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PHOSPHATE SOLUBILISING ACTIVITY OF *SYNCEPHALASTRUM* SP. ISOLATED FROM IRON MINE WASTE SOIL OF BARBIL, KEONJHAR

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ABSTRACT

Phosphate solubilising efficacy of microorganisms is influenced greatly by medium composition especially the N and C sources and pH of the medium used. The test organism Syncephalastrum was isolated from the iron mine waste soil of Barbil in Keonjhar district of Odisha during February 2013-July 2015. In the present investigation, phosphatase activity of the isolate Syncephalastrum sp., having phylogenetic relationship between zygomycetes and ascomycetes varied greatly when Pikovskaya's medium was supplemented with carbon sources(sucrose, dextrose, starch, manitol, sorbitol and fructose) at varied concentration(0.5,1, 1.5 and 2%) separately. Optimal activity for both acid and alkaline phosphatase was observed at 2% dextrose followed by sucrose and maltose in the medium, at 29^{0}C after 7 days of incubation. While different nitrogen sources (ammonium nitrate, sodium nitrate, calcium nitrate, Yeast-extract, potassium nitrate and peptone) at varied concentration (0.1, 0.3, 0.5 and 0.7%) were used separately to study their effect on phosphatase activity, it was observed that the isolate showed optimum solubilising activity at 0.7% ammonium sulphate followed by 0.1% sodium nitrate and 0.1% Yeast-extract at 29^{0}C after 7 days of incubation. The study underscores the importance of Syncephalastrum sp. as a potential biofertilizer and could be exploited as bio-inoculants in reclamation of mine waste soil.

Key words: S*yncephalastrum* sp, mine waste soil, phosphatase activity, RSM

INTRODUCTION

Soil microbes have a big impact on plant productivity. In soil, special group of microorganisms are known as plant growth promoting rhizobacteria (PGPR) that colonize in plant roots and promote growth of plants (Lucy *et al.* 2004). These organisms enhance crop productivity by increasing soil fertility in different ways such as nitrogen fixation, increasing supply of nutrients (phosphorus, sulphur, iron, copper etc.), producing plant hormones, controlling various fungal and bacterial diseases. Next to nitrogen, phosphorus plays an important role in nutrition of plants such as, photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration (Khan *et al.* 2010). Phosphate is present in soil in insoluble forms and it can be made available by different biogeochemical cycles through various group of bacteria and fungi (Harris *et al.* 2006; Perez *et al.* 2007). The concentration of bioavailable phosphate in soil is very low reaching the level of 1.0g - 1kg soil (Ezawa *et al.* 2002) in comparison, in case of mining soil available phosphate is also much lower than the standard value. This low level of phosphate in mining soil could be attributable to low microbial interaction in these environments, due to low availability of inorganic carbon and other nutrients. High temperature, low pH and presence of heavy amount of iron pyrite regulate not only microbial diversity also microbial activity (Arumanayagam and Arunmani 2014). In view of the above, the fungal isolate *Syncephalstrum* sp. isolated from mining soil of Barbil, Keonjhar, Odisha, India was subjected for studying its phosphate solubilising activities in order to explore its use as a phosphate solubiliser in these mine soil.

MATERIALS AND METHODS

Study site and sample collection

Barbil a municipal township in the Keonjhar district of the state of Odisha, India, lies between 22^0.10' N latitude, 85^0.37' E longitude and 477 metre altitude. The surrounding region of Barbil has the fifth largest deposit of iron ore and manganese ore in the world. A total of 10 soil samples were collected

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from different sites of Barbil from explored and unexplored mining area (Forest area) following the method adopted by Baruah and Barthakur (1998) during Feb 2013 – July 2015. All the soil samples were subjected to physico-chemical analysis (Parida *et al.* 2014).

Isolation and identification of source organism

The fungus (Isolate DB34) was isolated from mining soil of Barbil Keonjhar, Odisha through standard viable culture technique on Potato dextrose agar medium. Based on colony morphology, growth characters and microscopic features the isolate was identified as *Syncephalstrum* sp. [National Centre for Fungal Taxonomy (NCFT), New Delhi]. Molecular identification of the isolate was carried out through 18s rDNA sequencing. Evolutionary analyses were conducted by MEGA5 (Tamura *et al.* 2011). Total 22 sp of Zygomycotina and Ascomycotina with *Syncephalstrum* sp. (DB-34) were taken into account for preparation of the phylogenetic tree following the method of maximum parsimony. The assembled sequence of ITS region of 18S RNA of the isolate was submitted to NCBI, having accession number, KU 343134.

Screening for phosphate solubilizing activities

Phosphate solubilization activity of the isolates was tested on Pikovskaya's agar (PA) plate containing tricalcium phosphate as insoluble phosphate source. Pikovskaya's agar plates were prepared as per manufacturer's (Yeast extract 0.500 gm/L, Dextrose 10 gm/L, Calcium phosphate 5 gm/L, Ammonium sulphate 0.5 gm/L, Potassium chloride 0.2 gm/L, Magnesium sulphate 0.1 gm/L, Manganese sulphate 0.0001 gm/L, Ferrous sulphate 0.0001 gm/L, agar 15gm/L) instructions. Freshly grown culture of the isolate was spot inoculated on PA plates by the help of a sterile needle. Inoculated plates were incubated at 30^0 C for 72 hrs. Formation of a halo zone around the colony is indicative of positive phosphatase activity. Solubilizing index (SI) of the isolate was determined by using the formula of Arun and Sridhar (2005) as follows:

$$SI = \frac{\text{Halo zone + Colony diameter}}{\text{Colony diameter}}$$

Assay for phosphatase activity of the isolate

The isolate was cultured in Pikovskaya's broth, at 28 ± 2^0C, for 7 days. After the incubation period, the culture was filtered by Whatman filter paper. The filtrate was centrifuged at 1000 rpm for 10min and the supernatant was used for phosphatase assay, spectrophotometrically using p-nitrophenyl phosphate (p-NPP) as substrate. The p-nitrophenol was used to make standard curve for determination of phosphatase activity. Enzyme activity was indicated by an increase in the absorbance of light at λ = 410 nm. Phosphatase activity was transformed to absolute units using a standard curve based on increasing concentrations of p-nitrophenol (Berman *et al.,* 1990). The presence of acid phosphatase and alkaline phosphatase were determined as: 0.4 ml filtrate was incubated for 20 min with 0.8 ml of p-NPP at 70^0C for alkaline phosphatase, adding 0.4 ml of 0.05M tris-HCl buffer (pH 9.5) and at 65^0C for acid phosphatase in 0.25M sodium acetate buffer (pH 6.0). Reaction was terminated by adding 2 ml of 1N of NaOH to measure the freely dissolved phosphatase activity (acid and alkaline). The activity was expressed in n mol p-NP released/ml/ 20 min. (Lo pez *et al.* 2006).

Condition optimization for phosphatase activity of the Isolate

Incubation period: The phosphatase activity by the isolate was studied at different incubation period. Isolate was cultured at 29^0C in the medium as described above. The phosphate solubilizing activity was assayed at different time intervals i.e. 3, 4, 5, 6, 7, 8, 9,10,11,12 and 13 days, following the procedure described earlier.

Temperature: The isolate was cultured on Pikovskaya's medium at different temperatures (15^0C, 22^0C, 29^0C, 36^0C, 43^0C and 50^0C). The phosphatase activity was assayed after 7 days of incubation following the procedure as described earlier.

pH: The phosphatase activity was determined at different pH by following the method as described earlier, after 7 days of incubation at 29^0C by growing the isolate in Pikovskaya's medium at different pH (4, 5, 6, 7, 8, 9, 10, 11 and 12) separately.

*Carbon sources***:** An experiment was designed to study the effect of different carbon sources on phosphatase activity of the isolate. Briefly, Pikovskaya's medium was supplemented with

different carbon sources (sucrose, dextrose, starch, manitol, sorbitol and fructose) at varied concentration (1, 1.5 and 2%) separately. The isolate was incubated in the carbon supplemented medium at 29^0C for 7 days and the phosphatase activity was studied as described previously.

***Nitrogen sources*:** The Pikovskaya's medium was supplemented with different nitrogen sources (ammonium nitrate, ammonium sulphate, sodium nitrate, calcium nitrate, Yeast, potassium nitrate and peptone) with varied concentrations (0.1, 0.3, 0.5 and 0.7%) separately. The isolate was incubated at 29^0C for 7 days and the phosphatase activity was studied.

Optimization of incubation period, temperature and pH using Response Surface Methodology (RSM):

RSM is a statistical tool by which effect of different factors on microbial production can be summarized. Here, the effect of different factors (Incubation period, pH and Temperature) on phosphate solubilising activity of the isolate was optimized by statistical modelling and it was carried out by using Central Composite Design (CCD) (Stat-Ease, version 8 of Design-Expert).

Phosphatase (Y, n mol p-NP released/ml/ 20 min) was used as the dependent output variable. The three independent factors were investigated at five different coded levels (-1.682, -1, 0, +1,+1.682) (Table 2). A 2^3 factorial Central Composite Experimental Design, with six axial points and six replications at the centre points leading to a total number of 20 experiments was employed. Equation-1: $Y=b0+b1X1+b2X2 +b3X3 +b11X21 +b22 X22 +b33 X23 +b12 X1 X2 +b23 X2X3 +b13 X1X3$. Whereas, Y is the predicted response (Phosphatase) of X1, X2 and X3 are the coded levels of the independent variables, b0 intercept, b1, b2 and b3 the linear coefficients, b11, b22 and b33 are the squared coefficient and b12, b13 and b23 are the interaction coefficients. Statistical significance of the model equation was determined by Fisher's test value and the production of variance explained by the model was given by the multiple coefficient of determination, R squared (R^2) (Manikandan and Viruthagiri, 2010; Jena and Rath, 2013).

Statistical analysis

One way ANOVA was performed using SPSS (version 16) software, to test whether the mean sensory scores of the effect of carbon and nitrogen sources on phosphatase activity differed significantly from each other or not.

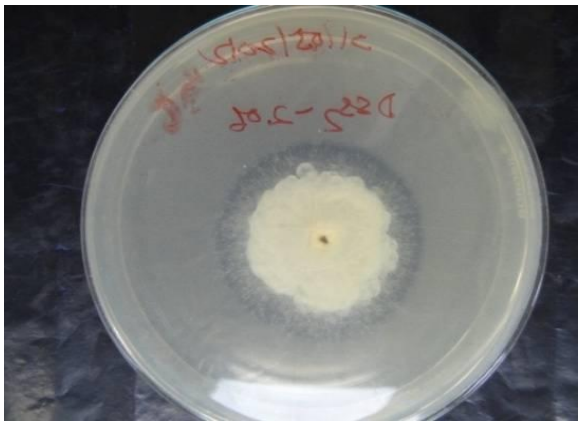
RESULTS AND DISCUSSION

Identification of the isolate

Primarily, the isolate was identified by studying their colony morphology on Potato Dextrose Agar medium and microscopic analysis of reproductive structure. The growth was very fast; initially the colony appeared whitish and later on light grey, cottony to fluffy, finally becoming dark grey with the development of sporangia. Sporangiophores are erect, stolon-like, often producing adventitious rhizoids, and show sympodial branching (racemose branching) producing curved lateral branches. The main stalk and branches form terminal, globose to ovoid vesicles which bear finger-like merosporangia directly over their entire surface (Fig. 1). The phylogentic relation of the isolate with zygomycotina and ascomycotina was evaluated by using the Maximum Parsimony method. The consistency index was observed to be 0.839437 (0.648148), with retention index 0.831858 (0.831858), and composite index 0.698292 (0.539167) for all sites and parsimony-informative sites. The MP tree was obtained using the Close-Neighbour-Interchange algorithm (Nei and Kumar, 2000). The analysis involved 26 nucleotide sequences (Fig. 2).

Table 1: Range of the values for the Response Surface Methodology of phosphatase activity

Independent variables	Coded levels				
	-1.682(-α)	-1	0	+1	+1.682 (+α)
Incubation Period (day)	1.9	5	7	10	12.04
Incubation Temperature (^{o}C)	5.5	15	29	43	52.5
pH	3.6	5	7	9	10.36



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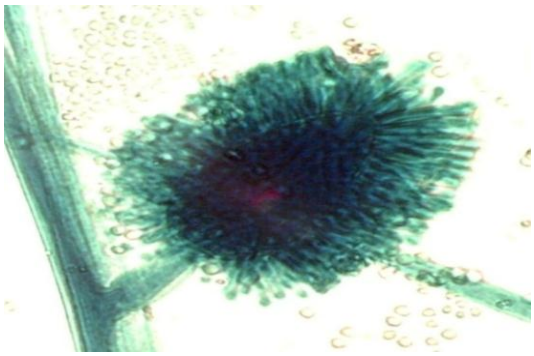

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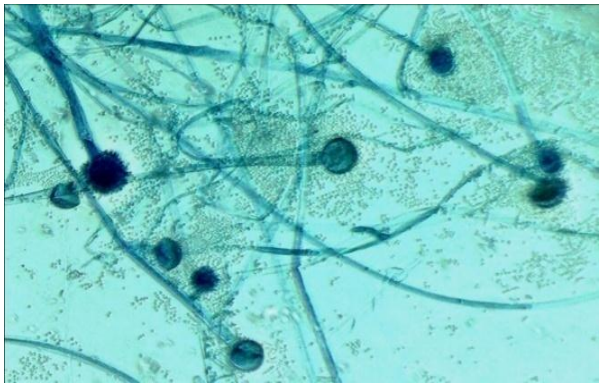


(c)

Fig. 1: Morphological identification of the isolates: (a) colony morphology and phosphate solubilisation (presence of halozone around the colony) on Pikovaskya's agar plate, (b) & (c) reproductive structure: sporangiophore with meroporangia [cover glass mount with lactophenol cotton blue staining (10X40x Magnification)

Effect of physical parameters (incubation period, temperature, pH) on phosphatase activity

The phosphatise activity of the isolate was assayed using P-NPP as the substrate following the method of Berman *et al.* (1990) and Lo pez *et al.* (2006). While studying the effect of incubation period on phosphate solubilising activity of the isolate, it was observed that the isolate showed maximum phosphatase activity after 7 days of incubation at 29^0C (12.4±0.3 and 9.22±0.5 n mol p-nitrophenol/mi/20 min of acid and alkaline phosphatase respectively). Surprisingly, while comparing the dry biomass with respect to phosphatase activity, it was found that the isolate did not grow after 7 days of incubation. Hence, all experiments were further conducted after 7days of incubation. On the other hand, the production of acid phosphatase was found to be more than alkaline phosphatase. The pH was reduced with the days of incubation from 4.18 to 3.4 (Fig. 3).

The effect of temperature on phosphatase activity was studied on Pikovskaya's medium by incubating the culture flasks at different temperatures (15^0C, 22^0C, 29^0C, 36^0C, 43^0C, 50^0C) after 7 days of incubation. The maximum phosphatase activity was observed to be 10.68±0.9 and 8.5±0.4 n mol p-nitrophenol/ml/20min for both acid and alkaline phosphatase respectively, at 29^0C. A decrease in both alkaline and acid phosphatase was reported at higher temperatures. The pH and dry biomass was found to be 4.26 and 0.43 g respectively at 29^0C (Table 2 and Fig. 3).

When the phosphate solubilizing activity of the isolate was studied at different pH (pH 4-12), maximum activity was observed at pH 7 (10.07±0.4 and 8.62±1 n mol p-nitrophenol/ml/20min of acid and alkaline phosphatase respectively) and a degree in reduction in activity was recorded at alkaline pH range. The pH was changed 4.26 from pH 7 after incubation and the dry biomass was observed to be 0.47 g.

Optimization of incubation period, temperature and pH by using RSM

The results of Central Composite Design (CCD) experiments were taken to study the effect of three independent variables (incubation period, temperature and pH). The actual phosphate solubilising activity of the isolate obtained in the experiments and the yields predicted by the model equation (1) in Table 2. The ANOVA result of quadratic regression model for Y of phosphate solubilizing activity is described in Table 3, where, Y is the phosphate

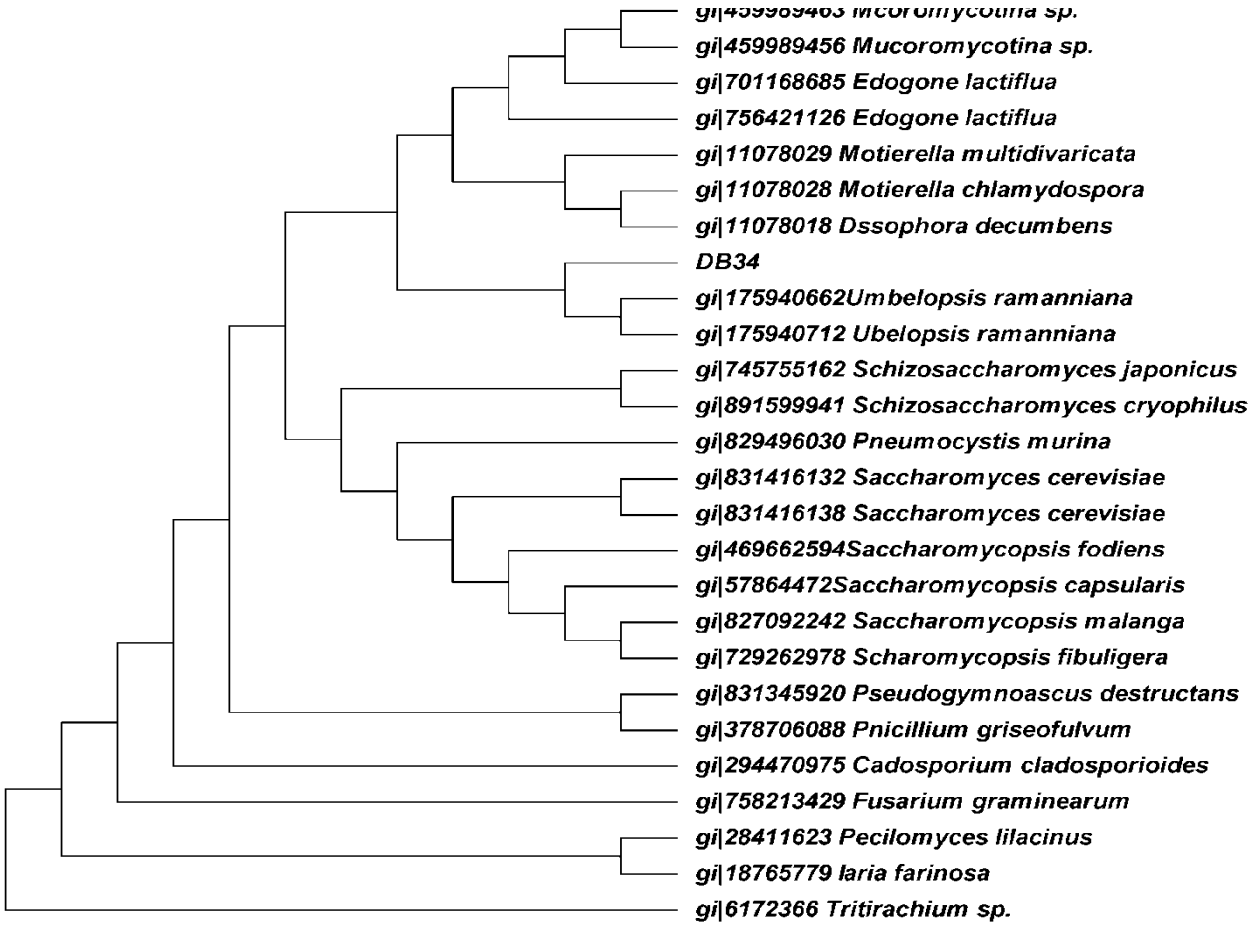
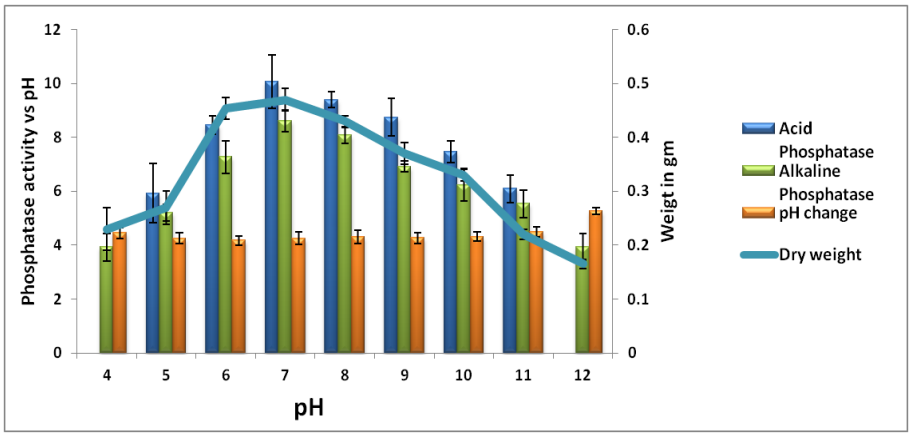


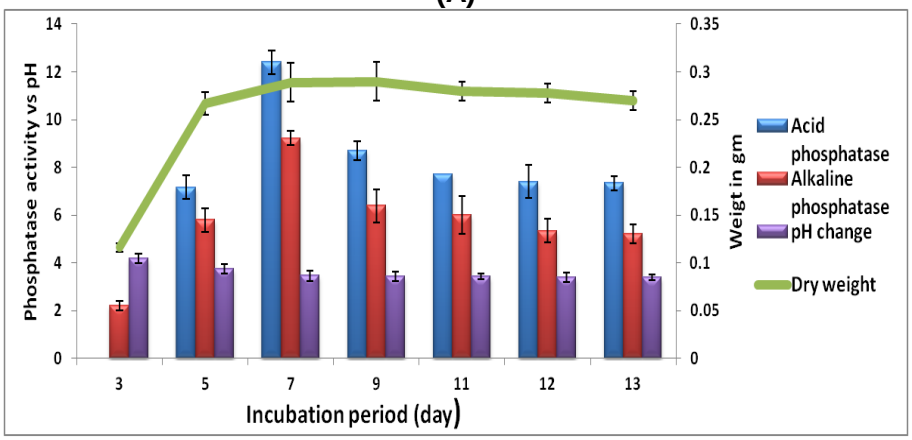
Fig. 2: Phylogenetic tree generated by maximum Parsimony showing evolutionary relationship of *Syncephalastrum* (DB34) along with 25 closely related species of of Zygomycotina and Ascomycotina sp.

Table 2: Experimental design and result of CCD of response methodology of phosphatase activity (n mol p-nitrophenol/ml/20mm)

Standard order	A: Incubation period (day)	B: Temperature (^{0}C)	C: pH	Phosphatase activity	
				Experimental value	Predicted value
1	7	29	3.65	2	0.58
2	7	5.4	7	4	3.19
3	10	15	5	3	4.19
4	4	15	9	4	6.8
5	7	29	7	5	1.43
6	4	43	9	6	4.03
7	7	29	7	8	8.04
8	1.9	29	7	10	10.64
9	7	29	7	4	5.86
10	10	43	5	11	10.24
11	7	29	7	0	4.25
12	10	15	9	16	12.85
13	7	29	7	0	-1.42
14	4	43	5	0	2.52
15	7	29	7	20	19.8
16	7	52.5	7	20	19.8
17	7	29	10.3	20	19.8
18	12.04	29	7	20	19.8
19	4	15	5	20	19.8
20	10	43	9	19	19.8



(A)



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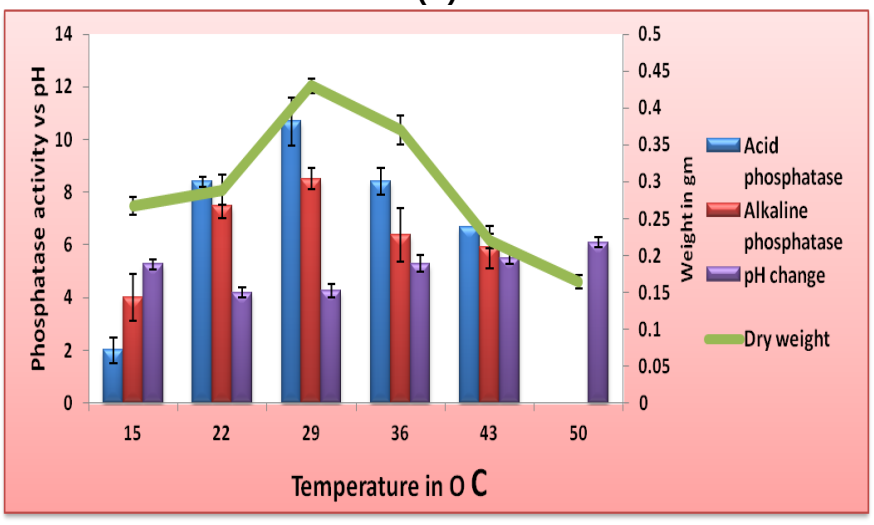

(C)

Fig. 3: Effect of different environmental conditions on phosphatase activity (n mol p-nitrophenol/ml/20min) of the isolate *Syncephalastrum*: (A) pH; (B) Incubation period; (C) Temperature

solubilizing activity. The coefficient of determination (R2) for phosphate solubilizing activity was found to be 0.94, indicating that the statistical model can explain 94% of variability in the response.

The R square value ranged between 0 and 1, the closer the R^2 to 1.0, the stronger is the model and the better it predicts the response (Ray *et al.* 2010). In adequate precision of 11.324 for phosphate solubilizing activity was calculated. The lack of fit F-value of 83.3 means

that lack of fit is significant. The model F-value is 17.56, prob >F (< 0.05 indicated that the model terms are significant) indicating that the model is significant for phosphate solubilizing activity. The predicted R^2 0.5518 are in reasonable concurrences with the adjusted R-Squared 0.8870 respectively. This indicated a good accord between the experimental and predicted value for phosphate solubilizing activity of the isolate *Syncephalastrum*. Response surface was generated by plotting the response of phosphate solubilizing activity on the Z axis against any two independent variables while keeping other independent variable at zero level. The response surface 3D curves and counter plots (Fig. 4) explained the interactions of independent variables and determine the optimum phosphate solubilizing activity of dependent variable. The maximum orientation of the principal axes of the response surface plot between incubation period and temperature, pH and temperature, temperature and incubation period, indicated that the mutual interactions between independent variables had a significant effect on dependent variable. In the present study the three response surface plots for phosphate solubilizing activity proved to be significant i.e. incubation period with temperature, incubation period with pH and temperature with pH for phosphate solubilizing activity in the medium by the isolate. A linear increase of phosphate solubilizing activity was found with the increase of incubation period up to 7days thereafter, it was declined. A similar result was found in case of pH and temperature for phosphate solubilizing activity. Thus, temperature at 29^0C and pH (7.0) were adequate for attaining maximum phosphate solubilizing activity (Fan *et al.* 2011). The experimental values were found to be very close to the predicted values and hence, the model was successfully validated and also reflected the accuracy and applicability of RSM to optimize the process for enzyme production in submerged fermentation (Table 2 and 3) Validation of the statistical model and regression equation was performed by taking [X1(7 days), X2 (29^0C), X3 (pH 7)] in the experiments.

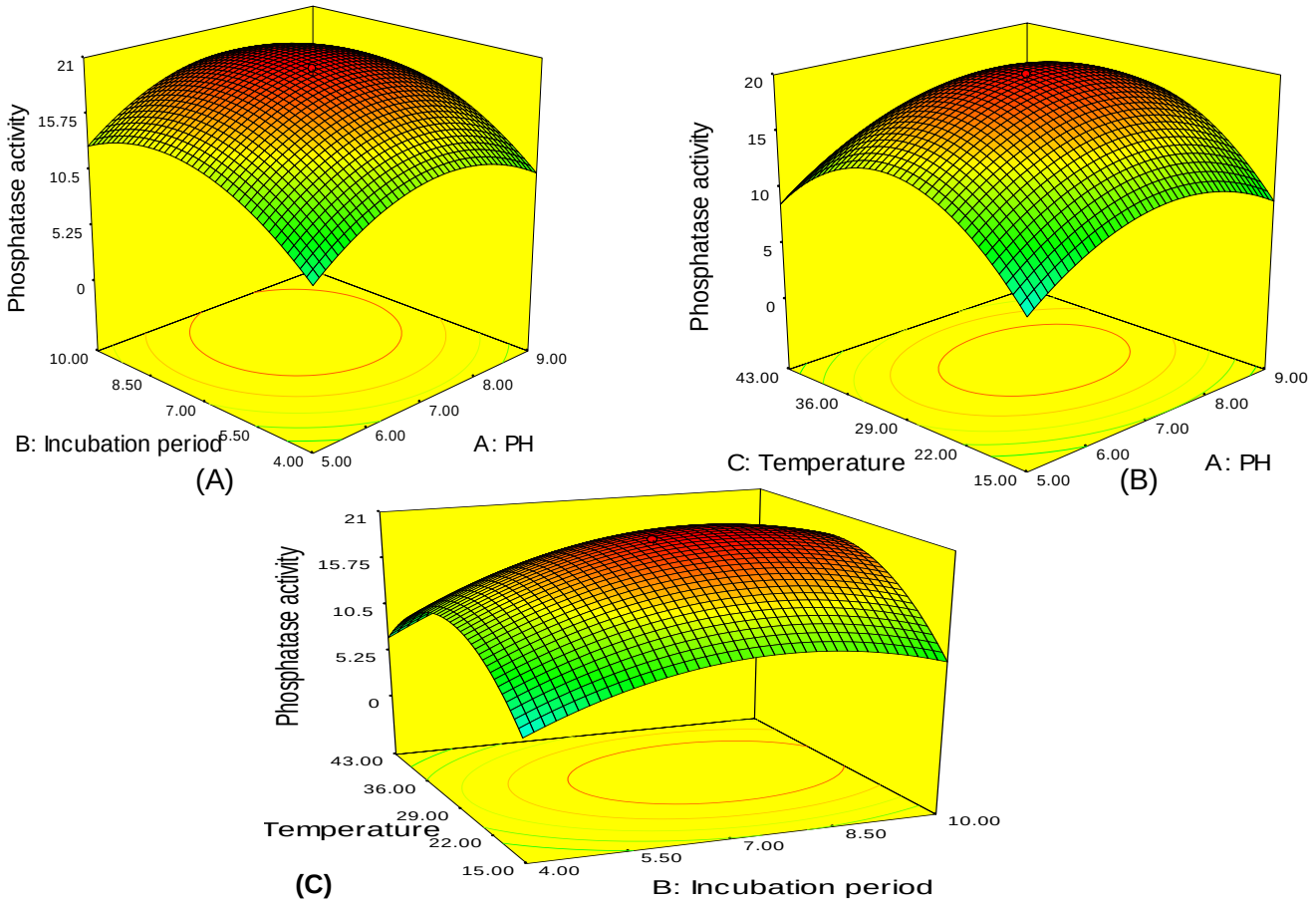

Fig. 4: Statistical optimization of Phosphatase activity (n mol p-nitrophenol/ml/20min) of the isolate *Syncephalastrum* using RSM: (A) pH and Incubation period; (B) pH and Temperature; (C) Temperature and Incubation period

Table 3: ANOVA for phosphates activity (n mol p-nitrophenol/ml/20mm) in batch culture of the isolate

Sources	Sum of squares	Degree of freedom	Mean square	F- value	p- value
Model	1110.548	9	123.3942	17.56447	< 0.0001
Lack of Fit	69.41885	5	13.88377	83.30262	< 0.0001
Pure Error	0.833333	5	0.166667		
Total	1.234401	19			

R-Squared 0.9405; Adj R-Squared 0.8870; Pred R-Squared 0.5518; Adeq Precision 11.324

Effect of carbon and nitrogen on phosphatase activity of the isolate

The phosphatase activity of the test isolate varied greatly with respect to carbon sources used in the experiment. The isolate showed optimum solubilizing activity at 2% dextrose followed by sucrose 2% and maltose 2% at 29^{0}C after 7 days of incubation. The activity at 2% dextrose was reported to be 23.65±0.32 and11.33±0.08 n mol p-nitrophenol/ml/20 min of acid and alkaline phosphate respectively. The pH changed from neutral to 4.51 after 7 days of incubation and the dry mass was 1.8 g (Fig. 5).

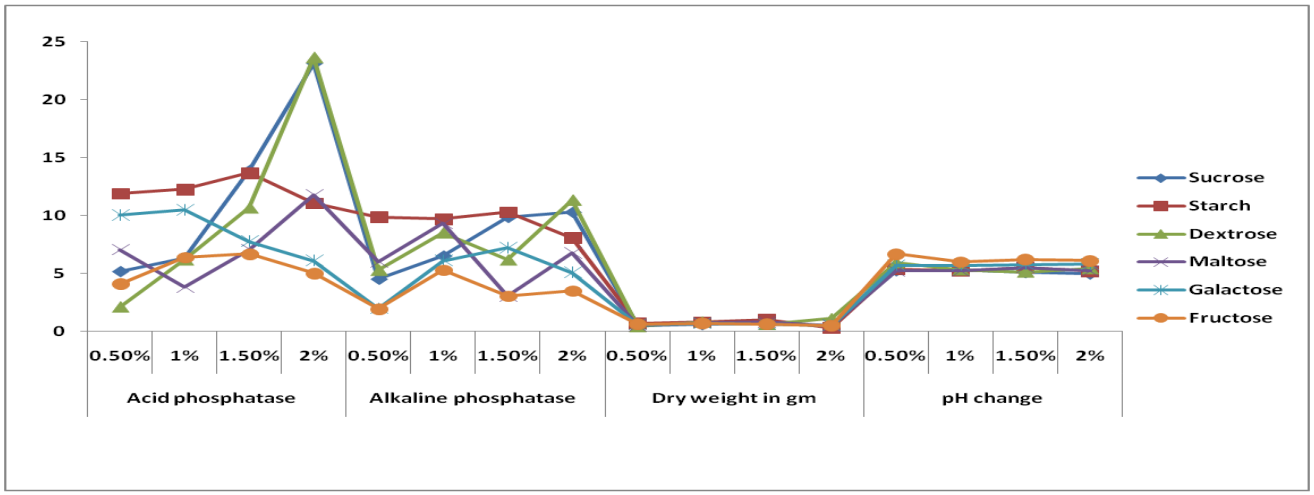

Fig. 5: Effect of different carbon sources on phosphatase activity (n mol p-nitrophenol /ml /20min) of the isoalte

It was observed that the isolate showed optimum solubilizing activity at 0.7% ammonium sulphate. It was found to be followed by 0.1% sodium nitrate and 0.1% yeast extract at 29^{0}C after 7 days of incubation. The activity at 0.7% ammonium sulphate was reported as 23.28±2 and 4.78±0.7 n mol p-nitrophenol/ml/20 min of acid and alkaline phosphatise, respectively. However, the pH changed from neutral to acidic 5.45 after 7 days of incubation with a dry mass was 0.33 g (Fig. 6).

Statistical modeling of different carbon and nitrogen sources on phosphatase activity in flask culture

The analysis of ANOVA showed that all the six carbon sources differed significantly from each other. Post-hoc analysis revealed that 2% dextrose followed by sucrose, and maltose have significant effect on phosphate solubilizing activity production (P< 0.05; Tukey's LSD). Further, all other carbon sources were less significantly differred from each other. While, the mean scores of six nitrogen sources were subjected to One-way ANOVA, the Post-hoc analysis revealed that, only three nitrogen sources i.e ammonium nitrate at 0.7% followed by peptone (0.1%), yeast (0.5) have highly significant effect on phosphate solubilizing activity (P<0.05; Tukey's LSD) of the test fungus.

In the present investigation, an attempt was made to study the effect of incubation period, incubation temperature, pH, carbon and nitrogen sources on phosphate solubilizing

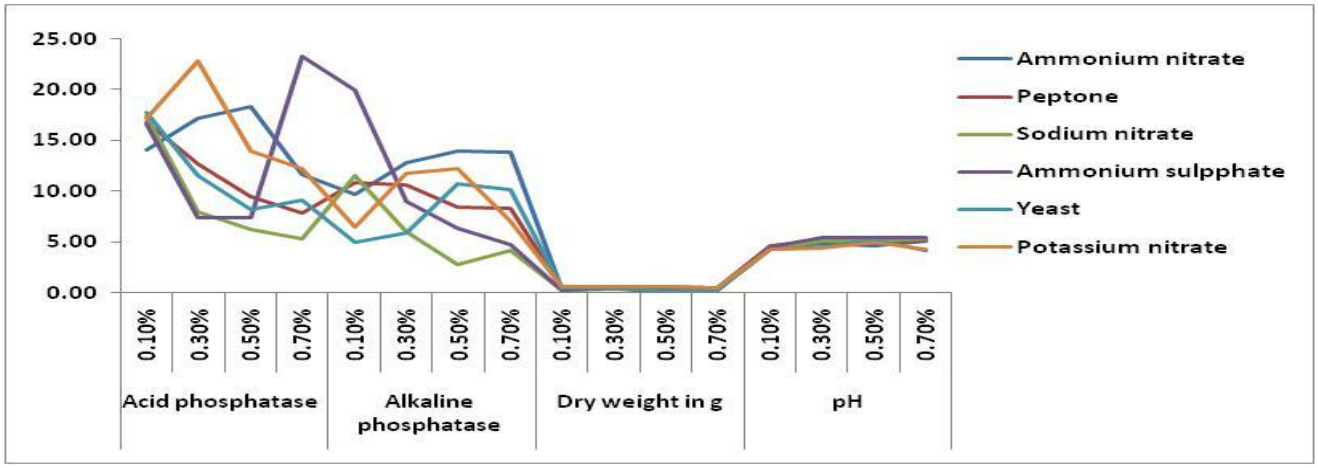

Fig. 6: Effect of various nitrogen sources on phosphatase activity (n mol p-nitrophenol /ml /20min)

activity of the isolate *Syncephalastrum* sp isolated from mining soil of Barbil, Keonjhar. Barbil is an industrialized area with mining activities throughout the year. Parida *et al.* (2014) reported that moisture content of explored mining soil was comparatively lower and it varied from 5.8 to 8.6. But, highest percentage of moisture (13.4%) was recorded from unexplored area or vegetation area. Soil temperature of the studied area varied from 34 – 44^0C. The percentage of organic carbon varied from 0.14 – 1.18 among the sites. Salinity of the soil samples was found to be even. Phosphorus content of the soil was observed to be very less, varied from 2.4 to 4.4 kg ha^{-1}. In contrast potassium content was found to be highest in unexplored area (351 kg ha^{-1}), lowest (50 kg ha^{-1}) of the explored area (Parida *et al.* 2014). Observation of above soil properties idicated that, the isolate have the ability to tolerate different adverse condition during their life process.

The phosphatase activity of the isolate, *Syncephalastrum* sp. was in a wide range of pH (4-12). But maximum phosphatase activity was reported at neutral pH. Our findings are in agreement with Oyeleke and Oduwole (2009) who stated that the most microbial enzymes function between a pH range of 6 and 8. Further, the retention of phosphatase activity by *Syncephalastrum* at a wide range of pH, suggested that it is a potential isolate that could be exploited for biofertilizer in mining soil environment. At all the stages, the isolate produced more acidic phosphatase in comparison to alkaline phosphatase. Jena and Rath (2013) reported that decrease of the pH in growth medium was due to the production of different titrable acid and could be attributable to the acidic phosphatase activity by the species as observed here. Further, it could be due to the production of different organic acids in the medium, as organic acids play a key role to solubilizing both inorganic and organic insoluble phosphates (Henri *et al.* 2008).

The phosphatase activity of the isolate was within 3 to 13 days of incubation. The isolate also showed phosphatase activity at a wide range of temperature (15-50^0C). It could be attributable to the variation of soil temperature 10-50^0C of the study site, and the viability of the isolate at this wide temperature variation. However, the optimal activity was reported on 7^{th} day of incubation at 29^0 C. Phosphate solubilizing efficacy of microorganisms is influenced greatly by medium composition, especially the N and C sources, and the pH of the medium used (Mehta and Nautiyal, 2001, Pradhan and Sukla, 2005). Maximum phosphatase activity of the isolate was observed while the medium was supplemented with dextrose, as dextrose is the simplest carbon source and used by fungi for growth and development. The isolate, *Syncephalastrum* sp. showed maximum phosphate solubilizing activity with ammonium sulphate and dextrose in the medium as N and C sources respectively. Furthermore, it could be due to the production of

inorganic acid by proton exchange mechanism in presence of NH_4^+ in the medium that accelerated phosphate solubilization (Sridevi *et al.* 2007) as NH_4^+ and NO_3^- are absorbed by heterotrophic organisms as the nitrogen sources.

It is indicated that, our isolate *Syncephalastrum* sp., is a rarely isolated soil fungus and this species is phylogeneticaly linked Zygomycetes and Ascomycetes. Interestingly, it was isolated from a mining soil rich with iron and showed phosphatase activity during the investigation. The isolate could retain its phosphate solubilizing activity even at high temperature and low pH hence; it could be exploited biotechnologically for their possible development as a biofertilizer for use in agriculture of mining soil. However, studies are essential to examine the toxicity of the strain towards plants and animals and their crop specificity (if any) before its use in agriculture.

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