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Bioprocess for Solubilization of Rock Phosphate on Starch Based Medium by *Paecilomyces marquandii* Immobilized on Polyurethane Foam

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Abstract *Paecilomyces marquandii*, a phosphate-solubilizing, starch-utilizing filamentous fungus, was immobilized on polyurethane foam (PUF). The immobilized fungus was applied in a repeated batch (six batches) fermentation process to solubilize Hirapur rock phosphate. The fungus was immobilized on PUF cubes and was used for phosphate solubilization in shake flask repeated batch cultivations. The fungus was also immobilized on PUF sheet and utilized in an airlift bioreactor in a repeated batch process. Maximum soluble phosphate (370 μg/ml) was recorded after third batch with 8 g rock phosphate/l. After 12 days of fermentation, a total production of 1,643 μg phosphate/ml was achieved.

Keywords Bioprocess · *Paecilomyces marquandii* · Polyurethane foam · Rock phosphate · Starch

Introduction

Immobilized biocatalysts have a variety of advantages over free cells in various biotechnological processes [1]. Entrapped cell systems in packed bed reactors are useful when non-particulate substrates are involved. Open reactors are of particular importance for the treatment of particulate substrates. Earlier studies from our laboratory have contributed significantly to the development of techniques for the immobilization of cells through surface adhesion and their use in open systems like cloth-strip bioreactor [2, 3] and annular bioreactor [4].

Research in solubilization of rock phosphate has been conducted in laboratory conditions, both in liquid media and soil–plant systems using a large number of phosphate solubilizers. Most of the research on rock phosphate solubilization using liquid media is confined to shake flask studies, although a few have been carried out for large-scale bioreactors. Goldstein et al. [5] have reported the development of a continuous stirred tank bioreactor for the solubilization

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of rock phosphate using free cells. However, the major disadvantage of mechanical agitated reactors is attrition effect that can be eliminated in an airlift bioreactor. The turbulence caused by the fluid flow ensures adequate mixing of the liquid in an airlift bioreactor. Several groups have reported the use of glucose as a carbon source in phosphate solubilization [6–9]. This makes the bioprocess economically undesirable. Immobilization of cells is desirable for an efficient and economically attractive bioprocess [10, 11]. Immobilizing the microbial cells in preformed porous carriers such as polyurethane foam (PUF) offers various advantages. Immobilization occurs as a result of microbial growth and thus is very suitable for filamentous fungi, which exhibit typical mycelial growth. Further PUF is available in a variety of shapes, sizes, and thickness. Fungus immobilized on PUF sheets as a carrier material can be used in loop reactors such as airlift, propeller, and jet-loop reactors. A phosphate-solubilizing fungus was isolated and identified in our laboratory as *Paecilomyces marquandii*. The 18S rRNA sequence of the isolate has been deposited in the GenBank database under accession number DQ083546. Earlier work from our laboratory [12] has shown that the isolate could solubilize rock phosphate using a wide range of carbon (fructose, galactose, glucose, glycerol, lactose, maltose, mannose, sorbitol, starch, and sucrose) and nitrogen sources (ammonium chloride, ammonium sulfate, asparagine, calcium nitrate, potassium nitrate, sodium nitrate, and urea). We have reported that *P. marquandii* secreted 2.2 mM citrate and 0.27 mM oxalate [12]. However, the level of organic acids leading to significant phosphate solubilization is in the order of 10–100 mM [6, 13]. Acidification of the medium by proton extrusion during NH_4^+ assimilation was involved in phosphate solubilization by this fungus [12]. The phosphate-solubilizing efficiency of the fungus was comparable to that of well-known P solubilizers viz. *Aspergillus niger*, *Penicillium* sp., and *Pseudomonas* sp. The purpose of the present work was to obtain an efficient immobilization of *P. marquandii* and to study the potential application of this system for rock phosphate solubilization in an airlift bioreactor. One of the main advantages of the strain was its ability to use starch as an economical carbon source.

Materials and Methods

Microorganism and Culture Media

P. marquandii isolated earlier in our laboratory [12] was used for this study. The fungus was maintained on potato dextrose agar slants at 4 °C. Pikovskaya's broth [14] was modified by replacing glucose with 10 g/l starch and $Ca_3(PO_4)_2$ with 1 g/l KH_2PO_4. This was used as growth medium for immobilization. The production medium used for repeated batch fermentations consisted of modified Pikovskaya's broth wherein glucose was replaced with starch (10 g/l) and $Ca_3(PO_4)_2$ with 4 or 8 g/l Hirapur rock phosphate (23% P_2O_5, 0.152 mm mesh), respectively.

Immobilization and Shaken Flask Repeated Batch Fermentation

The PUF cubes (2×2×1 cm) were used for immobilization of *P. marquandii.* Pre-washed foam material was submerged in 100 ml growth medium in 250-ml Erlenmeyer flasks and autoclaved for 20 min at 121°C for sterilization. The carrier cubes, inoculated with 5-day-old fungal spores (1×10^6), were cultivated for 24 h on a rotary shaker at 100 rpm. The immobilized mycelium was then separated from the liquid, washed with sterile distilled water, and transferred into100 ml sterile production medium (containing 8 g/l Hirapur rock phosphate) in 250-ml Erlenmeyer flasks. Fermentation was carried out under agitation of

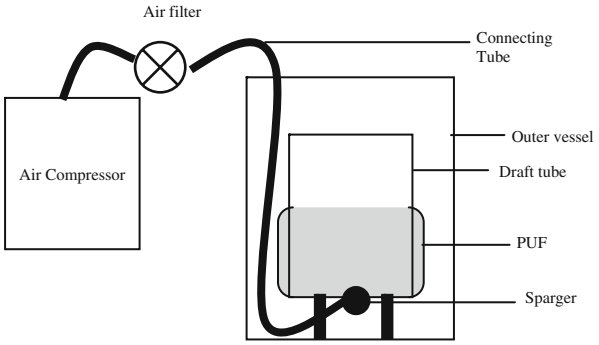

Fig. 1 Schematic diagram of the biorereactor

100 rpm at 30°C. The fermentation liquid was centrifuged (1,600×*g*/10 min) and was further used for analysis after each batch cycle. Production medium was changed following the same procedure every 48 h for six batches.

Airlift Bioreactor and Culture Conditions

Airlift bioreactor was designed and fabricated in our laboratory. The reactor was made of glass 29 cm in height and 21.6 cm in diameter containing a concentric draft tube of 12 cm in diameter. Pre-washed PUF sheet of thickness 3 cm and height 12 cm was stitched around the central draft tube. Growth medium (8 l) was added to the bioreactor, and the entire assembly was autoclaved for 20 min at 121°C. The PUF sheet was inoculated with 8×10^7 spores of *P. marquandii* obtained from 5-day slants. After 24 h, growth medium was removed from the bioreactor and 8 l sterile production medium (containing 4 or 8 g/l Hirapur rock phosphate) was added. After 48 h of fermentation, the exhausted medium was removed, and sterile production medium was added to refill. The process was repeated after 48 h for six batches. During the processes, samples taken after every 48 h were centrifuged (1,600×*g*/10 min) and used for phosphate estimation. The working volume of the reactor was initially kept at 4 l and was later scaled to 8 l. Air input in the bioreactor was through a 10-mm rubber tube with one of its ends coupled to the air filter for sterile air input (Millex-FG_{50}, 0.2 μm). The air sparger was located at the bottom of the draft tube. The air was introduced in the reactor at the rate of 18 l/min. A schematic diagram of the airlift bioreactor, used in the present study, is presented in Fig. 1.

Phosphate Estimation

Dissolved phosphorus concentration in the culture supernatant was determined by the ascorbic acid reductant method described by Watanabe and Olsen [15].

Results and Discussion

Solubilization of Rock Phosphate During Repeated Batch Cultivation of Immobilized *P. marquandii* in Shake Flask

Many researchers have reported the existence of microorganisms capable of solubilizing inorganic phosphate materials [8, 16]. Goenadi et al. [8] have reported accumulation of

142 μg phosphate/ml using *A. niger*. We have shown earlier that free *P. marquandii* could release 180 μg/ml phosphate from medium containing 1 g Hirapur rock phosphate/l and utilizing starch as the carbon source [12]. Experiments were carried out with immobilized *P. marquandii*, which showed that maximum rock phosphate solubilization was achieved after 48 h at 8 g rock phosphate/l in shake flask repeated batch process (data not shown). Therefore, the fermentation time for each batch was fixed at 48 h. The solubilization of Hirapur rock phosphate by *P. marquandii* immobilized on PUF cubes was studied in 6×48 h repeated batch fermentation. Maximum accumulation of soluble phosphate (440 μg/ml) was registered after second batch using production medium supplemented with 8 g rock phosphate/l (Fig. 2). Vassilev et al. [16] have reported the accumulation of 824 μg phosphate/ml using immobilized *A. niger* at 5 g rock phosphate/l. However, they have used 100 g glucose/l as the carbon source. In our process, though the yield is less, the use of starch makes it highly economical.

Solubilization of Rock Phosphate by Immobilized *P. marquandii* in Airlift Reactor

Preliminary experiments to determine the fermentation time for achieving maximum solubilization using immobilized cells cultivated in airlift reactor under repeated-batch conditions revealed the same pattern (48 h) as seen for shake flask fermentation (data not shown). Thus, each batch was run for 48 h. Initial studies were carried out with a bioreactor of 4 l volume. The bioreactor could be run at 4 and 8 g rock phosphate/l. The maximum solubilization of 294 μg phosphate/ml was recorded after third batch with 4 g rock phosphate/l and of 395 μg phosphate/ml with 8 g rock phosphate/l. This corresponded to 31.9% and 21.5% of the total amount of phosphate present in the rock phosphate, respectively. The bioreactor volume was increased to 8 l, and studies were run at 4 and 8 g rock phosphate/l. Data from these studies showed that scale up had a minimal effect on the solubilization process. At 4 g rock phosphate/l, maximum solubilization of 267 μg phosphate/ml was registered. Twenty-nine percent of available rock phosphate was solubilized. At 8 g rock phosphate/l, the maximum solubilization was 370 μg phosphate/ml that corresponded to 20.1% of rock phosphate being solubilized (Fig. 3). Using cells immobilized on PUF, the total amount of soluble phosphate reached 1,643 μg/ml in 12-day

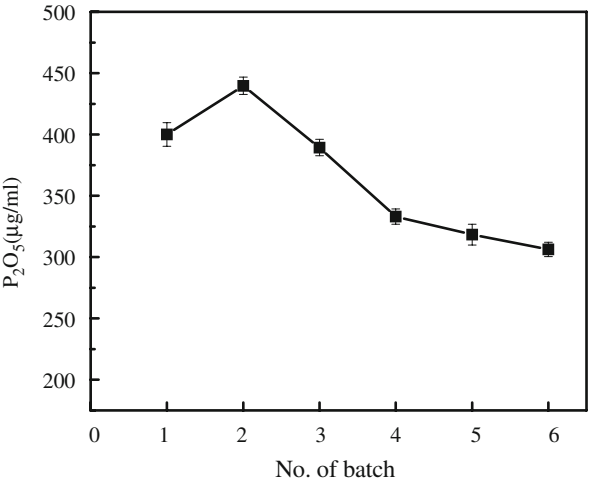

Fig. 2 Release of soluble phosphate from rock phosphate during batch culture in shake flask by immobilized *P. marquandii* at 8 g Hirapur rock phosphate/l. *Bars* indicate the standard deviations of the means of three independent experiments

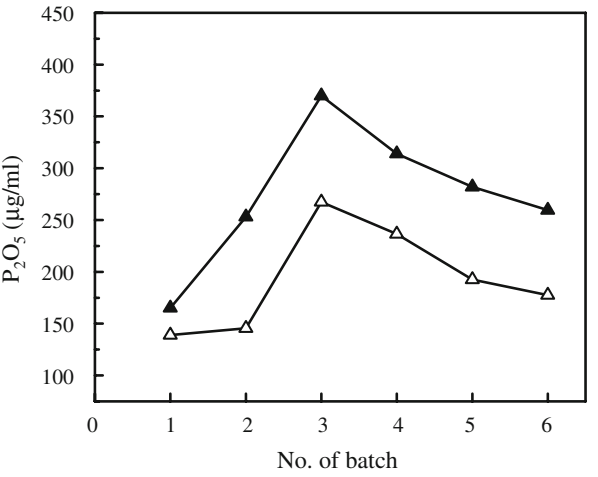

Fig. 3 Solubilization of rock phosphate in airlift bioreactor by immobilized *P.marquandii* at 4 (*open triangle*) and 8 (*filled triangle*) g Hirapur rock phosphate/l. The experiment was repeated twice and mean value is presented

repeated batch fermentation in the airlift bioreactor. There have been reports on solubilization of rock phosphate using microbes immobilized on PUF cubes for use in shake flask fermentation [16]. However, in this paper, we report the immobilization of our isolate on PUF sheet that can be used for phosphate solubilization in a variety of reactor geometries such as airlift, propeller and jet-loop.

Conclusion

Solubilization of Hirapur rock phosphate was achieved by using immobilized *P. marquandii*. The fungus was immobilized on PUF cubes and sheet and used in shake flask and airlift bioreactor, respectively. The microorganism effectively solubilized Hirapur rock phosphate in both the systems using starch as the carbon source.

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