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Research article

SHAKE FLASK OPTIMIZATION FOR SOME PHOSPHATE SOLUBILIZING BACTERIA

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ABSTRACT: Microbial growth is greatly influenced by environmental parameters; some favoring rapid microbial growth while some diminishing or slowing it. Each organism, when cultivated, has a specific optimum range for environmental parameters – temperature, pH, agitation/aeration, etc. Reports on optimization of shake flask conditions for growth of phosphate solubilizing bacteria (PSB) and phosphate solubilization are limited. In the present study, work on optimizing culture conditions for growth of some PSB isolates is presented. Three different PSB species (*Burkholderia cepacia*, *Rhizobium radiobacter* and *Ralstonia pickettii*) isolated from relatively unexplored sourceswere subjected to different conditions of temperature, pH and mixing/aeration at the shake flask level, to assess their effect on the production of biomass as well as phosphate solubilization. The effects of carbon and nitrogen sources and C/N ratios were evaluated. It was found that maximum biomass production was shown by *Burkholderia cepacia*/CC2 and *Ralstonia pickettii*/PC2 at pH 7.0; 36°C at 250 rpm. However, optimum phosphate solubilization was observed at 28°C under identical conditions. In case of *Rhizobium radiobacter*/KC3, the culture grew best and solubilized P maximally at 20°C at pH 4.0.The optimum medium composition for growth was observed to be when Glucose was the C source and yeast extract was the nitrogen source. The biomass production was optimum when C/N ratio was 60:1.Best solubilization of phosphate under optimized set of conditions was shown by *Ralstonia pickettii*/PC2 (863±78 ppm in 96 h) followed by *Burkholderia cepacia*/CC2 (763±92 ppm in 24h) and *Rhizobium radiobacter*/KC3 (690±62ppm in 48h).

Key words: Shake flask optimization; Phosphate solubilizing bacteria (PSB), *Ralstonia pickettii*; *Burkholderia cepacia*; *Rhizobium radiobacter;* environmental factors; C:N ratio.

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INTRODUCTION

Work on isolation of novel phosphate solubilizing bacteria (PSB) has been carried out for several decades; however, few comprehensive reports are available that describe optimization of culture conditions for optimum growth of PSB. Microbial growth significantly varies with environmental parameters; some conditions augment rapid microbial growth while others do not permit any growth (Archunan, 2004). The growth conditions reported in scientific literature so far are mainly concerned with work on isolation and screening for PSB (Jung et al., 2002; Ghosh et al., 2008; Sharma et al., 2013; Reena et al., 2013). There are relatively fewer reports available on optimization of culture conditions for growth of PSB under the laboratory conditions (Sagervanshi et al., 2012);most reports have cited generalized growth conditions for all isolates regardless of the source from which they were isolated (Pandey et al., 2006; Kannapiran and Ramkumar, 2011).

There are even fewer reports on effect(s) of change in environmental parameters on growth of PSB and their subsequent effect on the phosphate solubilization (Sagervanshi et al., 2012). However, effect of temperature fluctuations occurring in soil surface on microbial activities have been rather well documented (Haghiri, 1973; Krishnan and Rao, 1979; Cassman & Munns, 1980; Davidson et al., 1998; Nichols, 2005).Fuentes-Ramirez et al. (2005) have claimed that, the true efficiency of a PSB would be proved when it is able to actually sustain the dynamic variations in soil pH and temperature and retain its potential shown under lab conditions.Studies have also indicated that solubilization of phosphates in soil by cultures is an aerobic process and thus P solubilization can be affected by the mixing conditions (Sharma et al., 2013). It can thus be expected that phosphate solubilization by microbes in soil may be influenced markedly by changes in temperatures, pH and mixing conditions. Additionally, the phosphate solubilization efficiency of microorganisms is also dependent on factors like carbon and nitrogen sources (Scervino et al., 2011; Kumari, 2013). The availability and nature of nutrients is important to the mechanism of P solubilization because the concentration of organic acid production is influenced greatly by the substrates (Nahas, 2007). As a result, media composition and C:N ratios are important aspects to be evaluated for PSB during shake flask optimization (Cunningham and Kuiack, 1992; Whitelaw, 1999; Mehta and Nautiyal, 2001; Pradhan and Shukla, 2005).

The present study was aimed at optimization at shake flask level, for three promising PSB isolatesfor theirgrowth as well as solubilization potential forinorganic phosphate. The PSB isolates obtained from different sources were subjected to different conditions of temperature, pH, mixing/aeration; different media components as C and N sources and their concentrations.

MATERIALS AND METHODS

All chemicals and reagents were procured from Merck Chemicals Ltd, Mumbai, India or SRL Chemicals Ltd., Mumbai, India.Microbiological media components were procured from HiMedia Labs, Mumbai.

Medium Composition:

Pikovskaya's broth having (g/L): Dextrose 10.0, $(NH_4)_2SO_4$ 0.5, KCl 0.2 $MgSO_4.7H_2O$ 0.1, and trace quantities of $MnSO_4.H_2O$ and $FeSO_4.7H_2O$ and Yeast Extract 0.5 (Pikovskaya, 1948; Nair and Vakil, 2015) was used for the study. pH was adjusted to 7.0 using 1N NaOH before autoclaving and it was not controlled during growth.

Phosphate Solubilizing Microbes and Preparation of Cultures for Studies:

Three potentially efficient PSB (identified as *Burkholderia cepacia*/CC2, *Rhizobium radiobacter*/KC3, and *Ralstonia pickettii*/PC2) that were isolated from rhizospheres of coconut, cashew nutand pomegranate were selected for the study,as described previously (Nair and Vakil, 2015).Actively growing 24h old cultures ($O.D_{540nm}$ adjusted to ~10^8 cells/cm^3) were inoculated (1% v/v) in 50ml Pikovskaya's broth in 250 ml Erlenmeyer flasks and placed on orbital shaker with temperature and agitation control (Scigenics, India Orbitek shaker model 400 having stroke of 25mm).

Assessment of Growth

Dry cell weight estimation (DCW) was done in triplicate from broth to assess the growth (Mills and Lee, 1996).

Assay for Phosphate Solubilization

Phosphate solubilizationwas determined by spectrophotometrically using chloromolybdic acid and chlorostannous acid reagents as per the method described in Gaur A.C. (1990) with slight modifications (Nair and Vakil, 2015). All the assays were performed in triplicate.

Effect of Variation in Temperature, pH and Agitation

For determining the effect of temperature, cultures were incubated at 20°C, 28°C, and 36°C while the pH of the medium was adjusted to pH 7.0 before sterilization and mixing/aeration conditions were set at 180 rpm. Culture growth was recorded and phosphate solubilization was measured as described above.For studying the effect of pH, cultures were inoculated in Pikovskaya's broth with pH adjusted to 4.0, 7.0 and 8.0.For evaluating the effect of agitation, inoculated flasks were incubated at 100 rpm, 180 rpm and 250 rpm.

Effect of C and N Sources

For studying the effect of different carbon sources, the original Pikovskaya's medium was altered by replacing Glucose with Sucrose, Glycerol and a mixture of Glucose and Glycerol while maintaining the C:N ratio constant. Effect of change in nitrogen source was studied by replacing yeast extract from the original Pikovskaya's medium with peptone without changing the C:N ratio.

Effect of C:N Ratio

The original Pikovskaya's medium has C:N ratio of ~30:1. For this study, this ratio was altered to ~60:1. The medium composition thus became (g/L): Dextrose 25.0, $(NH_4)_2SO_4$ 0.5, KCl 0.2 $MgSO_4.7H_2O$ 0.1, trace quantities of $MnSO_4.H_2O$ and $FeSO_4.7H_2O$ and Yeast Extract 1.0.

RESULTS AND DISCUSSION

Effect of Temperature

Across the three temperature conditions, the highest growth (biomass as judged by DCW) was observed at 36°C, followed by at 28°C for *Burkholderia cepacia*/CC2 (1.2 ± 0.08g/L DCW) and *Ralstonia pickettii*/PC2 (1.4±0.16g/L DCW). However, it was observed that these isolates demonstrated highest ability for phosphate solubilization at 28°C followed by at 36°C.*Rhizobium* radiobacter/KC3 showed optimum growth and solubilization at 20°C.The trends of phosphate solubilization by each culture at 20, 28 and 36°C are shown in Figure1a, Figure 1b and Figure1c. Highest solubilization of phosphate was effected *by Ralstonia pickettii*/PC2 (574±43 ppm in 120h at 28°C); fastest solubilization was seen with *Burkholderia cepacia*/CC2 (374±33 ppm in 24h at 28°C)and least by *Rhizobium radiobacter*/KC3 (191±22 ppm in 72h at 20°C).

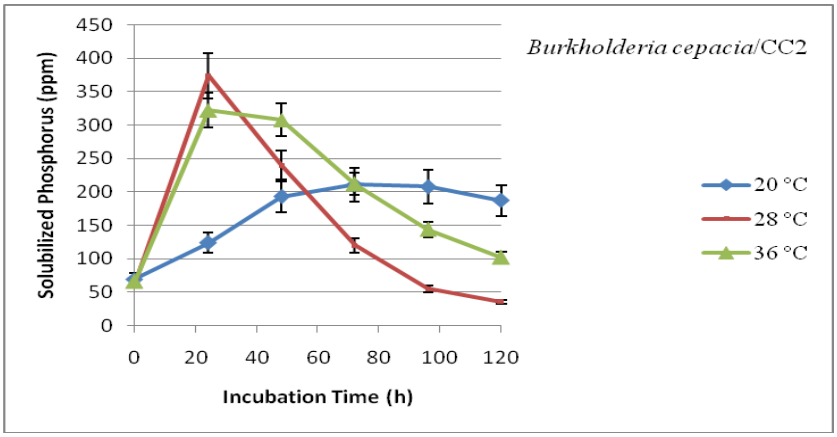

Figure 1a.

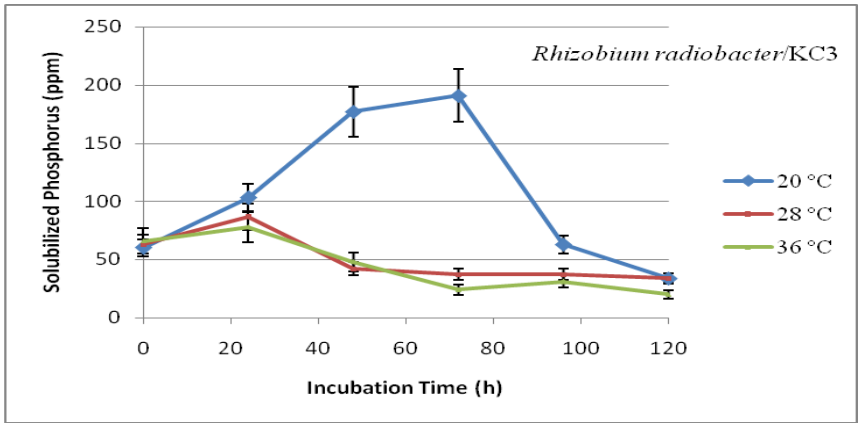

Figure 1b.

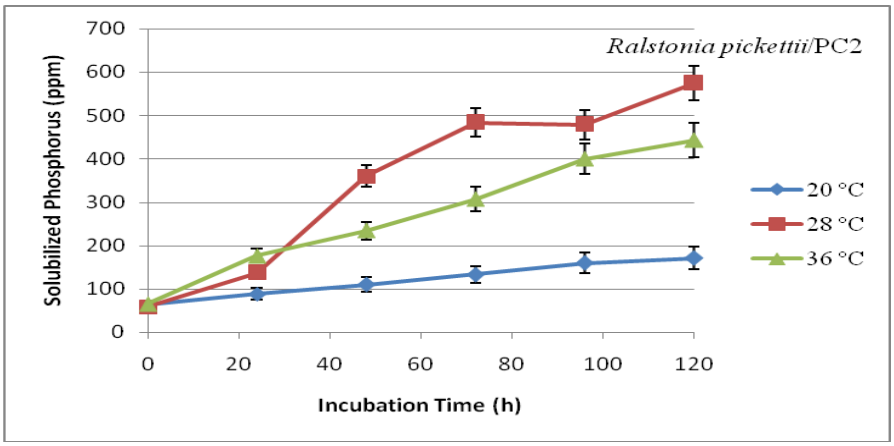

Figure 1c.

Figure 1a, b and c. Effect of temperature on phosphate solubilization by isolates *Burkholderia cepacia*/CC2, *Rhizobium radiobacter*/KC3 and *Ralstonia pickettii*/PC2. The cultures were inoculated in a medium of C:N ratio 30:1 (with pH of the medium was adjusted to 7.0 before sterilization), Flasks incubated at 180 rpm agitation.

The temperature conditions used for the study were selected on the basis of literature survey (Sharan and Jadhav, 2002; Krishnan and Rao, 1979). As shown in the Fig. 1, P solubilization by *Burkholderia cepacia*/CC2 and *Ralstonia pickettii*/PC2 was found to be better at 28°C than at 20 and 36°C. At 36°C, even though the biomass obtained was greater than that at 28°C, the amount of P solubilized was lesser. This indicates that in this case though higher temperatures are better for increase in cell number; this growth was not accompanied by better solubilization of insoluble phosphorus. Only *Rhizobium radiobacter*/KC3 showed a different trend - it showed greater ability to solubilize P at 20°C.

The phosphate solubilization trend is reasonably in agreement with findings of other researchers who observed that maximum growth of PSB cultures was obtained between 25-35°C (Sulbarán et al., 2009; Collavino et al., 2010, Ghosh et al., 2008). Hu et al. (2010) worked on a novel PSB *Pantoea* sp. and the effect of temperature conditions on the culture. They observed that *Pantoe astewartii* grew better between 20-35°C - the stable phase was attained after 24h, whereas, at 15°C, the stable phase was attained after 48-72h. However, none of the reports mention, different conditions for optimum growth and solubilization of phosphate though there are some stray reports on effect of temperature on the efficiency of phosphate solubilization (Zhu et al., 2011).

Effect of pH

During pH studies it was found that except *Rhizobium radiobacter*/KC3, the cultures showed optimal growth when the pH of the medium was ~7.0. At this pH, the cultures accumulated maximum biomass (1.2±0.08g/L for *Burkholderia cepacia*/CC2 and 1.4±0.16 g/L for *Ralstonia pickettii*/PC2). Growth was drastically affected for these isolates when the pH was 4.0 and 8.0. *Burkholderia cepacia*/CC2 and *Ralstonia pickettii*/PC2 showed optimum phosphate solubilization (374±33 ppm and 574±43 ppm respectively) at pH 7.0 whereas, *Rhizobium radiobacter*/KC3showedoptimum growth (1.2±0.08 g/L) and solubilization of phosphate (191±22 ppm)at pH 4.0 as observed in Figure 2a, Figure 2b and Figure 2c.

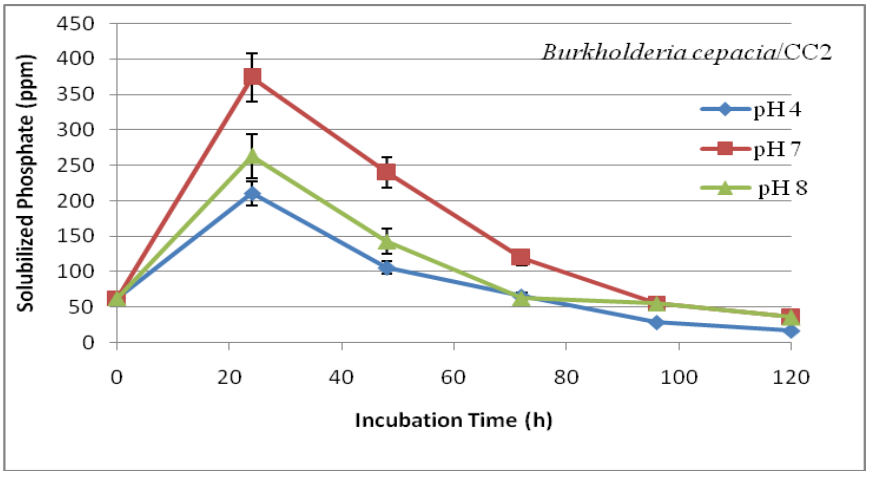

Figure 2a.

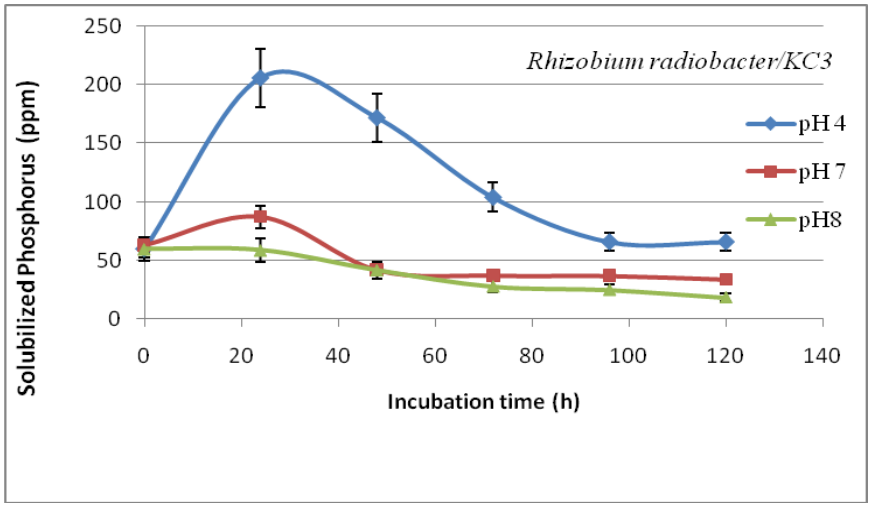

Figure 2b.

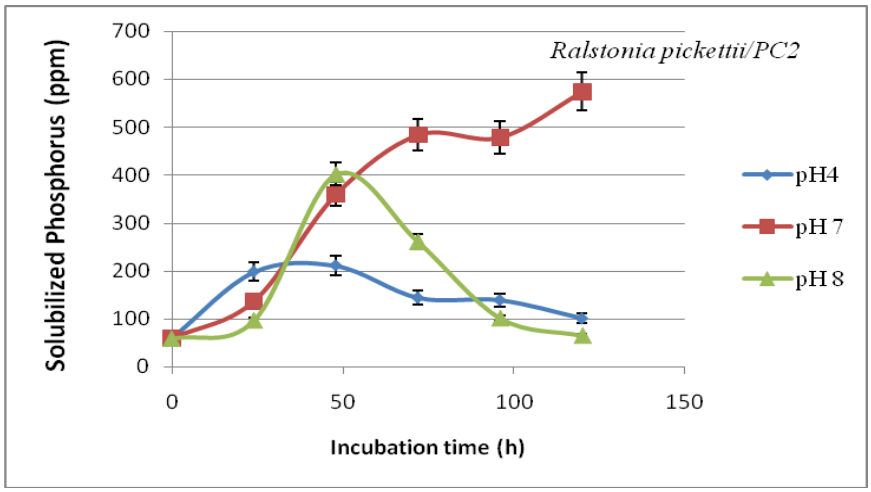

Figure 2c.

Figure 2a, b and c. Effect of pH on phosphate solubilization by isolates *Burkholderia cepacia*/CC2, *Rhizobium radiobacter*/KC3 and *Ralstonia pickettii*/PC2 respectively. The cultures were inoculated in a medium of C:N ratio 30:1 and incubated at 28°C with 180 rpm agitation.

Bhattacharya et al. (2013) have reported detailed studies regarding properties of different types of soils from different regions across India and reported pH values ranging from as low as 4.0 to as high as 9.3 in certain regions. However, mostly soil pH in India lies in the range of 4.7-7.9 (Bhattacharya et al. 1993; Pal et al. 2003). Our results related to role of pH highlight the ability of the 3 PSB isolates to grow under different pH conditions. . It can be seen from the graphs that *Burkholderia cepacia*/CC2 and *Ralstonia pickettii*/PC2 grew optimally at pH 7.0 while *Rhizobium radiobacter*/KC3 showed sustained growth at pH 4.0. Our results are in agreement with earlier published research. Islam et al. (2007) reported that PSB exhibited almost similar and rapid growth at pH 5 and 7; and very slow or no growth at pH 3 i.e. the cultures are less tolerant to acidic conditions. Gupta et al. (2007) have studied the effect of pH on the efficiency of P solubilization by certain PSB. They reported that the efficiency of phosphate solubilization was very low when the cells were inoculated in a broth of acidic pH compared to that at neutral pH.

Effect of Variation in mixing conditions

It was found that when the cultures were agitated at 250 rpm on the orbital shaker, the biomass accumulated by all cultures as well as solubilization of inorganic phosphate was higher compared to values seen at 180 or 100 rpm – *Burkholderia cepacia*/CC2 (1.6±0.13g/L), *Rhizobium radiobacter*/KC3 (1.5±0.12g/L) and *Ralstonia pickettii*/PC2 (1.9±0.2g/L). Fastest solubilization was seen with *Burkholderia cepacia*/CC2 (475±52 ppm at 24h/250 rpm) as can be seen in Fig.3a. Highest solubilization was seen with culture *Ralstonia pickettii*/PC2 (670±49 ppm at 120h/250 rpm) as is evident in Fig.3b.Even *Rhizobium radiobacter*/KC3 exhibited more than double increase in phosphate solubilization (up to 300±28 ppm in 24 h) at 250 rpm as depicted in Fig.3c.

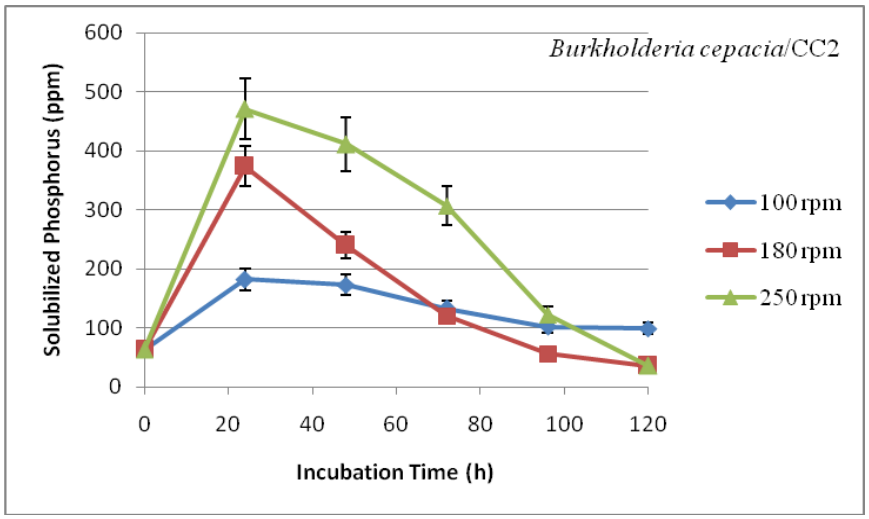

Figure 3a.

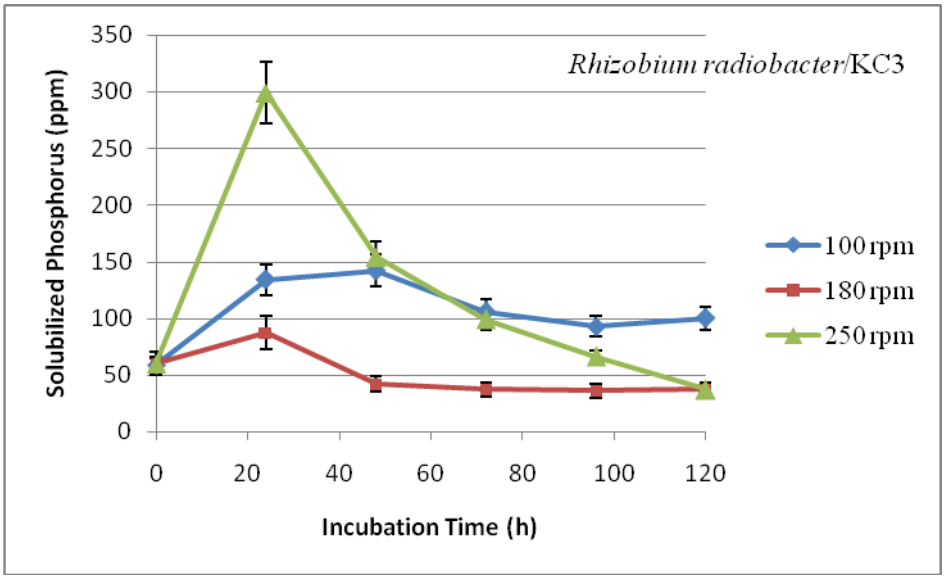

Figure 3b.

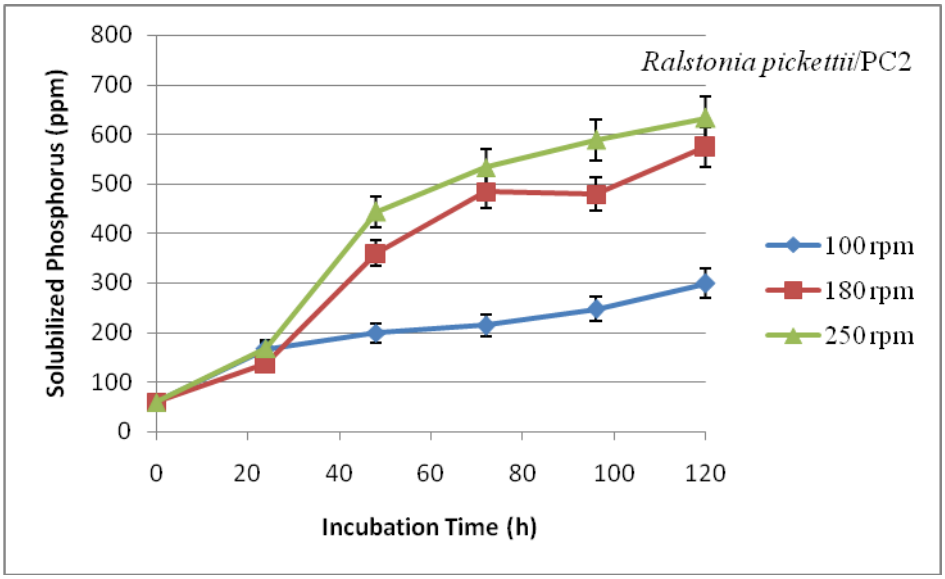

Figure 3c.

Figure 3 a, b and c. Effect of rate of agitation on phosphate solubilization by isolates *Burkholderia cepacia*/CC2, *Rhizobium radiobacter*/KC3 and *Ralstonia pickettii*/PC2, respectively. The cultures were inoculated in a medium having C:N ratio of 30:1 and incubated at 28°C.

Burkholderia cepacia/CC2 and *Ralstonia pickettii*/PC2 were grown in media having pH 7.0 (before sterilization) while medium pH *Rhizobium radiobacter*/KC3 was adjusted to 4.0 prior to sterilization. As reported by several scientists, the principal mechanism of phosphate solubilization by bacteria is through production of organic acids via the direct oxidation pathway (Rodriguez and Fraga, 1999; Khan et al. 2009; Panhwar et al. 2013; Sharma et al. 2013). Thus, it may be postulated that since production of organic acids by PSB is a product of oxidative metabolism, phosphate solubilization is also dependent on oxidative metabolism. However, literature survey did not reveal any reports that have explored the effect of varying agitation conditions on the phosphate solubilization by cultures - most of the published works have not reported the actual aeration/mixing conditions employed for growth of PSB (Harrison et al. 1972; Kannapiran and Ramkumar 2011; Sagervanshi et al. 2012; Dastager et al. 2013); whereas those reports in which aeration/mixing conditions have been reported, the values appear to be arbitrarily chosen (Jung et al. 2002; Pandey et al. 2006). Ours is probably the first report showing beneficial effect of increased mixing and aeration on growth and solubilization by PSB.

Thus the optimum environmental conditions for the 2 of the 3 PSB isolates, *Burkholderia cepacia*/CC2 and *Ralstonia pickettii*/PC2 are incubation at 28°C, mixing at 250 rpm on an orbital shaker in Pikovskaya's broth with initial pH of 7.0. The third culture *Rhizobium radiobacter*/KC3 must be incubated at 20°C at 250 rpm in Pikovskaya's broth at pH 4.0 so as to achieve good biomass along with maximum solubilization of inorganic phosphates.

Effect of C-Source

After optimization of environmental growth conditions, the effects of different C-sources on growth and phosphate solubilization by the PSB isolates were determined. It can be seen from Figure 4a that all the cultures showed similar growth in presence of glucose, glycerol as well as mixture of glucose and glycerol. Only *Rhizobium radiobacter*/KC3 showed marginally higher growth in presence of sucrose. While maximum DCW accumulation was obtained for cultures *Burkholderia cepacia*/CC2, and *Rhizobium radiobacter*/KC3 (by 72h of incubation), culture *Ralstonia pickettii*/PC2 showed maximum biomass by 96h. With respect to phosphate solubilization, it was observed that all cultures showed best solubilization when glucose(10g/L) was used as the carbon source (Figure 4b).Maximum solubilization was effected by *Ralstonia pickettii*/PC2 (at 120h) followed by *Burkholderia cepacia*/CC2 (at 24h).

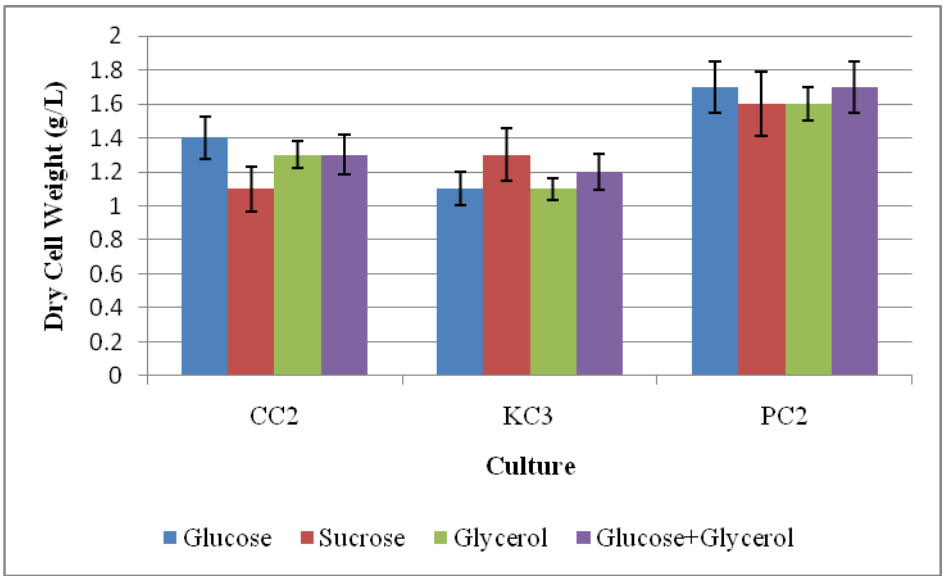

Figure 4a.

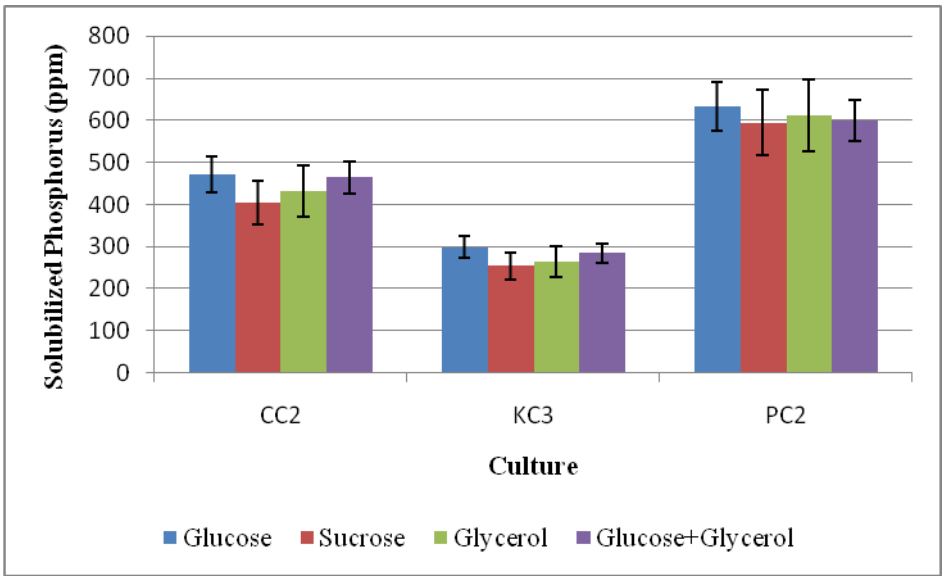

Figure 4b.

Figure 4 a and b. Effect of C-source on biomass (DCW) and phosphate solubilization by isolates *Burkholderia cepacia*/CC2, *Rhizobium radiobacter*/KC3 and *Ralstonia pickettii*/PC2 respectively. All the cultures were grown in the medium with pH 7.0 (adjusted before sterilization) and incubated at 28°C and agitation at 250 rpm.

Effect of N-Source

The cultures were studied by using peptone and yeast extract as the N-source to evaluate the effect on growth and P solubilization. Figure 5a shows dry cell weight accumulated by the cultures in presence of yeast extract and peptone.In figure 5b, the effect of N-source on P-solubilization is seen. It was found that peptone was almost as good source of organic nitrogen as was yeast extract, and P solubilization was also comparable. As was seen in the previous study, the cultures *Burkholderia cepacia*/CC2 and *Rhizobium radiobacter*/KC3 showed maximum biomass at 72h. Maximum P solubilization for these isolates was seen by 48h, whereas cultures *Ralstonia pickettii*/PC2 showed maximum growth and solubilization by 120h.

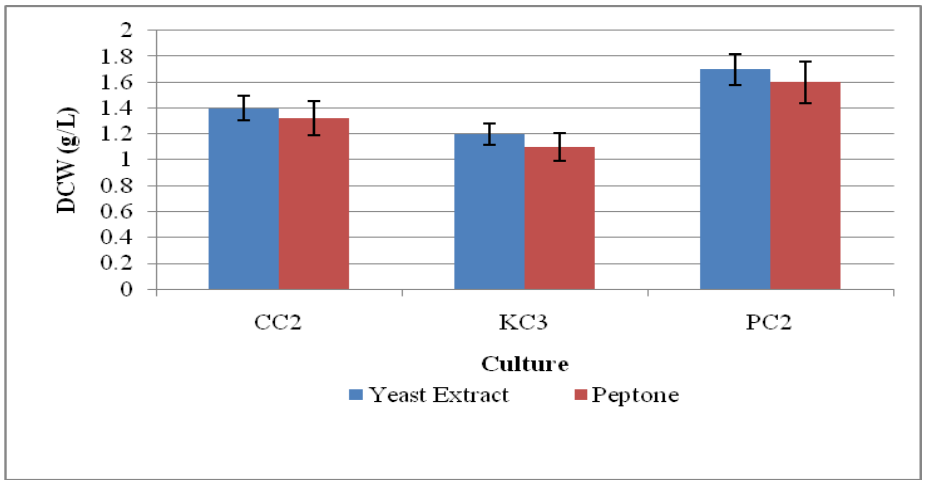

Figure 5a.

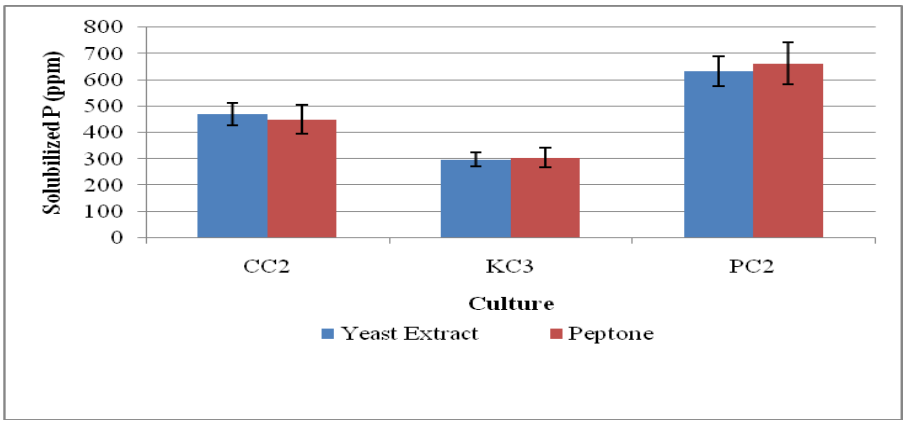

Figure 5b.

Figure 5 a and b. Effect of N-source on biomass (DCW) and phosphate solubilization by isolates *Burkholderia cepacia*/CC2, *Rhizobium radiobacter*/KC3 and *Ralstonia pickettii*/PC2, respectively. All the cultures were grown in the medium with pH 7.0 (adjusted before sterilization) and incubated at 28°C and agitation at 250 rpm.

The results obtained are in agreement with the reports of other workers who attempted medium optimization for PSB/PSM. Whitelaw et al. (1999) reported that PSM utilize a variety of carbon compounds as energy sources, but the amount of soluble phosphate varies significantly with different sources of energy. The nature and amount of organic acids excreted by fungi are mainly influenced by medium pH and buffering capacity, carbon source and the balance of nitrogen and phosphate (Mattey 1992; Reyes et al., 1999). Reyes et al. (1999) reported that in case of glucose, the total organic acid production was higher as compared to other C sources. Seshadri et al. (2004) also found that glucose was the best Carbon source for *Aspergillus* sp.

Our results for N-source did not reveal any major relation between source of nitrogen and extent of growth and P-solubilization though some researchers have found some correlation. (Asea et al., 1988; Vora and Shelat, 1998; Whitelaw et al., 1999; Sagervanshi et al., 2012). Whitelaw et al. (1999) reported that more phosphate was solubilized when ammonium source was used rather than a nitrate source. Similarly, Vora and Shelat (1998) tested a variety of N sources and found that ammonium sulphate promoted the most phosphate solubilization for bacterial species, *Bacillus circulans*, *Bacillus brevis* and *Bacillus coagulans*. For some microorganisms, the release of H^+ ions due to the assimilation of NH_4^+ seems to be the sole mechanism promoting phosphate solubilization.

Effect of Change in C:N Ratio:

Figure 6a and 6b depict the effect of change in C:N ratio on growth (as DCW) and P solubilization by the cultures. Increase in C:N ratio of the medium brought about a dramatic rise in the maximum biomass accumulated by the cultures. All cultures showed approximately 2-fold increase in biomass under the improved conditions. In case of P solubilization, it can be clearly seen that the altered C:N ratio brought about a tremendous increase in the amount of P solubilized by the cultures. Maximum P solubilization was effected by *Ralstonia pickettii*/PC2 which showed up to 863±97 ppm P solubilization i.e. >85% of the total P was solubilized by the culture in this new medium. Culture *Rhizobium radiobacter/*KC3 which solubilized 300 ±28ppm P at C:N ratio 30:1 was now capable of solubilizing almost up to 700±52 ppm P at C:N ratio of 60:1.

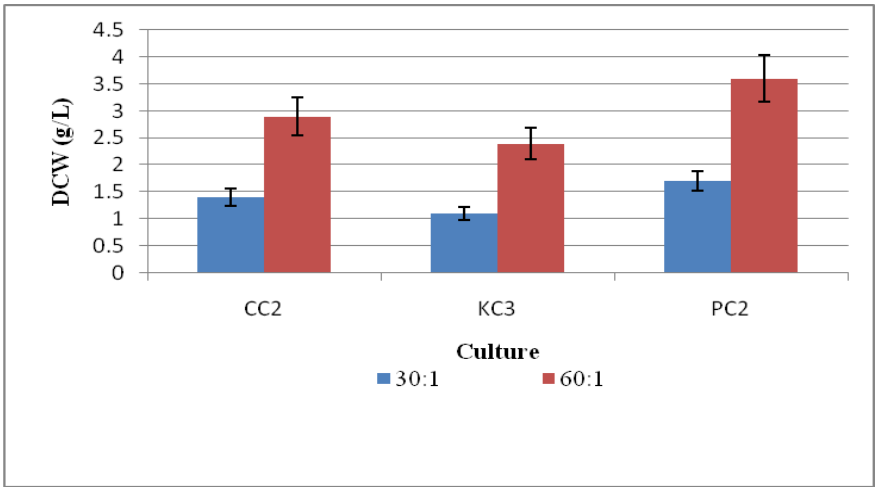

Figure 6a.

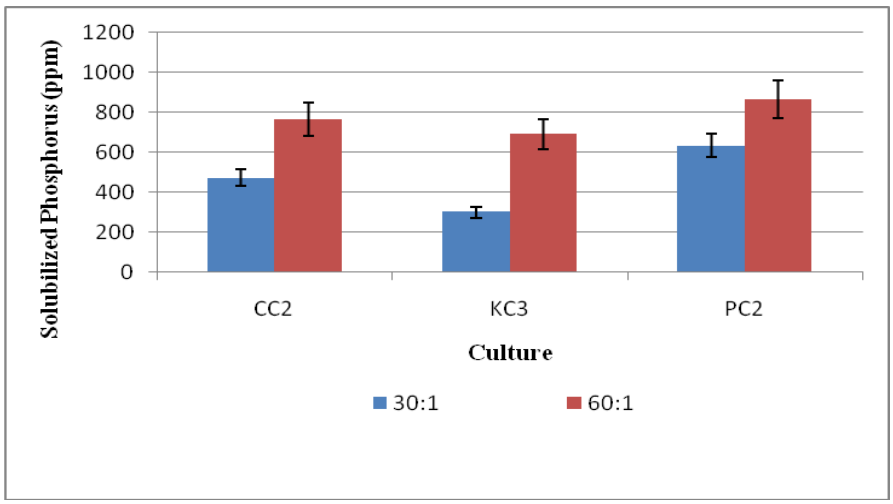

Figure 6b.

Figure 6 a and b. Effect of change in C:N ratio on biomass (DCW) and phosphate solubilization by isolates *Burkholderia cepacia*/CC2, *Rhizobium radiobacter/*KC3 and *Ralstonia pickettii/*PC2 respectively. All the cultures were grown in the medium with pH 7.0 (adjusted before sterilization) and incubated at 28°C and agitation at 250 rpm.

As may be noted from the fig. above, biggest benefit of changed C:N ratio was seen with *Rhizobium radiobacter/*KC3 which solubilized 690±52 ppm phosphate in 24h even at pH 7.0, indicating that this culture is capable of growing well at pH 7.0 as well at 4.0, This culture did not previously show very good growth and solubilization at pH 7.0. This improvement in the efficiency of the cultures is probably due to the improved balance of carbon and nitrogen and their relative proportion to the amount of phosphate in the medium.

These factors have been proved to be important in the amount of organic acid produced by the cultures (Mattey, 1992).Thus the three isolates were optimized to obtain increased biomass and phosphate solubilization by successfully manipulating the process conditions like temperature, pH, agitation rate as well as media optimization with respect to C and N source as well as the C:N ratio.. The optimized parameters for growth and solubilization of phosphates for each of the three cultures of PSB have been depicted in Figure 7.

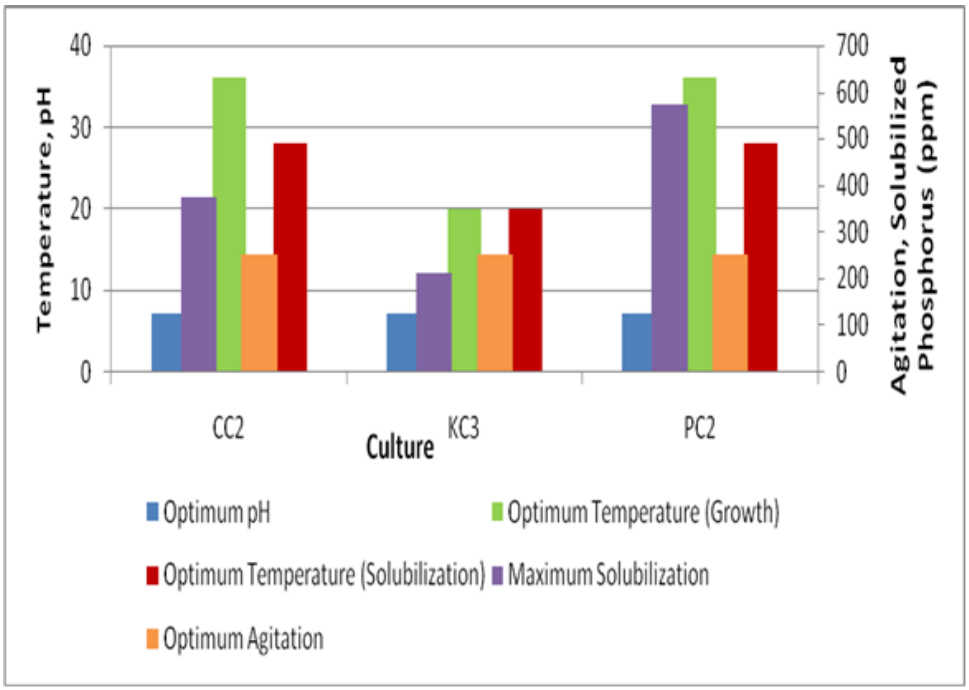

Figure-7: Optimized process conditions for growth and phosphate solubilization for *Burkholderia cepacia*/CC2, *Rhizobium radiobacter*/KC3 and *Ralstonia pickettii*/PC2.

CONCLUSION

The primary aim of the present study was to identify the optimum process conditions and also to alter the media composition including C: N ratio under which the isolates were able to grow optimally and demonstrate maximum P solubilization.

It may be concluded that the selected 3 PSB isolates were shown to be capable of growing very well coupled with highly enhanced ability to solubilize inorganic phosphate under optimized conditions. Most reports show solubilization potential for PSB around 300-400 ppm under laboratory conditions (Chen et al., 2006; Zhu et al., 2011; Gupta et al., 2012) as against what we have demonstrated –maximum of 863±97ppm

The 3 cultures under study have also showed ability to solubilize phosphate at different rates. This is an important aspect that can be utilized for devising a versatile microbial consortium that may be effective under various and variable soil conditions. It has been shown that these cultures also produce important plant growth promoting factors (Nair and Vakil, 2015). Hence, the high phosphate solubilization potential combined with plant growth promoting characteristics make these cultures, *Burkholderia cepacia*/CC2, *Rhizobium radiobacter*/KC3 and *Ralstonia pickettii*/PC2, excellent candidates to be combined and used as an effective phosphate solubilizing consortium capable of surviving and sustaining growth under different conditions that may prevail in soil. A similar trend can be expected when the culture(s) would be grown in suitable bioreactor and then deployed as a consortium under real soil/field conditions.

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