and protect them from pathogen. *P. indica* confers resistance against drought and salinity. Thus *P. indica* has great potential in agriculture, forestry, horticulture and viticulture [2, 3]. Better understanding of *P. indica* symbiosis would open up numerous opportunities for the optimization of plant productivity in both managed and natural ecosystem, while minimizing risk of environmental damage. Hence the mass cultivation of *P. indica* is an important criterion which can be achieved by response surface methodology. Classical method, factorial combination of medium optimization involving one variable at a time by keeping others at fixed level fails as it is laborious, time consuming; moreover, it does not guarantee the optimal conditions [4]. Hence statistical tools such as placket burmann and response surface methodology can be employed to achieve optimization of nutrient components. Fungal enzymes such as amylase and proteases have numerous applications in starch processing, brewing, alcohol production, dairy industries and textile industries, pharmaceutical and detergent industries [5, 6]. The present paper investigates the optimization of nutrients for the production of high biomass, amylase and protease enzyme by *P. indica* using response surface methodology.

MATERIALS AND METHODS

Microorganism and culture maintenance: The culture of *P. indica* was maintained on potato dextrose agar at optimum conditions and stored at 4^{o}C for further studies.

Experimental Design: Based on the results obtained by Placket Burmann design [4], glycerol, peptone and pH was found to be influential for the production of high biomass. Hence optimization was carried out using response surface methodology taking the above said three significant variables into account. Twenty experiments involving three significant variables glycerol, peptone and pH in the medium (Table. 1) were performed according to CCD matrix using MATLAB version 7 [7].

Table 1 Experimental codes and levels of the variables chosen for the model

Variables Chosen	Code	Actual factor levels at coded factor levels of				
		-2	-1	0	1	2
Glycerol Concentration	C1	0	0.05	0.1	0.15	0.2
Peptone Concentration	C2	0	0.05	0.1	0.15	0.2
pH value	C3	4.5	5.5	6.5	7.5	8.5

Submerged fermentation: Inoculum size of 5 x 10^5 spores' ml^{-1} of the culture was inoculated to all the flasks (21 runs) designed as per the statistical experimental design and these flasks were incubated at 30^{o}C under continuous shaking conditions (120 rpm) and the response was measured in terms of biomass, amylase and protease production.

Measurement of cell growth: Mycelium was filtered through pre-weighed Whatman No. 1 filter paper, dried in a hot air oven for 48–72 h and the growth of *P. indica* was expressed in terms of dry cell weight per liter of the culture broth [4].

α- amylase activity: a-Amylase activity was performed using 1 % soluble starch as substrate followed by the estimation of reducing sugars using dinitrosalicylic acid [8]. One unit of amylase activity was defined as the amount of enzyme that liberates reducing sugar equivalent to 1.0 mg glucose under specific assay conditions.

Protease activity: Protease activity using casein as substrate was performed according to Rodarte et al. [9] with some modifications. One unit of the protease activity was defined as the amount of enzyme required to liberate 1μg of tyrosine per hour under the experimental conditions.

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RESULTS AND DISCUSSION

P. indica forms flat creamish colony on potato dextrose agar and it forms pear shaped chlamydospores (Fig. 1a & b).

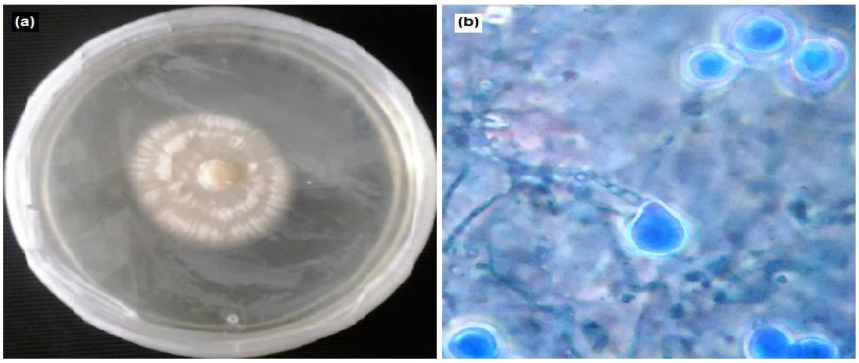

Fig. 1a & b

Figure 1a & b: Morphological and Microscopic View of *Piriformospora indica*

Response Surface Methodology: The interaction effect of variables on biomass yield was studied by plotting 3D surface plots against any 2 independent variables keeping other variable at its central (0) level.

Table 2: Full factorial Central Composite Design of three variables in natural units and the responses from mode

Runs	Glycerol	Peptone	pH	Biomass (gl^{-1})		Amylase Activity (U/ml)		Protease Activity (U/ml)	
				Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	0.05	0.05	5.5	4.0000	4.5534	0.1120	0.1004	0.1180	0.1211
2	0.15	0.05	5.5	3.0000	3.8284	0.1760	0.1529	0.1080	0.0987
3	0.05	0.15	5.5	3.0000	3.5284	0.1510	0.1364	0.1420	0.1440
4	0.15	0.15	5.5	1.6000	2.7034	0.1770	0.1499	0.1270	0.1371
5	0.05	0.05	7.5	5.0000	5.1784	0.0990	0.1296	0.1880	0.1982
6	0.15	0.05	7.5	6.8000	7.5534	0.1180	0.1361	0.1680	0.1863
7	0.05	0.15	7.5	5.0000	5.4534	0.1100	0.1366	0.1410	0.1706
8	0.15	0.15	7.5	7.0000	7.7284	0.0890	0.1041	0.1570	0.1742
9	0	0.1	6.5	5.2000	4.9841	0.0780	0.0643	0.1630	0.1507
10	0.2	0.1	6.5	7.6000	6.5341	0.0740	0.0843	0.1400	0.1320
11	0.1	0	6.5	6.2000	5.6841	0.0800	0.0748	0.1450	0.1440
12	0.1	0.2	6.5	5.6000	4.8341	0.0770	0.0788	0.1740	0.1547
13	0.1	0.1	4.5	1.8000	0.9341	0.2310	0.2710	0.1230	0.1302
14	0.1	0.1	8.5	7.0000	6.5841	0.2980	0.2545	0.2720	0.2445
15	0.1	0.1	6.5	5.4000	5.4864	0.1110	0.1091	0.1420	0.1371
16	0.1	0.1	6.5	6.0000	5.4864	0.1170	0.1091	0.1390	0.1371
17	0.1	0.1	6.5	5.6000	5.4864	0.1030	0.1091	0.1420	0.1371
18	0.1	0.1	6.5	5.8000	5.4864	0.1070	0.1091	0.1390	0.1371
19	0.1	0.1	6.5	5.5000	5.4864	0.1110	0.1091	0.1420	0.1371
20	0.1	0.1	6.5	5.9000	5.4864	0.1090	0.1091	0.1390	0.1371

Biomass: The equation of the model explaining 3 variables for biomass is as given below

$$-8.0437 - 97.45 \times \text{glycerol} - 40.9545 \times \text{peptone} + 4.8261 \times \text{pH} - 10 \times \text{glycerol} \times \text{peptone} + 15.50 \times \text{glycerol} \times \text{pH} + 6.5 \times \text{peptone} \times \text{pH} + 27.27 \times \text{glycerol}^2 - 22.72 \times \text{peptone}^2 - 0.4318 \times \text{pH}^2$$

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Interactive effect of peptone and glycerol on the biomass yield is as given in Fig. 2a. The biomass increased with the increase in concentration of glycerol while it remained almost constant with the increase in peptone concentration. The maximum biomass yield was obtained with maximum glycerol concentration but minimum peptone concentration. In case of glycerol and pH, the interactive effect of both the variables increased biomass with the maximum yield at the maximum concentration of both the variables. Although at low pH the yield decreased with increase in glycerol concentration. Similarly increase in pH didn't yield much at low glycerol concentration (Fig. 2b). The interactive effect of pH and peptone concentration also showed significant variation. The yield increased sharply with pH at higher peptone concentration. With the increase in pH and decrease in peptone concentration, favoured the growth of *P. indica* (Fig. 2c). Thus from the above obtained data it can be concluded that glycerol concentration and pH influences the growth of *P. indica*. Flask no. 10 containing 0.2g of glycerol, 0.1g of peptone with pH 8.5 was found to be best for the high biomass (7.6g/l) production.

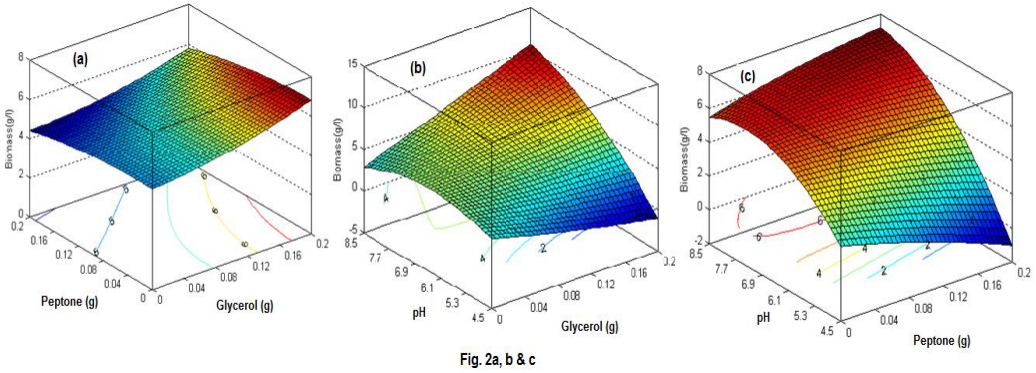

Fig. 2a, b & c

Figure 2a, b & c 3D: Response surface plot showing relative effect of glycerol, peptone and pH on biomass yield (gl^{-1}).

α amylase: The equation of the model explaining 3 variables for biomass is as given below

1.3973+ 2.6814 x glycerol+ 1.9989 x peptone- 0.4661 x pH- 3.90 x glycerol x peptone- 0.230 x glycerol x pH- 0.145 x peptone x pH- 3.4818 x $glycerol^2$- 3.2318 x $peptone^2$+ 0.0384 x pH^2

Table 3: Regression coefficient results for Biomass, α- amylase activity and protease activity from the model

Parameters	Biomass (gl^{-1})		**α- a**mylase Activity (U/ml)		Protease Activity (U/ml)	
	t - value	p value	t - value	p value	t - value	p value
Constant term	-0.85	0.41378	4.56	0.00105	1.94	0.08104
Glycerol	-2.23	0.04945	1.89	0.08781	-0.73	0.48227
Peptone	-0.94	0.36980	1.41	0.18881	1.40	0.19132
pH	2.02	0.07104	-6.00	0.00013	-2.26	0.04705
Glycerol × Peptone	-0.08	0.93584	-0.99	0.34527	0.60	0.55949
Glycerol × pH	2.56	0.02842	-1.17	0.26977	0.41	0.69119
Peptone × pH	1.07	0.30841	-0.74	0.47831	-1.97	0.07754
Glycerol × Glycerol	0.40	0.69819	-1.57	0.14801	0.29	0.77634
Peptone × Peptone	-0.33	0.74630	-1.46	0.17628	0.84	0.41826
pH × pH	-2.53	0.02998	6.92	0.00004	3.47	0.00604

The interaction effect of glycerol and peptone on amylase activity is as depicted in Fig. 3a. This typical scarf plot showed a maximum amylase activity of 0.08U/ml at mid concentration of the variables. The amylase activity increased quite significantly with increase in the concentration of either of the variable keeping other at its minimum level. The interactive effects of pH and glycerol concentration showed a channel type design with dip in amylase activity being observed at mid pH range. Similar trend was observed with the interactive effects of pH and peptone concentration. It shows that mid ranges of peptone and glycerol concentration and low pH is good for amylase activity.

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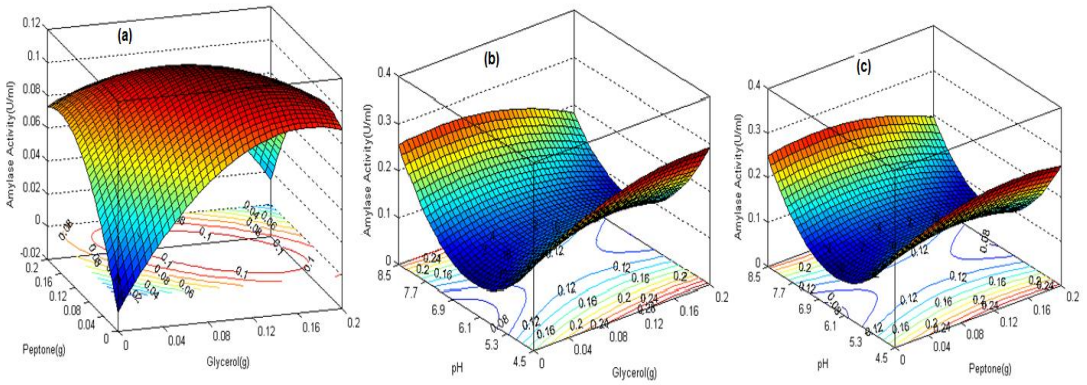

Fig. 3a, b & c

Figure 3a, b & c 3D: Response surface plot showing relative effect of glycerol, peptone and pH on α- amylase activity (Uml^{-1}).

Protease Activity: The equation of the model explaining 3 variables for biomass is as given below

$0.3879 - 0.6745 \times \text{glycerol} + 1.295 \times \text{peptone} - 14 \times \text{pH} + 1.55 \times \text{glycerol} \times \text{peptone} + 0.0525 \times \text{glycerol} \times \text{pH} - 0.2525 \times \text{peptone} \times \text{pH} + 0.4227 \times \text{glycerol}^2 + 1.2227 \times \text{peptone}^2 + 0.0126 \times \text{pH}^2$

The interaction effect of peptone and glycerol is shown in Fig.4, which didn't show much variation for protease activity. The increase in glycerol concentration didn't make any significant impact but the protease activity increased significantly with pH. A maximum activity of 0.27 U/ml was observed at pH 8.5 keeping other 2 variables glycerol and peptone at "0" level. Similarly pH had a positive impact in protease activity at lower peptone concentration with maximum activity of 0.3U/ml being observed at low peptone concentration and pH 8.5. Increase in concentration of peptone decreased the protease activity whereas increase in pH and decrease in glycerol concentration, highest protease activity of 0.25 U/ml was observed.

Table 4: Analysis of Variance results for RSM quadratic model for the three entities under study

	F Value	p-value	Mean Square	Sum of Squares	R^2	Adj. R^2
Biomass	6.98	0.00274	0.73	7.34	0.8626	0.7390
α- amylase Activity	7.52	0.00204	0.0008	0.0078	0.8712	0.7553
Protease Activity	6.411	0.00381	0.0003	0.0033	0.8523	0.7193

Note: Degree of Freedom for these models is 9

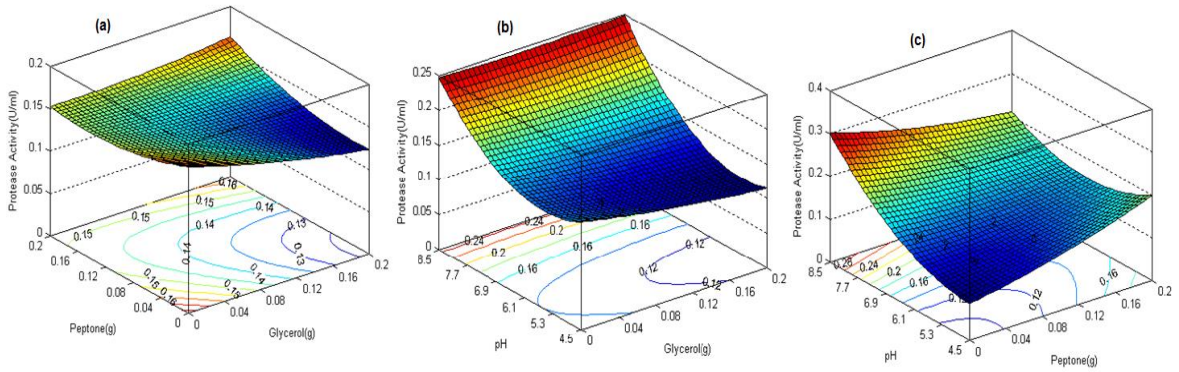

Fig. 4a, b & c

Figure 4a, b & c 3D: Response surface plot showing relative effect of glycerol, peptone and pH on Protease activity (Uml^{-1}).

Mass cultivation of *P. indica*, a multifaceted fungus is an important criterion in plant biotechnology which has been achieved employing RSM. Glycerol as a carbon source has favored the growth of *P. indica* at 0.2g concentration though it can induce osmotic stress. The above findings are in accordance with Dikshit and Tallapragada [10, 11], who have also reported the increase in biomass and pigment yield by Monascus spp. in presence of glycerol. In our previous study, we observed the effect of glycerol on the growth of *P. indica* [4]. Meinicke et al. [12] have also reported the positive influence of the glycerol on the biomass as well as pigment production by *Monascus ruber*. Hence it can be concluded that glycerol as a carbon source supports the growth of *P. indica*. pH is also one of the important parameter which plays an important role in the biomass production. Sreekumar et al. [13] have reported the enhancement of probiotic biomass yield by optimizing pH, ammonium citrate and peptone. Amylases and proteases occupy a large share of the enzyme market hence production of these enzymes is a thrust area of research. In the present study mid ranges of peptone and glycerol concentration at low pH favored the production of amylase enzyme. Akcan et al. [14] have reported the negative impact of glycerol and peptone on the production of amylase using *Bacillus subtilis* in submerged cultivation which is not true in our case. According to Aiyer et al. [15], peptone (484 Uml^{-1}) and glycerol (242 Uml^{-1}) has influenced the production of amylase enzyme by *Bacillus licheniformis* SPT 27 suggesting the importance of peptone in amylase production. Vidyalakshmi et al. [16] have reported that at pH 7 highest amylase enzyme was produced by Bacillus spp. At lower concentration of glycerol, peptone and increase in pH has influenced the production of protease enzyme by *P. indica*. Vidyasagar et al. [17] have reported that at neutral and alkaline pH, high protease enzyme was produced by Halogeometricum sp. TSS101. The novelty of present paper lies in the optimization of glycerol, peptone and pH to obtain higher biomass of *P. indica* as it has a remarkable application as a biofertilizer and biocontrol agent.

CONCLUSION

The present paper investigates the optimization of process parameters to achieve high biomass of *P. indica*. Three variables glycerol, peptone and pH played an important role on growth of *P. indica*. Glycerol concentration exhibited significant effect on the growth of *P. indica*. A maximum amylase activity of 0.08U/ml was observed at mid concentration of the variables. Increase in concentration of peptone decreased the protease activity whereas increase in pH and decrease in glycerol concentration, maximum protease activity of 0.25 U/ml was observed.

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