Ann Microbiol (2013) 63:173–178
DOI 10.1007/s13213-012-0458-z

ORIGINAL ARTICLE

Rock phosphate solubilization by four yeast strains

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Received: 21 January 2012 / Accepted: 20 March 2012 / Published online: 21 April 2012

Abstract The solubilization of rock phosphate (RP) by four yeast strains, *Rhodotorula* sp., *Candida rugosa*, *Saccharomyces cerevisiae* and *Saccharomyces rouxii*, which were isolated from wheat rhizospheric soils, was investigated in this study. The yeast isolates demonstrated diverse levels of soluble phosphate releasing abilities in modified Pikovskaya liquid medium containing RP as sole phosphate source. *C. rugosa* was the most effective solubilizer under different conditions, followed by *Rhodotorula* sp., *S. rouxii* and *S. cerevisiae*. Acidification of the broth seemed to be the major mechanism for RP solubilization by the yeast isolates, and the increase in soluble phosphate released was correlated significantly with an increase in titratable acidity and a drop in pH. The optimal composition for the solubilization of RP by the yeast isolates in the broth was 20 g L^{-1} glucose, 1 g L^{-1} yeast extract, 0.5 g L^{-1} $(NH_4)_2SO_4$, and 5 g L^{-1} RP, respectively. The yeast isolates were able to solubilize RP at wide range of temperature and initial pH, with the maximum percentage of soluble phosphate released being recorded at 30–35 °C and pH 5–6, respectively.

Keywords Rock phosphate · Solubilization · Yeast · Soluble phosphate

Introduction

Phosphorus is one of the most important nutrients limiting plant production. Despite phosphorus being distributed widely and abundantly in soil in both its inorganic and organic forms, many soils throughout the world are deficient in phosphorus. Therefore, large amounts of costly phosphate fertilizer are applied to soils to satisfy the demands of plant growth.

Natural rock phosphate (RP) has often been recognized as a cheap alternative source of phosphate fertilizer, especially for acid soils (Goenadi et al. 2000). Unfortunately, RP is not plant-available in soils with a pH greater than 5.5–6.5 and, even when conditions are optimal, yields are as a rule lower than those obtained with soluble phosphate (Rajan et al. 1996).

One very attractive approach to RP solubilization is the application of phosphate-solubilizing microorganisms (Vassilev et al. 2006). Many soil microorganisms, particularly those colonizing the rhizosphere of plants, are able to mobilize insoluble inorganic phosphates to the soil solution, making them available to plant roots (Hayat et al. 2010). These phosphate-solubilizing microorganisms render insoluble phosphate into a soluble form through the production of organic acids, phosphatases or other complex agents (Duponnois et al. 2005).

The use of phosphate-solubilizing microorganisms has been proposed as a low-cost and low-energy mechanism to help increase the agronomic effectiveness of insoluble inorganic phosphates, especially insoluble RP (Sahu and Jana 2000; Biswas and Narayanasamy 2006). Considering this factor, many phosphate-solubilizing microorganisms, including bacteria and fungi, have been isolated from different soils, and an increase in phosphorus availability of RP through the inoculation of these microorganisms has been reported under pot and field conditions (Hamdali et al. 2008; Xiao et al. 2009; Mamta et al. 2010). However, only a few studies have reported the isolation of phosphate-solubilizing yeasts from rhizospheric soils, although yeasts are widespread in soils, especially in acidic soils (Narsian et al. 2010; Mundra et al.

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2011). Therefore, as more studies are conducted, a wider diversity of phosphate-solubilizing yeasts is expected to be described.

In this context, this study was designed to isolate indigenous phosphate-solubilizing yeasts from wheat rhizospheric soils in Hubei province of China, and RP solubilization by these yeast isolates was investigated and characterized. The optimization of some parameters including substrate concentration, temperature and initial pH were also studied.

Materials and methods

Isolation of phosphate-solubilizing yeast strains

The yeast strains were isolated from soil samples collected from 15–25 cm depth from the rhizosphere of wheat in a farm located in the suburbs of Wuhan city (Hubei, China). For collection of rhizospheric soils, plants were uprooted and the soil attached to roots was then suspended in sterilized water, and mixed using a magnetic stirrer for 20 min to separate microorganisms completely from the soils. Serially diluted soil solution was plated on modified Pikovskaya agar medium (pH 5.5–6.5), which contained (per liter): 10 g glucose, 0.5 g yeast extract, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.0001 g $MnSO_4{\cdot}H_2O$, 0.0001 g $FeSO_4{\cdot}7H_2O$ and 20 g agar (Pikovskaya 1948). Tricalcium phosphate (5 g L^{-1}) was added to the medium as a sole phosphate source for selectively screening of microorganisms, which have the ability to release soluble phosphate from insoluble tricalcium phosphate. After 3–5 days of incubation at 30±0.5 °C, colonies with clear zones were purified further by replating on the medium maintained above supplemented with tricalcium phosphate. Four strains identified as *Rhodotorula* sp., *Candida rugosa*, *Saccharomyces cerevisiae* and *Saccharomyces rouxii* were selected for further analysis in this study.

Broth assays for RP solubilization by yeast isolates

The RP sample used in this experiment was obtained from Yichang phosphate mines (Hubei, China). The sample was ground to a particle size of 100–200 mesh. RP solubilization assays were carried out in shake flasks with 50 mL Pikovskaya liquid medium (without agar) containing 0.5 g RP sample as sole phosphate source. The initial pH of the medium was adjusted to 6. Mycelial discs (10 mm) of each isolate from actively growing colonies after 4 days on modified Pikovskaya agar medium were added as inoculum. Flasks were shaken under 160 rpm at 28 °C for 7 days. Autoclaved, uninoculated medium served as control. Flask was taken daily and the broth was centrifuged at 11000×*g* for 20 min, and the supernatant was filtered. The filtrate was then assessed for the soluble phosphate, titratable acidity and pH. All experiments were performed in triplicate.

Optimal substrate concentration, temperature and initial pH for RP solubilization by yeast isolates

Substrates in the broth was added in different concentrations (glucose: 5, 20, and 30 g L^{-1}, respectively; yeast extract: 0.1, 1, and 2 g L^{-1}, respectively; $(NH_4)_2SO_4$: 0.1, 1, and 2 g L^{-1}, respectively; RP: 1, 10, and 20 g L^{-1}, respectively) to study the effect of changing concentration of substrate on RP solubilization by the yeast isolates. To study the optimal temperature for RP solubilization by the isolates, flasks were shaken at different temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 °C), and the influence of initial pH was studied by adjusting the initial pH of the medium to 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. Each assay was carried out in shake flasks containing 50 mL Pikovskaya liquid medium inoculated with 10 mm mycelial discs of different yeast isolates from 4-day-old actively growing colonies on modified Pikovskaya agar medium. After shaking at 160 rpm at 28 °C for 7 days, flasks were taken and assessed for soluble phosphate in the broth. All experiments were performed in triplicate.

Analytical methods

Soluble phosphate in the filtrate was determined by using the vanadium-ammonium molybdate colorimetric method with a UV–vis 8500 spectrophotometer at 490 nm (Jiang et al. 2001). The pH was recorded with a pH meter equipped with a glass electrode. The titratable acidity was determined by titrating the filtrate with 85.7 mmol L^{-1} standard NaOH

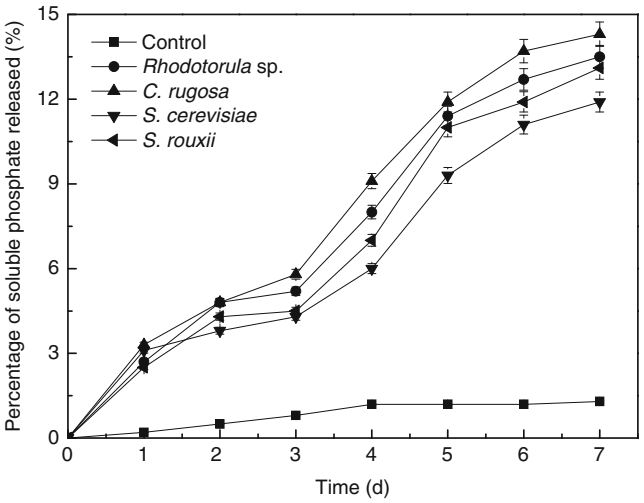

Fig. 1 Percentage of soluble phosphate released by different yeast isolates during 7 days of rock phosphate (RP)-solubilizing experiments (means ± SD, n=3)

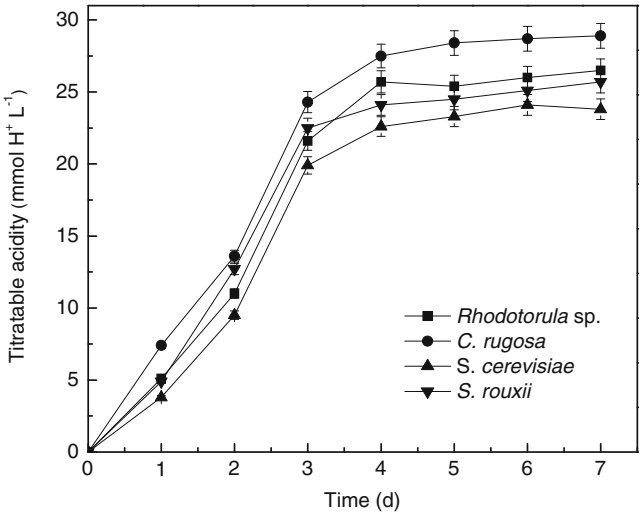

Fig. 2 Quantity of titratable acidity in the broth inoculated with different yeast isolates during 7 days of RP-solubilizing experiments (means ± SD, n=3)

solutions (Yi et al. 2008). Values were given as means ± standard deviation (SD) for triplicate samples. Data were analyzed by analysis of variance (ANOVA) and the means were compared with Duncan's Multiple Range Test (DMRT) at $P<0.05$ level.

Results and discussion

RP solubilization by different yeast isolates

The role of yeasts in agriculture has been a matter of interest due to their abundant population and potential as plant growth promoters (Vassilev et al. 2001a, b; Medina et al. 2004). In the present investigation, four strains identified as *Rhodotorula* sp., *C. rugosa*, *S. cerevisiae* and *S. rouxii* were

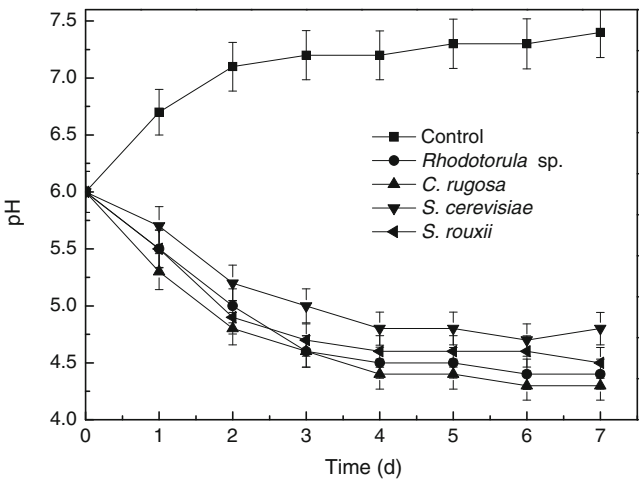

Fig. 3 Graph showing pH in the broth inoculated with different yeast isolates during 7 days of RP-solubilizing experiments (means ± SD, n=3)

Table 1 Effect of substrate concentration in the broth on the percentage of soluble phosphate released (%) by yeast isolates. Results represent means ± SD of three independent experiments. Means with the same letter in a row are not significantly different at $P<0.05$

Isolate	Glucose (g L^{-1})				Yeast extract (g L^{-1})				$(NH_4)_2SO_4$ (g L^{-1})				RP (g L^{-1})			
	5	10	20	30	0.1	0.5	1	2	0.1	0.5	1	2	1	5	10	20
Rhodotorula sp.	7.9±0.3 a	13.3±1.2 b	14.1±1.5 b	14.0±0.8 b	4.3±0.3 c	13.3±0.7 b	14.6±1.1 b	14.3±0.8 b	6.8±0.5 a	13.5±1.1 b	13.2±1.3 b	12.1±0.7 b	7.1±0.3 a	14.4±0.7 b	13.7±0.6 b	12.1±1.4 b
C. rugosa	8.5±0.6 a	13.7±1.3 b	14.5±0.3 b	14.3±0.4 b	5.1±0.4 c	14.0±1.3 b	15.2±0.7 b	14.7±1.1 b	6.9±1.0 a,c	14.0±1.3 b	14.0±1.2 b	12.5±0.7 b	7.2±0.6 a	14.3±1.7 b	14.0±0.9 b	12.7±0.5 b
S. cerevisiae	7.4±0.6 a	12.5±1.1 b	13.1±0.6 b	12.5±1.2 b	4.1±0.3 c	12.9±1.0 b	13.6±1.7 b	13.0±0.6 b	6.2±0.8 a,c	12.1±0.3 b	11.8±0.9 b	10.9±0.4 b	6.5±0.5 a	13.0±0.8 b	12.5±1.1 b	10.9±0.5 b
S. rouxii	8.0±0.7 a	13.1±1.8 b	13.8±0.6 b	13.3±1.2 b	4.6±0.4 c	13.4±0.6 b	13.9±0.9 b	13.8±0.7 b	6.5±0.9 a,c	13.1±0.7 b	13.3±1.3 b	12.7±1.1 b	7.0±0.6 a	13.9±1.2 b	13.6±1.2 b	12.1±0.7 b

Table 2 Effect of temperature on the percentage of soluble phosphate released (%) by yeast isolates. Results represent means ± SD of three independent experiments. Means with the same letter in a row are not significantly different at $P<0.05$

Isolate	Temperature (°C)									
	5	10	15	20	25	30	35	40	45	50
Rhodotorula sp.	1.3±0.1 a	3.2±0.4 b	6.9±0.9 c	10.8±0.9 d	11.8±1.1 d	13.7±1.0 d	14.0±1.1 d	9.6±0.4 c,d	3.7±0.5 b	1.1±0.7 a
C. rugosa	1.3±0.3 a	3.5±0.6 b	7.7±0.4 c	11.5±0.5 d	12.1±1.5 d	14.5±0.7 d	14.3±0.3 d	10.8±0.9 d	3.9±0.5 b	1.0±0.4 a
S. cerevisiae	1.2±0.5 a	2.9±0.2 b	6.7±0.3 c	10.5±0.7 d	11.4±0.6 d	13.1±0.6 d	13.7±0.2 d	10.2±0.4 c,d	2.9±0.7 b	1.5±0.3 a
S. rouxii	1.2±0.1 a	3.2±0.3 b	6.8±0.3 c	11.0±1.0 d	11.9±1.2 d	13.6±1.3 d	14.3±1.3 d	10.5±0.5 d	3.1±0.4 b	1.2±0.1 a

isolated from wheat rhizospheric soils, and the results of RP solubilization by these yeast isolates in modified Pikovskaya liquid medium are presented in Fig. 1. All the isolates could effectively solubilize RP in the broth, and the percentage of soluble phosphate released by the isolates increased significantly during 7 days of RP-solubilizing experiments, while there was no significant change in the percentage of soluble phosphate released under control conditions. The results indicate that these yeast isolates have a great potential for use as potential RP solubilizers for use in natural soils. Isolates varied with respect to levels of RP solubilization achieved. Among the four isolates, *C. rugosa* was the most efficient strain for RP solubilization and released the largest percentage of soluble phosphate (14.3%) after 7 days of RP-solubilizing experiments, followed by *Rhodotorula* sp. (13.5%), *S. rouxii* (13.1%) and *S. cerevisiae* (11.9%).

Figure 2 shows that the quantity of titratable acidity in the broth inoculated with the yeast isolates increased significantly during 7 days of RP-solubilizing experiments. Among the four isolates, *C. rugosa* produced the highest of 28.9 mmol $H^+ L^{-1}$ titratable acidity at the end of the experiment, followed by *Rhodotorula* sp. (26.5 mmol $H^+ L^{-1}$), *S. rouxii* (25.7 mmol $H^+ L^{-1}$) and *S. cerevisiae* (23.8 mmol $H^+ L^{-1}$).

The results in Fig. 3 show that the pH in the broth inoculated with the yeast isolates decreased gradually during 7 days of RP-solubilizing experiments, while the pH increased to more than 7 and remained almost constant for the duration of the control experiment. Among the four isolates, *C. rugosa* had the largest reduction of pH in the broth, from an initial value of 6 to 4.3 on day 7, compared to that of *Rhodotorula* sp., *S. rouxii* and *S. cerevisiae*, which showed a reduction to 4.4, 4.5 and 4.8, respectively, on day 7.

The results shown in Figs. 1–3 show that the increase in the percentage of soluble phosphate released in the broth was accompanied by an obvious rise in the quantity of titratable acidity and a significant drop in pH during 7 days of RP-solubilizing experiments. Simple regression analyses revealed a significant positive correlation between the percentage of soluble phosphate released and the quantity of titratable acidity ($r=0.81$; $P<0.01$) and a significant negative correlation ($r=-0.8$; $P<0.01$) between the percentage of soluble phosphate released and pH.

The major mechanism for the bio-solubilization of RP is reported to be the excretion of low-molecular weight organic acids by phosphate-solubilizing yeast (Vassileva et al. 2000). Results in this study were also in agreement with this finding. In this study, HPLC analysis detected multiple organic acids, including gluconic, citric, malic, tartaric, and lactic acids, in broth inoculated with different yeast isolates (data not shown). Presumably these organic acids play a vital role in the acidification of the broth, which can be illustrated by the increase of titratable acidity and decrease of pH, and thus further facilitate the solubilization of RP by yeast isolates.

However, the bio-solubilization of RP is a complex process, and may be determined by multiple mechanisms. The production of organic acid by phosphate-solubilizing microorganisms is a major, but not the sole, factor responsible for

Table 3 Effect of initial pH on the percentage of soluble phosphate released (%) by the yeast isolates. Results represent means ± SD of three independent experiments. Means with the same letter in a row are not significantly different at $P<0.05$

Isolate	pH									
	1	2	3	4	5	6	7	8	9	10
Rhodotorula sp.	1.8±0.2 a	3.8±1.1 b	7.2±0.9 c	9.9±1.3 d	13.5±0.7 e	13.7±1.7 e	12.2±1.1 e	8.0±0.4 c,d	5.7±0.6 b,c	2.9±0.3 a,b
C. rugosa	1.7±0.6 a	4.0±0.7 b	7.6±0.3 c	10.7±0.7 d	14.4±1.3 e	14.0±1.1 e	12.5±1.6 d,e	8.5±0.3 c	6.5±0.9 c	3.5±1.2 b
S. cerevisiae	1.6±0.3 a	3.3±1.0 b	6.3±0.9 c	10.5±0.9 d	13.0±0.4 e	12.9±0.9 e	10.7±0.6 d	7.1±0.6 c	4.7±0.2 b,c	2.4±0.2 a,b
S. rouxii	2.0±0.8 a	3.7±0.7 b	6.5±0.7 c	11.1±1.1 d	14.3±0.4 e	13.7±0.3 e	11.5±0.6 d	7.9±1.1 c	5.4±0.9 b,c	2.5±0.5 a

phosphate solubilization (Illmer and Schinner 1995). It has been reported that some phosphate-solubilizing microorganisms that could not excrete organic acids also have the capability to solubilize inorganic phosphates (Chen et al. 2006). Therefore, other factors must be responsible for the bio-solubilization of RP, such as the excretion of phosphatases (Achal et al. 2007; Park et al. 2011) or the cellular H^+ exudation to balance NH_4^+ uptake (Pandey et al. 2008). Given that the yeasts are such a large group of rhizospheric microorganisms, and that a wide diversity of soil yeasts have the potential to act as bio-fertilizers (Botha 2011), it is essential to further isolate phosphate-solubilizing yeasts from different soils and to understand their phosphate-solubilizing mechanisms.

Optimization of RP solubilization by different yeast isolates

The effects of different substrates at varying concentrations on RP solubilization are presented in Table 1. The percentage of soluble phosphate released was significantly higher in the case of 20 g L^{-1} glucose, 1 g L^{-1} yeast extract, 0.5 g L^{-1} $(NH_4)_2SO_4$, and 5 g L^{-1} RP, respectively. Higher or lower than these concentrations, the percentage of soluble phosphate released decreased. The percentage of soluble phosphate released gradually increased with as the concentration of glucose increased from 5 to 20 g L^{-1}, yeast extract from 0.1 to 1 mg L^{-1}, and $(NH_4)_2SO_4$ from 0.1 to 0.5 mg L^{-1}, respectively. On the contrary, there was a large decrease in solubilization of RP with increasing RP concentration from 5 to 20 g L^{-1}.

The effects of different temperature on RP solubilization by yeast isolates are presented in Table 2. All the yeast isolates were able to solubilize RP from 10 to 45 °C, and the highest percentage of soluble phosphate released was obtained between 30 °C and 35 °C. Negligible percentage of soluble phosphate released was liberated when the temperature was lower than 5 °C or higher than 50 °C.

Initial pH of the broth also had significant effect on RP solubilization by yeast isolates (Table 3). All the isolates were able to solubilize RP at initial pH range of 2–10. Negligible soluble phosphate was liberated when the initial pH was lower than 1.0, and the highest percentage of soluble phosphate released was observed at initial pH 5–6. Results also show that a pH value in the broth of lower than 7 was more favorable for solubilization of RP compared to pH greater than 7. The percentage of soluble phosphate released decreased considerably under alkaline conditions, which suggests that acidity tended to facilitate the solubilization of RP.

The results in Tables 2 and 3 have generated useful information about yeast isolates with tolerance to extremes of temperature and pH. These stress-tolerant traits are of significance in the growth and survival of these isolates in soils, and further benefits the solubilization of RP.

This study also demonstrated that the capacity to solubilize RP varies from isolate to isolate in Pikovskaya liquid medium under different conditions, including substrate concentration, temperature and initial pH. Among the isolates studied here, *C. rugosa* seemed to be the most powerful RP solubilizer, suggesting a higher soluble phosphate releasing ability and a good adaptation of this isolate to the solubilizing environment. However, since conditions in soils are much more complex than those in vitro, further studies are required in order to determine their efficiency in solubilizing RP under field conditions.

Acknowledgments This research work was kindly supported by National Natural Science Foundation of China (No. 51004078), Program for New Century Excellent Talents in University (NCET-11-0965), Program for Changjiang Scholars and Innovative Research Team in University (No. IRT0974), National Basic Research Program of China (No. 2011CB411901) and the Open Foundation of Key Laboratory for Green Chemical Process of Ministry of Education (GCP201104).

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