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Molecular Genetic Differentiation of Naturally Isolated Phosphorus Solubilizing yeasts.

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Abstract:

Phosphorus is one of the major nutrients for plants. A grater part of soil phosphorus, approximately 95-99%, is present in the form of insoluble phosphates and hence cannot be utilized by the plants. Phosphate solubilizing microorganisms (PSMs) play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers. Application of PSMs in the field has been reported to increase crop yield. In the present study, fifty six strains of natural yeasts were collected from different geographical regions in Egypt. Three selected isolates (R1, R2, and R3) showed good ability to solubilize inorganic phosphates based on inducing clear zones around their colonies were selected. Forming of clear zone around the colonies growing on the TCP-medium for 5 days of incubation at 25°C was donated as Solubilization Index (SI) which was ranged from 1.6 to 2.28. The results showed that the highest P-solubilization efficiency was demonstrated by R3 strain. The three coded selected strains were identified at molecular genetics technique as, *Pichia sp.*, *Candida sp.*, and *Rodotorula sp.* respectively. Additionally, the differentiation of these strains was possible at molecular level by using RAPD-PCR analysis, the results revealed that the tested strains can be characterized depending upon their profile pattern. The three selected strains were regrouped in two clusters; one of them included R3 and the second cluster contained R1 and R2.

Introduction

In addition to nitrogen, phosphorus is one of the most important nutrients for plant growth phosphorus contributes to the biomass construction of micronutrients, the metabolic process of energy transfer, signal transduction, macromolecular biosynthesis, and respiration chain reactions. Also, Phosphorus plays a significant role in several physiological and biochemical plant activities like photosynthesis, transformation of sugar to starch, and transporting of the genetic traits (Shenoy and Kalagudi, 2005).

Unfortunately, phosphorus is one of the least available and the least mobile mineral nutrients for plants in the soil (Takahashi and

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Anwar, 2007). Plants acquire phosphorus from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca2+, Mg2+, Fe3+ and Al3+, depending on the particular properties of a soil. In these forms, phosphorus is highly insoluble and unavailable to plants.

Phosphatic fertilizer, such as costly chemical fertilizers that contain large amounts of soluble phosphorus, have been applied to the agricultural fields to maximize production (Del Campillo, *et al.*, 1999; and Shenoy and Kalagudi, 2005). However, a large proportion of phosphorus fertilizer after application is quickly transformed to the insoluble form (Omar, 1998). Therefore, very little percentage of the applied phosphorus is available to plants, making continuous application necessary (Abd Alla, 1994).

The unmanaged use of phosphatic fertilizers has increased agricultural costs and instigated a variety of environmental problems (Del Campillo, *et al.*, 1999). Therefore, the concept of adding phosphate-solubilizing microbes to soluble phosphorus presents an economically and environmentally promising strategy. P-solubilizing microbes play fundamental roles in biogeochemical phosphorus cycling in natural and agricultural ecosystems. It can transform the insoluble phosphorus to soluble forms $[HPO_4]^{2-}$ and $[H_2PO_4]^-$ by acidification, chelation, exchange reactions, and polymeric substances formation (Delvasto, *et al.*, 2006). Therefore, the use of P-solubilizing microbes in agricultural practice would not only offset the high cost of manufacturing phosphatic fertilizers but would also mobilize insoluble phosphorus in the soils to which they are applied (Rodríguez and Fraga, 1999).

Several bacterial genera including *Pseudomonas, Bacillus, Enterobacter, Azotobacter, Agrobacterium, Achromobacter, Rhizobium, Burkholderia, Flavobacterium* and *Micrococcus* isolated from the temperate countries have been reported to solubilize phosphorous (Jeon, *et al.*, 2003; and Rodríguez and Fraga, 1999).

Although many authors reported yield increasing on wheat, onion, alfalfa and soybean through simple inoculation of P-solubilizing fungi (Reyes, *et al.*, 2002; Whitelaw, *et al.*, 1997; and Rodríguez and Fraga, 1999), little is known about P-solubilizing yeasts. Hesham and Hashem, (2010) reported that forty yeast strains isolated from soils have ability for P-solubilizing activities.

Accordingly, the objectives of this study were to isolate P-solubilizing yeasts from different geographic regions in Egypt and study their performance to solubilize insoluble inorganic phosphates. Three isolates with good P- solubilizing activities were selected for further molecular

genetic studies. Analysis of RAPD-PCR fingerprint was applied to discriminate the isolates at molecular level.

Materials and Methods:

Yeast Extract Peptone Medium (YEPD):

It was composed of 2% glucose, 2% peptone, 1% yeast extract, distilled water up to 100 ml. It was used as solid medium by adding 2% agar for maintenance of cultures and as a liquid when required. It was autoclaved at 120°C and 2.5 atmosphere/m^2 for 15 minutes.

Pikovskaya's Agar Medium:

It was composed of Glucose, 10 g; $Ca_3 (PO_4)2$, 5 g $(NH_4)2SO_4$, 0.5 g KCl, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4$ trace; $FeSO_4$ trace (pH 7) (Sundara Rao and Sinha, 1963). It was used as solid medium by adding 2% agar. It was autoclaved at 120°C and 2.5 atmosphere/m^2 for 15 minutes.

Sampling and Isolation of yeast strains:

Soil and fruit samples were collected from different locations in Egypt, and used for the isolation of naturally occurring yeast strains. About 1 g of soil or fruit was added to 20 ml of YEPD medium, and incubated at 28°C on a rotary shaker at 150 rpm for 1 day. Aliquots of 100 µl were then spread onto a YEPD agar supplemented with 50 mg/ml ampicillin to inhibit bacterial growth. After incubation for 3 days at 25°C, different colonies were randomly isolated from each sample according to the shape, color, and surface feature.

Determination of P-solubilization potential using yeast strains:

The ability of the yeast strains to solubilize insoluble phosphate was described by the solubilization index (SI): the ratio of the total diameter (colony + halo zone) and the colony diameter (Edi Premono, *et al.*, 1996) on Pikovskaya's agar medium. The halo zone formation around the yeast growth on Pikovskaya's agar medium was measured after incubation for 5 days at 25°C.

Quantitative measurement of phosphate solubilization by yeast strains:

The ability of the yeast strains to solubilize insoluble tricalcium phosphate was measured in 100 ml aliquots of Pikovskaya's liquid medium. The yeast strains were grown in 100 ml aliquots of the liquid medium for 10 days at 25 °C, then the cultures were filtered and centrifuged at 10,000 rpm for 10 minutes. Soluble phosphorus in the supernatant and blank sample of the medium was determined by the chlorostannous phosphomolybdic acid method (Jackson, 1973).

Extraction of genomic DNA for PCR

Cells used for DNA extraction were grown for approximately 24 h at 25°C in 50 ml YEPD broth medium on a rotary shaker at 200 rpm and harvested by centrifugation. The cells were washed once with distilled water, re-suspended in 2 ml of distilled water, and the suspension was divided into two 1.5 ml micro

centrifuge tubes. After centrifugation, the supernatant was decanted from the micro centrifuge tubes, and the packed cells were used for DNA extraction which was performed according to procedures described by Harju, *et al.*, (2004); and Hesham *et al.*, (2009).

RAPD-PCR analysis:

Primers and DNA ladder used in RAPD analysis:

RAPD assays were based on the polymerase chain reaction (PCR) amplification of random sites spread all over the genomic DNA. Five random ten-mer oligonucleotide primer sequences selected from a set of Operon kits (OPA-2, OPA-3, OPA-9, OPA-11, and OPA-15) were used in the present study (Martorell, *et al.*, 2005). Their codes and sequences are shown in Table (1).

Table (1): Primer sequences and codes used to study the variation among different yeast isolates.

Serial No.	Primer codes	Sequence (5' to 3')
1	OPA-2	5'- TGCCGCGCTG -3'
2	OPA-3	5'- AGTCAGCCAC -3'
3	OPA-9	5'- GGGTAACGCC -3'
4	OPA-11	5'- CAATCGCCGT -3'
5	OPA-15	5'- TTCCGAACCC -3'

1.5 Kb Ladder was used for calibration of the products of the five primers in RAPD analysis.

The PCR was performed in a final volume of 50 µl containing 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM $MgCl_2$, each dNTP at a concentration of 0.2 mM, 1.25 IU of Taq polymerase, each primer at a concentration of 0.2 mM, and 2 µl of the DNA template.

PCR program and gel run:

The amplification reactions were carried out in a thermocycler under the following conditions: initial denaturation at 94°C for 5 min, followed by 45 cycles of 92°C for 1 min, 36°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min and subsequently cooled to 4°C. Five µl of the amplified mixture was then analyzed using 1.5% 0.5×TBE agarose gel electrophoresis (Martorell, *et al.*, 2005). The gel was stained with ethidium bromide, visualized under UV light, and photographed. The molecular sizes of DNA fragments were obtained with comparison with a 100-pb molecular marker.

RAPD analysis

Agarose gel photos were scanned by the Gene Profiler 4.03 computer software program that uses automatic lane and peak finding for detecting the presence of banding patterns, and calibrat-

ing them for size and intensity. A binary data matrix recording the presence (1) or the absence (0) of bands was made. The software package MVSP (Multi-Variate Statistical Package) was used and genetic similarities were computed using the Dice coefficient of similarity. (Nei and Li, 1979):

$$\text{Similarity} = \frac{2 * n11}{(2 * n11) + n01 + n10}$$

Where:

n11 - designates the number of common bands for the two compared samples,

n10 - cases where the bands were visible only in the first sample,

n01- when bands were visible in the other sample only (Dice, 1945).

Cluster analysis was carried out on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA) software.

Results and Discussion:

Isolation and Screening of Phosphate-Solubilizing Yeasts and Estimation of Phosphate Solubilization

Fifty six yeast strains were isolated from soil and fruit samples (Banana and Guava) collected from different locations in Egypt. The purified isolates were screened for the solubilization of inorganic phosphates on Pikovskaya's agar medium. Three isolates showed good ability to solubilize inorganic phosphates based on inducing clear zones around their colonies were selected and identified as shown in Table (2).

Table (2): Different sources, locations, codes, and genus of the investigated isolates of wild yeasts.

No.	Source	Location	Code	Genus
1	Banana	Cairo	R1	*Pichia spp.*
2	Guava soil	El-Badary	R2	*Candida spp.*
3	Banana	Tanta	R3	*Rodotorula spp.*

The phosphate solubilization index and amounts of phosphorus solubilized from tricalcium phosphate (TCP) by the three selected yeast isolates are shown in Table (3) and Figure (1). The highest amount of P solubilized (49.66 µg/ml) was recorded for yeast strain R-3, which showed a phosphate solubilization index of 2.28 and pH of 4.72 in a 5-day culture Table (3). The results also showed that the lowest amount of P solubilized (21.33 µg/ml) were recorded by yeast strain R2, which showed a phosphate solubilization index of 1.6 and pH of 5.14 in a 5-day culture.

Table (3): Phosphate solubilization by isolated yeast strains

Isolated strain code.	Solubilizing index	Solubilized P in liquid culture (µg/ml)	pH of cultures
R1	1.6 ± 0.015	41 ± 0.00	4.51 ± 0.010
R2	1.6 ± 0.012	21.33 ± 0.667	5.14 ± 0.026
R3	2.28 ± 0.072	49.66 ± .333	4.72 ± 0.072

All values are the means of three replicates.

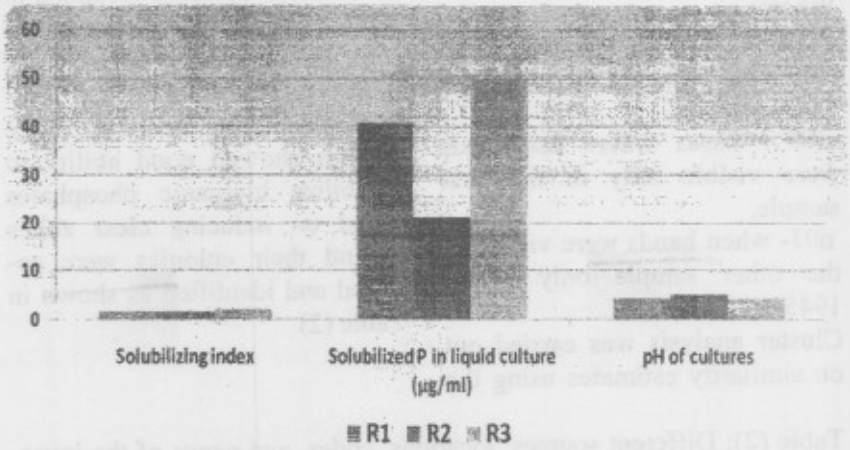

Figure (1): phosphate solubilization by isolated yeast strains.

The solubilized amounts of P and determined phosphate solubilization index for the yeast strains were compatible with the acidity produced in their respective cultures (Table 3), indicating that the organic acids produced from fermentation of the sugars in the media by the yeast strains were the main cause of the solubilization. Some species of bacteria and other microorganisms are well known to produce organic acids from sugar fermentation, and are already used as biofertilizers for solubilizing inorganic phosphate and increasing the phosphorus availability in soil. The solubilization of insoluble P by microorganisms is mainly due to the production of organic acids and chelating substances (Luo, *et al.*, 1993; and Surange and Kumar. 1993).

Many bacterial, fungal, and actinomycete species capable of solubilizing sparingly soluble phosphorus in a pure culture have already been isolated and studied (Halder, *et al.*, 1991; Hamdali, *et al.*, 2010; and Whitelaw, 2000). Hesham and Hashem (2010) reported that 9 isolates out of 40 isolated from Egyptian soil, had

the ability to P solubilize activities.

Genetic variation between the isolates by using RAPD-PCR analysis:

RAPD-PCR analysis was performed to determine the variation at molecular level within the three phosphate solubilizing yeasts.

Results obtained from RAPD analysis among the three isolates of yeast revealed that the number of fragments amplified for each primer varied between 8 and 18 fragments as in Table (4), and their size was ranged from 169 to 1974 bp based on the Figures (2 and 3). On the other hand, Table (4) illustrates also the total number of bands was of 70, whereas the total number of polymorphic bands was of 69 and the polymorphism percentage was (89.57 %).

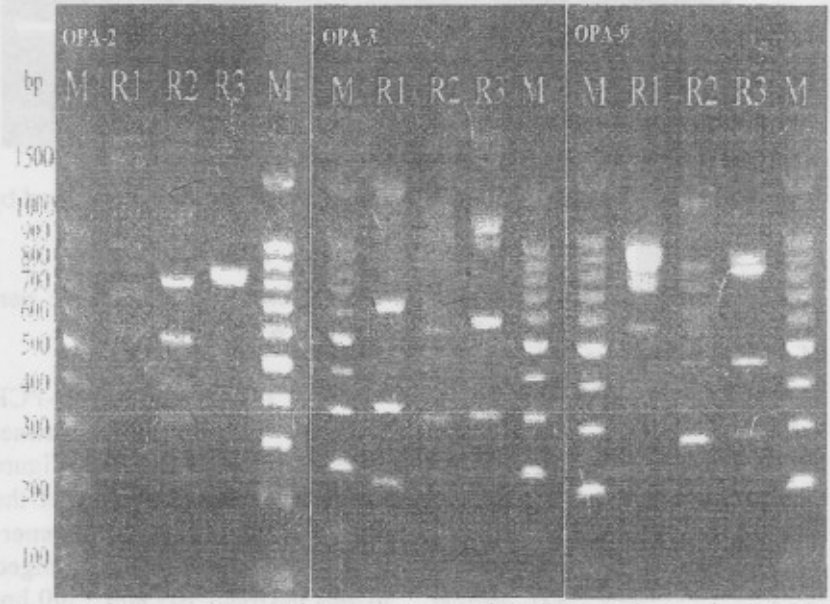

Figure (2): Agarose gel electrophoresis of RAPD products obtained by OPA-2, OPA-3, and OPA- 9 primers.

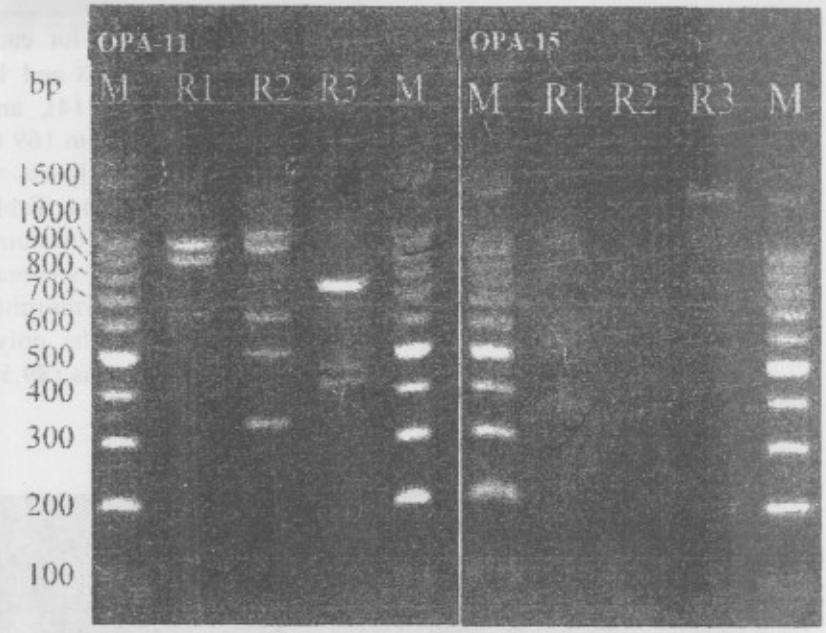

Figure (3): Agarose gel electrophoresis of RAPD products obtained by OPA-11 and OPA-15 primers.

The characterization of the fragments generated by the array of the five primers is surveying in figures (2 and 3), and the description of each primer, and generated bands are summarized in the following:

Primer (OPA –2)

The results of RAPD-PCR analysis obtained by primer OPA-2 are illustrated in Figure (2). The results showed that this primer reacted with all three isolates generating 15 fragments ranged in size between 289 and 1974 bp. The band of size 794 was detected in all isolates. The number of the bands ranged between 1 and 10 among the three isolates. It can be noticed that isolate R3 yielded only one band with size of 794 bp. Table (4) shows that the polymorphic percentage was 93.33%.

OPA-3:

The results of RAPD-PCR analysis obtained by primer OPA-2 are illustrated in Figure (2). The results showed that the number of total bands that generated with this primer is 18 ranged in size between 169 and 1500 bp. The number of the bands ranged between 4 and 13 among the three isolates. The band with size 1000 bp was specific to the isolate R1. Table (4) shows that the number of polymorphic bands is 18 and the polymorphic percentage is 100 %.

OPA-9:

The results of RAPD-PCR analysis obtained by primer OPA-9 are illustrated in Figure (2). This figure shows that the

number of total bands that generated with this primer is 13 ranged in size between 277 and 1327 bp. The number of the bands ranged between 4 and 8 among the three isolates. Table (4) shows that the number of polymorphic bands is 13 and the polymorphic percentage is 100 %.

OPA-11:

Data in Figure (3) illustrate results of RAPD-analysis obtained by this primer. This primer reacted with all three isolates and generated 16 bands ranged in size between 331 and 1403 bp. The number of bands ranged between 3 which related to the isolate R3 and 10 which related to the isolate R2. Table (4) shows that the number of polymorphic bands is 16 and the polymorphic percentage is 100%.

OPA-15:

Figure (3) illustrate the results of RAPD-analysis obtained by this primer. This Figure shows no reaction was detected, when the isolate R2 was tested by this primer. This primer reacted with the two isolates R1 and R3 generated 8 bands ranged in size between 298 and 1516 bp. The number of bands ranged between 2 which are specific to the isolate R3 and 6 which are specific to the isolate R1. Table (4) shows that the number of polymorphic bands is 8 and the polymorphic percentage is 100%.

Table (4) Polymorphism obtained by RAPD analysis among the three isolates.

Primer	Total number of bands (a)	Number of polymorphic bands(b)	Polymorphism -b/a * 100%
OPA-2	15	14	93.33
OPA-3	18	18	100
OPA-9	13	13	100
OPA-11	16	16	100
OPA-15	8	8	100
Total	**70**	**69**	**98.57**

Many authors concluded that the traditional phenotypic characterization of yeasts is of limited potential (Prillinger, *et al.*, 1999; and Andrighetto, *et al.*, 2000) and showed often an incomplete identification or misidentification of yeasts. Molnar, *et al.*, (1993) and data of Lopandic, *et al.*, (1996) indicated that only 53% of all investigated yeast strains were identified reliably using morphological, biochemical, and physiological criteria.

RAPD analysis makes comparable the whole chromosomal

DNA exhibiting bands of genotypically specific value, and additionally this method turned out to be a fast, reliable, highly sensitive, and convenient method (Molnar, *et al.*, 1993).

Thanos, *et al.*, (1996) proved that the numbers and sizes of amplification products were characteristic for each species. All yeast species tested could be clearly distinguished by their amplification patterns. With all primers, PCR fingerprints also displayed intraspecies variability. However, PCR profiles obtained from different strains of the same species were for more similar than those derived from different *Candida sp.*

Genetic similarity matrix and cluster analysis

Data of the presence / absence of DNA fragments (markers) phenotypically analyzed using MVSP program of (Nie and Li, 1979), and pair-wise comparisons between the tested isolates of yeasts were used to calculate the genetic similarity. Then, based on the calculated genetic similarity presented in Table (5), an estimation of the relationship between different isolates could be concluded. Data showed also that the lowest genetic similarity was observed between isolate R1 and R3 (12.5%), while the highest value was found between R1 and R2 (30.1%).

Table (5): Genetic similarity values calculated from the DNA fragments amplified from the three isolates of yeasts using five RAPD primers.

Isolates	R1	R2	R3
R1	1		
R2	0.301	1	
R3	0.125	0.182	1

All three isolates of wild yeasts were regrouped in two clusters based on genetic similarity given in Table (5), and dendrogram as in Figure (4). The first group included isolate R3 formed an independent group that was distinct from all other isolates. The other two isolates R1 and R2 formed the second group.

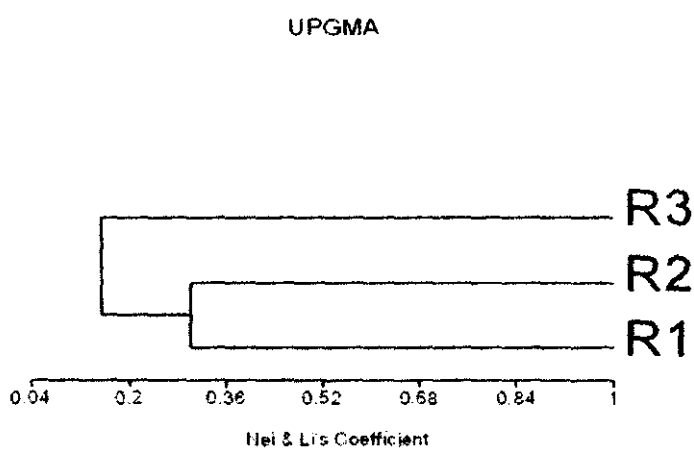

Figure (4): Dendrogram demonstrating the relationship among three isolates of wild yeasts based on data recorded from polymorphism of RAPD markers.

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التمييز الوراثى الجزيئى للخمائر المذيبة للفوسفور المعزولة من الطبيعة

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الفوسفور هو أحد العناصر الغذائية الرئيسية للنبات ، والجزء الأكبر من فوسفور التربة الذي يصل الي حوالي 95-99% يكون موجود في صورة فوسفات غير ذائب وبالتالي لا يمكن للنبات ان يستفيد منه. والكائنات الدقيقة المذيبة للفوسفات تلعب دورا هاما في امداد النبات بالفوسفور وبالتالي تدعم استخدام الأسمدة الفوسفاتية. ان استخدام هذه الكائنات في الحقل اقر بانها تزيد من كمية المحصول. وقد تم فى هذا البحث تجميع ستة و خمسون عزلة من الخميرة من مناطق جغرافية مختلفة فى مصر، ومن ثم انتخاب ثلاث عزلات (R1, R2, R3) والتى أظهرت قدرة عالية على إذابة الفوسفات الغيرعضوى بناءاً على قدرتها فى تكوين منطقة رائقة حول المستعمرات النامية على بيئة الفوسفات ثلاثى الكالسيوم بعد التحضين لمدة 5 ايام على درجة حرارة 25°C. وقد اشير اليه بدليل الإذابة وقد تراوح بين (1.6الى 2.28). وكانت أعلى كفاءة فى إذابة الفوسفات للعزلة R3 هذا وقد أمكن تعريف الثلاث عزلات R1, R2, R3 على المستوى الوراثى الجزيئى وثبت ان الأول ينتمى لنوع الخميرة بيشيا والثانية كانديدا والثالثة رودتريولا. هذا بالإضافة الى أنه امكن التفرقة بين العزلات الثلاثة على المستوى الجزيئى بإستخدام تقنية RAPD-PCR من خلال الأنماط المختلفة للحزم الناتجة من كل منهم. وقد وضعت فى مجموعتين، اشتملت الأولى على السلالة R3، فى حين ضمت الثانية R1, R2.