Molecular Identification of Phosphate-Solubilizing Yeast Isolate KR.1BP.4 From Citatah Karst Area

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Abstract Citatah Karst Area has diverse microorganisms, including phosphate-solubilizing yeasts. Phosphate-solubilizing yeasts in the soil play an important role in solubilizing organic phosphates into inorganic ones absorbable by plants for photosynthetic process and root development. Phenotypical and biochemical identification of phosphate-solubilizing yeast isolate KR.1BP.4 has been reported, but molecular identification should be performed to ensure accurate results. The aim of this research was to identify the species of phosphate-solubilizing yeast isolate KR.1BP.4. This research was carried out by a descriptive method using the following ITS primers: forward primer ITS1(5'-TCC GTA GGTGAACCTGCG G-3') and reverse primerITS4 (5'-TCCTCCGCT TAT TGA TAT GC-3'). The method consisted of three stages, namely extraction with Genomic DNA Mini Kit (Blood and Cultured cell), PCR with KAPA2G Robusta PCR MasterMix, and electrophoresis and sequencing. Data analysis was performed using BLAST, and alignment was performed using MEGA 6.0. The phosphate-solubilizing yeast isolate KR.1BP.4 was identified as *Trichosporonasahii*with an ITS fragment length of 774 bp. Based on the phylogenetic tree and genetic distance, the *T. asahii*isolate KR.1BP.4 had the closest relatedness with *T. asahii*strain V9, *T. asahii* strain V3, *T. asahii*YCH116, *T. asahii*KDLYL 4-1, *T. asahii*V1, *T. asahii*AP.MSU6, and *T. asahii* PMM08-1100L at a distance value of 0%. From this research, it can be concluded that the isolate KR.1BP.4 belongs to the species *Trichosporonasahii*.

Keywords: Internal Transcribed Spacer (ITS), Karst, Phosphate, Yeast, *Trichosporonasahii*, PCR

1 Introduction

Karst areas are rarely encountered for its lengthy formation time. The formation of karst areas through dissolution process may cause degradation which takes place at a very slow rate. The degradation rate varies depending on the air temperature and annual rainfall [1]. Citatah Karst Area, Rajamandala spans 27 km from Rajamandala (adjacent to the Regency of Bandung Barat, Cianjur) to Padalarang [2]. Given the rarity of karst encounter, it is necessary to conduct research on Citatah Karst Area to make the best use of its potentials. According to Yondri [3], Pawon Cave that sits within Citatah Karst Area is rich of phosphategenerated from bat guano sedimentation that is potential to be used as organic fertilizer.

Guano phosphate is composed of nitrogen (N), phosphate (P), calcium (Ca) and a little potassium (K), magnesium (Mg), and sulphur (S) [4]. Phosphate (P) plays a significant role in

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photosynthesis process and root development, but it is available in a meagre amount, usually less than 0.01% of the total phosphate (P), and it is mostly bound to soil colloids, thereby unabsorbable by plants [5]. Phosphate-solubilizing microorganisms, therefore, are needed for phosphate solubilization [6].

The aim of this research was to identify the species of the isolate KR.1BP.4t hrough molecular method using ITS (internal transcribed spacer). The isolate KR.1BP.4 was identified phenotypically and biochemically. However, phenotypical and biochemical identification comes with some shortcomings. For example, not all strains of certain species have general characteristics, and biochemical tests are not optimal in identification as little changes can affect the outcomes [7]. Moreover, the same strain may exhibit varying characteristics at every reiteration of test [8]. Other instances are that existing databases or guidebooks do not list appropriate species and that test results will depend on every individual's expertise and interpretation, providing non-objective and unclear results [9]. For the reasons described above, a further, focused identification of the isolate KR.1BP.4 from Citatah Karst Area using internal transcribed spacer (ITS) is deemed necessary.

The use of molecular technique in organism identification is advantageous in that the results can be obtained faster and in a higher accuracy, and in the case of microbes, this technique can cover all types of microbes, including the uncultured ones. Fungi are eukaryotic organisms that have conserved regions flanking 18S, 5.8S, and 28S rRNA genes [10]. Amidst the encoding genes, there lies ITS (internal transcribed spacer) in the fungi rDNA.

ITS refers to a region of non-coding DNA, which is an RNA sequence derived from prime transcription process that takes place between ribosomal subunit precursors and removed during splicing process when RNA precursor marker of molekul structural is processed into ribosom. Eukariotic organisms have two ITS: ITS1, which is located between 18S and 5.8S genes, and ITS2, which is located between 5.8S and 28S genes. The three ribosomal genes have an extremely high conservation degree [10]. 5.8S gene is only applicable in the identification to phylum and class levels, while 28S gene have a higher variety of nucleotide as compared with 18S and 5.8S genes, allowing it to be applicable in the identification to genus and special levels [11].

ITS, a non-coding region, has higher rates of mutation as compared with coding regions (SSU, 5.8S, and LSU) [12], permiting a wide variety of nucleotide sequences for every species [13]. Sequences of ITS region are highly variable between species, allowing them to be used in yeast identification to species level [14] and even in the identification of some closely related yeast species [12]. ITS is miniscule, approximately 700 bp in size, and has a great number of copies in the core genome. In yeast group, ITS exhibits more effective results than do other sequences and is a recommended universal DNA barcode marker for all fungi in the consortium barcode of life (CBOL). ITS sequences might also be used for protists [15].

2 Materials And Methods

2.1 Material

Isolate

Isolate KR.1BP.4 obtained from Genetic and Molecular Laboratory, Isolated From Citatah Karst Area.

Media

Pikovskaya agar.

Methods

DNA extractionwas done using Genomic DNA Mini Kit (blood and cultured cell) (RBC lysis buffer, GT buffer, GB buffer, W1 buffer, wash buffer, elution buffer, GD column, and collection tube 2 ml) and zymolase per yeast DNA isolation procedure from the Genomic DNA Mini Kit (blood and cultured cell) protocol.

Amplification was conducted using PCR with KAPA2G Robusta PCR master mix (KAPA2G Robusta HotStar, KAPA2G buffer A, KAPA2G buffer B, KAPA2G GC buffer, KAPA enhancer, KAPA MgCl2, KAPA dNTP mix), forward primer ITS1(5'-TCC GTA GGTGAACCTGCG G-3') andreverse primerITS4 (5'-TCCTCCGCT TAT TGATAT GC-3') (Sugita et al.,1998) per ITS1 and ITS4 fragment amplification procedure from the KAPA2G Robusta PCR master mix protocol.

A PCR cycle consisted of pre-determination at 95 oC for 5 minutes, denaturation at 94 oC for 15 seconds, annealing at 52 °C for 15 seconds, extension at 72 °C for 15 seconds, and completion at 72 °C for 3 minutes. PCR was performed for 35 cycles. Electrophoresis using 1% agarose at 100 V for 1 hour.

2.1.1 Data Analysis

PCR result was sequenced using the service provided by 1st BASE Malaysia. From the sequencing, raw data were obtained and processed using the program BLAST (Basic Local Alignment Search Tool) by comparing them against the sequences listed on GenBank by the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) based on which a phylogenetic tree was generated and the genetic distance was calculated using the application MEGA6 [16].

3 RESULTS

Result from Molecular Identification

IsolateKR.1BP.4 from Citatah Karst Area.

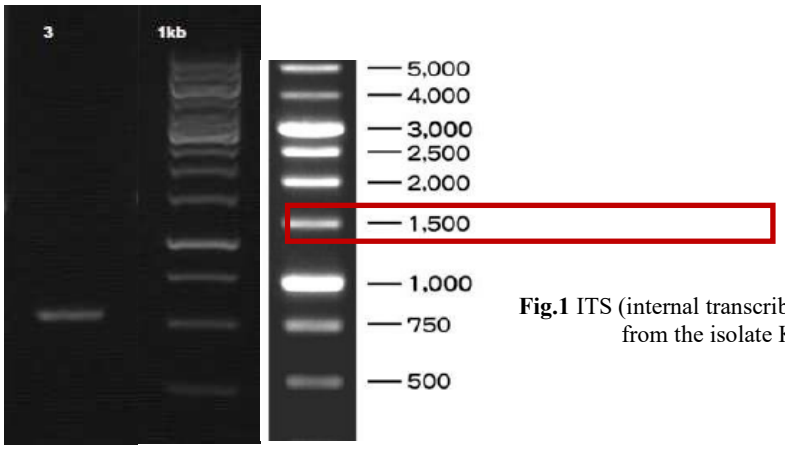

Fig.1 ITS (internal transcribed spacer) fragment from the isolate KR.1BP.4

From the electrophoresis, it was found that the ITS fragment of the isolate KR.1BP.4 was 774 bp long with a bright, thick ribbon and an absence of smear (fire type).

Result from Sequencing

The sequencing result shows the nucleotide sequence:

GCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAAT
TCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTGAAAGTTTTATT
TTGTTATAATAAAACGACGTTCATTACACATTGTTTGTAAAAATACTCGACTTGCG
TCAAGTAGTAGAACAGTTCACAGGTGTAAGTGGATATAGTTATAAGCCTATAAAG
GCAATCACTAATGATCCTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGATTG
CCTTTATAGGCTTATAACTATATCCACTTACACCTGTGAACTGTTCTACTACTTGA
CGCAAGTCGAGTATTTTTACAAACAATGTGTAATGAACGTCGTTTTATTATAACAA
AATAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCG
AATTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC
GCAGCTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTTCAGTGTCATGAAATC
TCAACCACTAGGGTTTCCTAATGGATTGGATTTGGGCGTCTGCGATTTCTGATCGC
TCGCCTTAAAAGAGTTAGCAAGTTTGACATTAATGTCTGGTGTAATAAGTTTCACT
GGGTCCATTGTGTTGAAGCGTGCTTCTAATCGTCCGCAAGGACAATTACTTTGACT
CTGGCCTGAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAA

Phyologenetic Tree Resulted

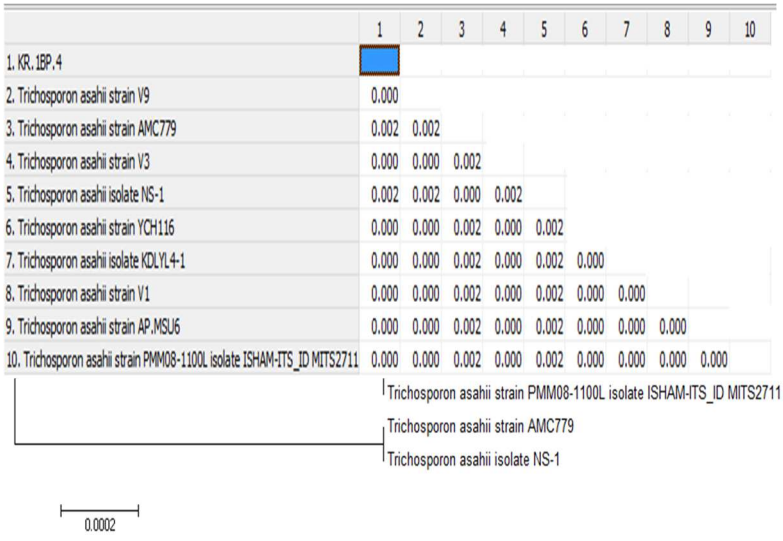

Figure 2.

Phylogenetic tree respresentsKR.1BP.4 as *Trichosporonasahii*at a boostrapping value of 65%.

Fig 3. Isolate KR.1BP.4 has the shortest genetic distance (0%) from *T. asahii*strain V9, *T. asahii*strain V3, *T. asahii*YCH116, *T. asahii*KDLYL 4-1, *T. asahii*V1, *T. asahii* AP MSU6, and *T. asahii*PMM08-1100L and the longest genetic distance (0.2%) from *T. asahii* AMC779 and *T. asahii* isolate NS-1.

4 Discussion

From the electrophoresis, it was found that the ITS (internal transcribed spacer) fragment derived from KR.1BP.4 was 774 bp long, bright and thick, and had no smear (fire type). DNA fragments of *Trichosporonasahii* are 250–1,000 bp long [17]. *Trichosporonasahii* was found to have 500-bp-long fragments[18]. Meanwhile, DNA fragments in ITS region are normally 300–900 bp in length [19].

The bootstrapping value in the phylogenetic tree was 65%. This suggests poor stability as according to Osawa [20], bootstrap is performed to evaluate the stability of a branch, where a branch is said to be stable if the bootstrapping value exceeds 95% and unstable if the value stands below 70%. KR.1BP.4 was at the shortest genetic distance (0%) from *T. asahii* strain V9, *T. asahii* strain V3, *T. asahii* YCH116, *T. asahii* KDLYL 4-1, *T. asahii* V1, *T. asahii* AP MSU6, and *T. asahii* PMM08-1100L and the longest genetic distance (0.2%) from *T. asahii* AMC779 and *T. asahii* isolate NS-1. According to Tamura [21] , these distance values are reliable because a distance value is usable if it stands below 3%.

Trichosporon asahii yeasts, which belong to the genus *Trichosporon*, are characterized by cream color in the colony's morphology, moist or dry texture, and either the presence or absence of flour coating [12]. They have hyphae and are rich of arthroconidia. Some of them also have clavate and globose. Sometimes appresorria, sarcinae, fused form of a big cell, and endoconidia are also spotted in them. The hyphae have septa, which may or may not be accompanied by tubular or vesicular parethesome.

*T. asahii*is a yeast pathogen most commonly associated with clinical specimens such as urine, peritoneal fluid, saliva or phlegm, soft tissues, catheter case, and the tip of catheter tube identified using PCR [22]. It is also found to be the most responsible for trichonosporonosis [23]. *T. asahii* has the capability of transmitting trichonosporonosis and infecting patients with acute myeloblastic leukemia [24]. From the molecular identification performed by El-Mashad et al.[25], 13 of 16 samples from fragments of nail and skin were found to contain *T. asahii* which was the clinical cause of most trichosporonosis cases. *T. asahii* was also found in the houses of patients with summer-type hypersensitivity pneumonitis (SHP) [18]. *T. asahii* was reported to have infected the skin of lower extremity in immune competent patients without any slight symptom, leading to the assumption that *T. asahii* possibly attacks normal flora on the skin [26]. *T. asahii* implication as a pathogen that attacks cancer patients has been reported to increase within the last 10–15 years, but *T. asahii* infections are not restricted to only cancer and neutropenia patients because they are categorized as opportunistic yeast infections and trichonosporonosis has appearance skin to candidiasis [27]. Aside from clinical specimens or body tissues, *T.* asahii is also present in natural environments, especially in the soil. Over the course of the research, the environmental condition was hot and humid, which was suitable for

*Trichosporon*growth [28] . Not only as a pathogen, *T. asahii* was found in a study to be able to produce 104 U/mL of lipase, which is useful in biotechnology sector [29]. In addition, the *T. asahii* which is isolated from the petroleum-contaminated soil has the capability of producing biosurfactant on a mineral salt medium that contains diesel oil as a carbon source and is efficient in degrading diesel oil (95%)[30].

5 Conclusion

Based on molecular identification using ITS (Internal Transcribed Spacer), isolate KR.1 BP.4 isolated from Citatah karst area identified as *Trichosporon asahii* with 774 bp fragment length. Kinship of isolate KR.1 BP.4 with *T. asahii* strain V9, *T. asahii* strain V3, *T. asahii* YCH116, *T. asahii* KDLYL 4-1, *T. asahii* V1, *T. asahii* AP.MSU6, *T.asahii* PMM08-1100L with a distance value of 0%.

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