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Intestinal *Pichia burtonii* associated with Qiweibaizhusan

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ABSTRACT: *Qiweibaizhusan is a millennium ancient prescription of traditional medicine and is commonly used to treat pediatric diarrhea. Qiweibaizhusan has shown to enhance the growth of fungi, but which fungi species are associated with Qiweibaizhusan treatment remains unknown. Here we isolated a yeast strain Y1 from mouse's intestine whose growth was enhanced by Qiweibaizhusan and identified the strain by morphologic, biochemistry and molecular biological methods. The yeast colonies were milky white, homogeneous texture. The strain could form pseudohyphae, budding breed in multiterminal buddings. It could ferment glucose and D-xylose but not form amyloid substance, assimilate glucose, sucrose, D-xylose, fructose and sorbitol. It couldn't use potassium nitrate and urea for nitrogen source. A phylogenetic tree constructed by comparing with the published 18SrDNA and 26SrDNA sequences of relative yeast species suggested that the yeast Y1 strain was the closest relative to Pichia burtonii with more than 99% sequence similarity. So the yeast Y1 strain was Pichia burtonii. It was the first report that yeast strain in mouse intestine associated with the Traditional Chinese medicine, Qiweibaizhusan.*

KEY WORDS: *Chinese medicine, Qiweibaizhusan, Intestinal yeast , Phylogenetic tree, Pichia burtonii*

I. INTRODUCTION

Yeast strains are commonly used in forage production. Under specific process condition, they can be developed into micro-ecological products. In narrow concept, yeast refers to *Saccharomyces cerevisiae*, which belongs to the ascomycete subphylum imperfect fungi. The generalized yeasts refer to those which can not form the mycelium of fungi, belonging to ascomycotina, deuteromycotima etc. They can survive and reproduce in aerobic and anaerobic environments and also can produce plentiful nutrient substances such as B-complex, GSH, engosterin, gamma-aminobutyric acid and some unidentified growth factors [1, 2]. Yeast has been the most beneficial fungi with the abilities to promote digestion and absorption of nutrient in animals, and to significantly inhibit some harmful bacterial species. Cultured yeast is a kind of pure natural raw materials for animal feeding. It plays an important role in improving palatability, the rate of digestion, and the feeding efficiency, which promote animal growth [3-7]. It had been shown that the reproductive capacity and vigor were strengthened after the yeast entering into the gastrointestinal tract [8, 9]. Yeast can effectively inhibit the proliferation of pathogenic microorganisms, participate in the pathogenic microflora survivability competition, exclude adhesion of pathogenic bacteria on the gastrointestinal mucosa surface, and help the body eliminate toxins and pathogens metabolites. Yeast can also prevent toxins and waste absorption, enhance both immunity and

resistance to diseases. The intestinal yeast metabolism also consumes oxygen in the digestive tract, and compete with *Escherichia coli* for limited oxygen, while creating an anaerobic environment for anaerobic lactic acid bacteria and *Bifidobacteria*, inhibiting the growth of harmful bacteria such as *Escherichia coli*, *Salmonella spp*., and promoting the reproduction of some beneficial bacteria such as lactic acid bacteria and *Bifidobacteria* [10, 11]. Qiweibaizhusan is composed of ginseng, costustoot, poria cocos, roasted rhizoma atractylodis macrocephalae, pueraria, agastache and liquarice according to the Chinese Pharmacopoeia. It is a millennium ancient prescription to treat pediatric diarrhea, and it has stringent design and accurate appropriate compatibility. It has excellent effects on spleen and stomach deficiency, vomiting diarrhea, loss of appetite, and thinness and weakness. Both ultra-Qiweibaizhusan and jiawei Qiwebaizhusan showed effects on the intestinal microorganisms and enzyme activities [12] . The amounts of intestinal fungi were significantly increased in Qiweibaizhusan treated mice, suggesting that Qiweibaizhusan could enhance fungi growth. In this project, we are investigating which fungi species promoted by Qiweibaizhusan. We isolated a yeast strain from the intestine of Qiweibazhusan treated mice. The growth of the yeast strain was enhanced by the medicine. We identified the yeast strain as *Pichia burtonii*.

II. MATERIAL AND METHODS

Media [13]

Potato Dextrose Agar (PDA) medium and Yeast Extract Peptone Dextrose (YEPD) solid medium was used for culturing yeast. McNamara medium, Assimilation of carbon sources basal medium, Assimilation of nitrogen sources basal medium, The assimilation ethanol basal medium, Production of amyloid medium, Decomposition of urea medium and Mashing water medium was used for physiological and biochemical tests.

Reagents

Glucose, Sucrose, Lactose, D-xylose, D-mannitol, Urea, Iodine solution, Tris, SDS, Potassium acetate, Chloroform-isoamyl alcohol (23:1), Isopropanal, Phenol-chloroform-isoamyl alcohol(24:23:1), 70% ethanol, dNTPs, 10×Taq Buffer， Taq polymerase, Ethidium bromide, Agarose, 6×Loading Buffer，DL3000 DNA Marker. They were bought from Beijing Biological Science and Technology Co Ltd.

Isolation of yeast strain

Mice were treated with Qiweibaizhusan according to our previous description [10]. The intestinal contents from the jejunum to the ileum of the treated mice were taken and placed in the sterilized water bottle filled with glass beads, microorganisms were fully dispersed by shaking for 30 mins at 120 r/min. Yeast strains were cultured on YPD solid medium. .

The growth of yeast strain in medium containing Qiweibaizhusan

1% (v/v) of yeast strain were added into a liquid medium with 0%, 5%, 10%, 15%, 20%, 25% or 30% Qiweibaizhusan, and inoculated for 36 h at 28 ℃ with shaking at 200 rpm/min. The mycelia were collected by filtration and dried for 12 h at 50 °C, the weights were measured.

Morphological characteristics

The yeast strain was cultured on the PDA solid medium for 3-7 d at 28°C, the colony morphology was observed under a microscope, the reproduction and cell shape of the budding yeast were recorded. In order to investigate the ultra-morphology of the yeast strain, the strain was cultured in a thin PDA culture medium on a glass slide for 3 d at 28 ℃, and subjected to scanned electron microscopic observation was done according to previous description [14] .

Carbohydrate fermentation

0.1 mL yeast solution was added into 4 mL culture medium containing 2% glucose, sucrose, lactose, D-xylose, or D-mannitol, a fermentation Duchenne tube was put upside down in the culture medium. The culture medium with yeast strain was cultured at 28 ℃ with shaking, gas accumulation in the fermentation Duchenne tube was a sign of carbohydrate fermented.

Assimilation of carbon and nitrogen compounds

Assimilation of carbon sources (glucose, sucrose, fructose, lactose, D-xylose, D-mannitol, rhamnose and sorbitol) and nitrogen sources (peptone, urea, ammonium sulphate and potassium nitrate) was analyzed as previous description [15].

Amyloid-like compound formation

The yeast strain was inoculated in PYG liquid medium for 1-2 h at 28 ℃. A drop of iodine solution was added, if the color of medium changed to blue, purple or green, suggesting amyloid-like compounds formed.

Urine enzyme activity

The yeast strain was inoculated in urea liquid medium at 37 ℃, if the color of the medium became red, indicating the production of urine enzyme.

Extraction of DNA

The yeast strain was cultured at 28 ℃ and collected by centrifuge. The yeast pellet was grinned in liquid nitrogen. The genomic DNA was extracted DNA using CTAB buffer (Sigma, H6269) according to the manufacture's instruction and dissolved in TE buffer. The quality of extracted DNA was analyzed by 1% agarose gel electrophoresis.

Polymerase chain reaction (PCR)

Primers used for PCR are fungal universal primers including 18S rDNA forward primer: 5'-GTAGTCATATGCTTGTCTC-3', reverse primer: 5'-TCCGCAGGTTCACCTACGGA-3'; 26S rDNA forward primer: 5'-GCATATCAATAAGCGGAGGAAAAG-3' and reverse primer: 5'-GGTCCGTGTTTCAAGACGG-3'. The PCR reaction condition for 18S rDNA contained denaturing at 94 °C for 10 min, 35 cycles of denaturing at 94 °C for 45 s, annealing at 55 °C for 90 s, and extension at 72 °C for 90 s, and a further extension cycle at 72 °C for 10 min. The PCR reaction condition for 26S rDNA contained denaturing at 94 °C for 10 min, 35 cycles of denaturing at 94 °C for 60 s, annealing at 52 ^{o}C for 60 s and extension at 72 °C for 90 s, and a further extension cycle at 72 °C for 10 min. The amplified products were analyzed by agarose gel electrophoresis and subjected to sequencing.

Bioinformatic analysis

18S rDNA and 26S rDNA sequences of other species were obtained from the GenBank, sequence homology to other species was analyzed by using CLUSTAL x program [15], the phylogenetic trees were constructed by using MEGA Neighbor-Joining [16].

III. RESULTS

The effects of Qiweibaizhusan on the growth of the isolated yeast strain

A yeast strain was isolated from intestinal contents of mice treated with Qiweibaizhusan.

In vitro experiments showed that the growths of the isolated yeast strain Y1 could be enhanced by Qiweibaizhusan. The weight of yeast (g/100 mL) increased continuously along with the concentration of Qiweibaizhusan, the highest increase was under the treatment of 15% Qiweibanzhiusan, the increase kept steady under the treatment of 20%, 25% and 30% of Qiweibaizhusan, respectively (Fig. 1).

Morphology of the isolated yeast strain

The yeast strain was cultured in PDA medium and observed with an optical microscope, the strain showed open-ended, multiple budding (Fig.2A) and formed hyphea (Fig.2B). The yeast strain displayed multiple budding, spherical, oval, occasionally into a cone, but do not form spires under electron microscopic observation (Fig.2C and 2D).

Characteristics of yeast colonies

The yeast strain was cultivated in PDA medium at 28 °C, the colonies were formed after 3 days. As shown in Fig.3, the colonies were creamy white, round and convex, the color of the colony edge ring was light. A slight concave was localized in the center of the colony and was opaque with smooth surface. The colonies were easy to pick up by needles, displayed same color from the front to the back sides and from the center to the edge. The colonies exhibited hairy (class mold) from the front surface and expanded around with the neat edges. The colonies displayed radial growth when observed from the backside.

Physiological and biochemical characteristics of the yeast strain

The Carbohydrate fermentation experiments suggested the yeast strain could ferment glucose and D-xylose, but not sucrose, lactose or D-mannitol. The strain could assimilate glucose, sucrose, D-xylose and sorbitol, but not fructose, lactose, D-mannitol, or rhamnose. It also assimilated peptone and $(NH4)_2SO_4$, but not potassium nitrate or KNO_3. The strain did not form amyloid-like compound and t produce urease.

PCR and sequencing

A 1706 bp fragment for 18S rDNA (Fig4.A) and a 572 bp fragment for 26S rDNA (Fig4.B) were cloned by PCR, and analyzed by agarose gel electrophoresis. PCR products were purified and subjected to sequencing. Sequences of both PCR products were submitted to GenBank, the accession number for 18S rDNA is JQ217075 and for 26S rDNA is KC192660.

18S rDNA and 26Sr DNA phylogenetic tree analysis

BLAST searching showed that the 18S rDNA sequence is homologous to the pichia yeast (*Pichia burtonii*) 18S rDNA (Accession number: AB158656) with identity of 99%, and the 26S rDNA sequence is also homologous to the pichia yeast (*Pichia burtonii*) 26S rDNA (accession number: U45712) with identity of 100%.To further confirm the species of isolated yeast strain, the phylogenetic trees of 18S rDNA and 26S rDNA were constructed with the Neighbor-Joining method in the MEGA 4.0 software and shown in Figures 5 and 6. The sequence of 18S rDNA from the isolated yeast strain had the closest genetic relation with 18S rDNA sequence from the yeast species, *Pichia burtonii* (AB158656) (Fig. 5). The 26S rDNA phylogenetic tree showed the sequence of the isolated yeast strain 26S rDNA also had the closest genetic relation with 26S rDNA from the yeast species, *Pichia burtonii* (U45712). Therefore, the isolated yeast strain was identified as *Pichia burtonii*.

IV. DISCUSSION

The traditional way of identification of microorganisms is using biochemical and physiological methods. DNA sequence and database (e.g. RDP and GenBank) provide new approaches for analyzing microbial diversity of microorganism. The rDNA gene research makes it possible to study the evolutionary relations of eukaryotes and identify microbial species of specific environments. rDNA contains variable areas, which are available to track specific organisms and organic stocks. rDNA analysis can be used to investigate classification and evolution of fungi [17]. Qiweibaizhusan has been shown to promote the growth of fungi species [10]. Here we isolated a yeast strain, whose growth was enhanced by Qiwenbaizhusan. The colonies of the strain exhibited milky white, round and convex with propagation for multi-bud colonization. The strain can ferment glucose and D-xylose, and assimilate glucose, sucrose, D-xylose, fructose, sorbitol, peptone and $(NH_4)_2SO_4$. Bioinformatic analyses of 18S rDNA and 26S rDNA sequences indicate that the isolated yeast strain is *Pichia burtonii* [18-20], Which is known as *Endomycopsis burtonii* [21]. *Pichia burtonii* only require simple nutrition and has fecundity, it can make full use of sugars and organic acids, and can grow and reproduce in an environment with high concentration of reducing substances. It also is resistant to both higher temperature and acidic environment. *Pichia burtonii* is thought to be non-pathogenic species. It is the first time to identify *Pichia burtonii* in mouse intestines, and Qiweibaizhusan could enhance the growth of the yeast species. The strain may involve in the regulation of intestinal physiological flora by Qiweibaizhusan and have a role in pediatric diarrhea.

V. ACKNOWLEDGEMENTS AND FUNDING

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FIGURES

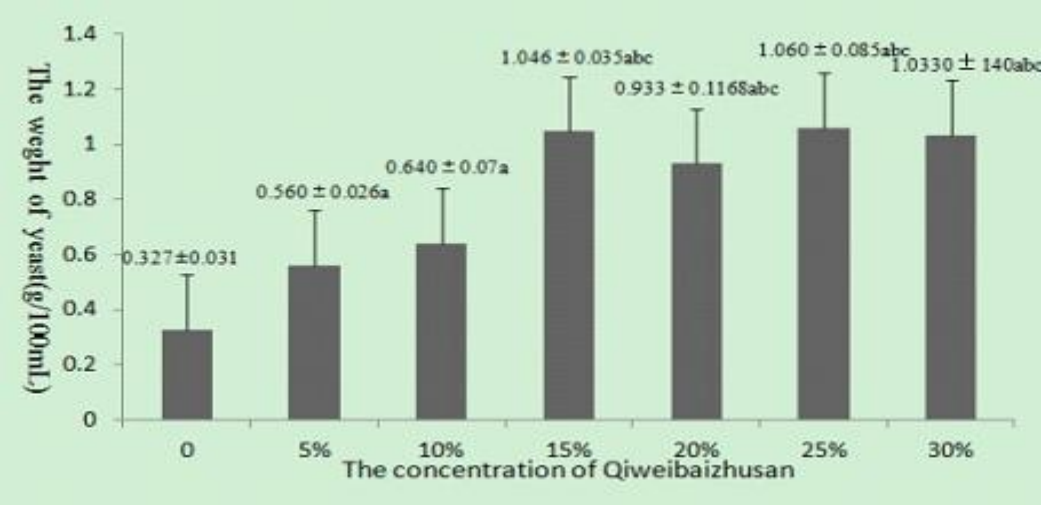

Fig.1 The effect of Qiweibaizhusan on the growth of yeast

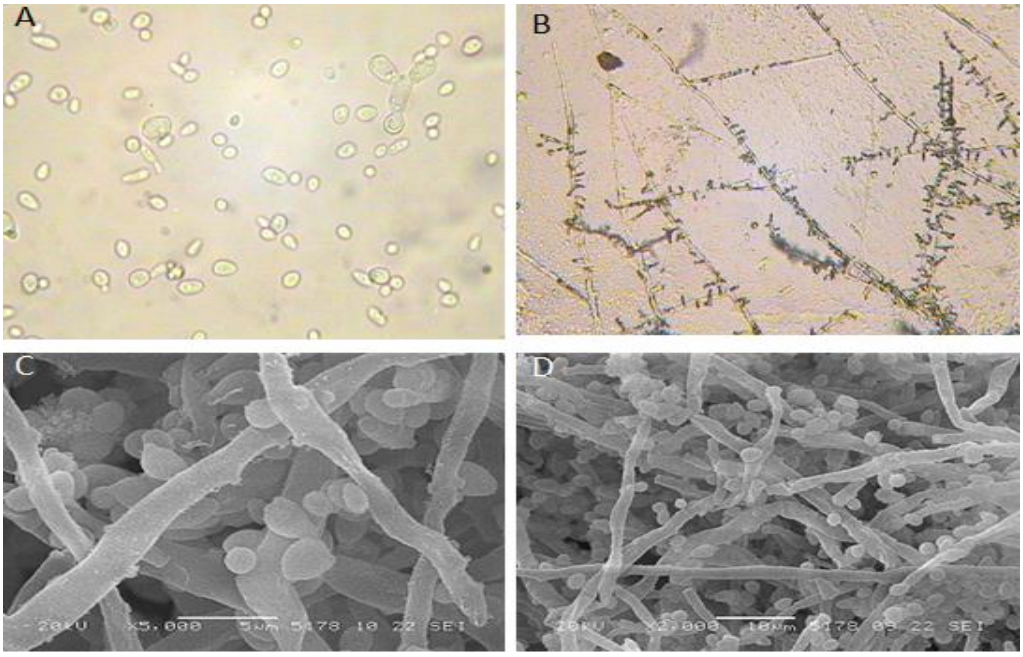

Fig.2 Morphology of the isolated yeast strain. (A,B) morphology under optical microscopical observation (400); (C,D) ultra morphology (5000 for C and 2000 for D).

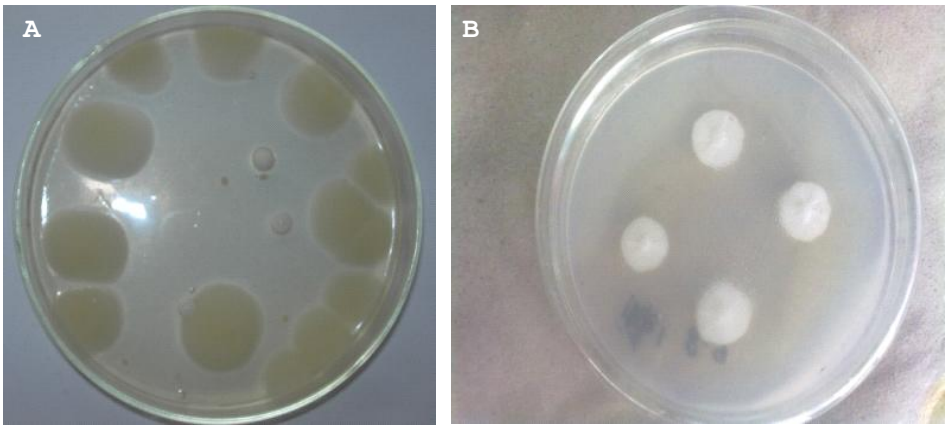

Fig. 3 Colonies characteristics of yeast

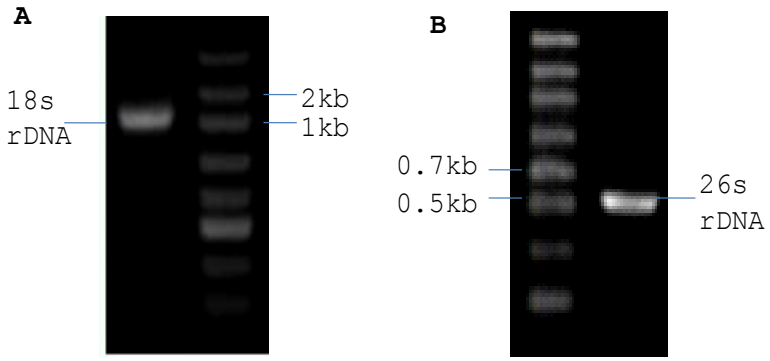

Fig.4 PCR products of 18S rDNA and 26S rDNA

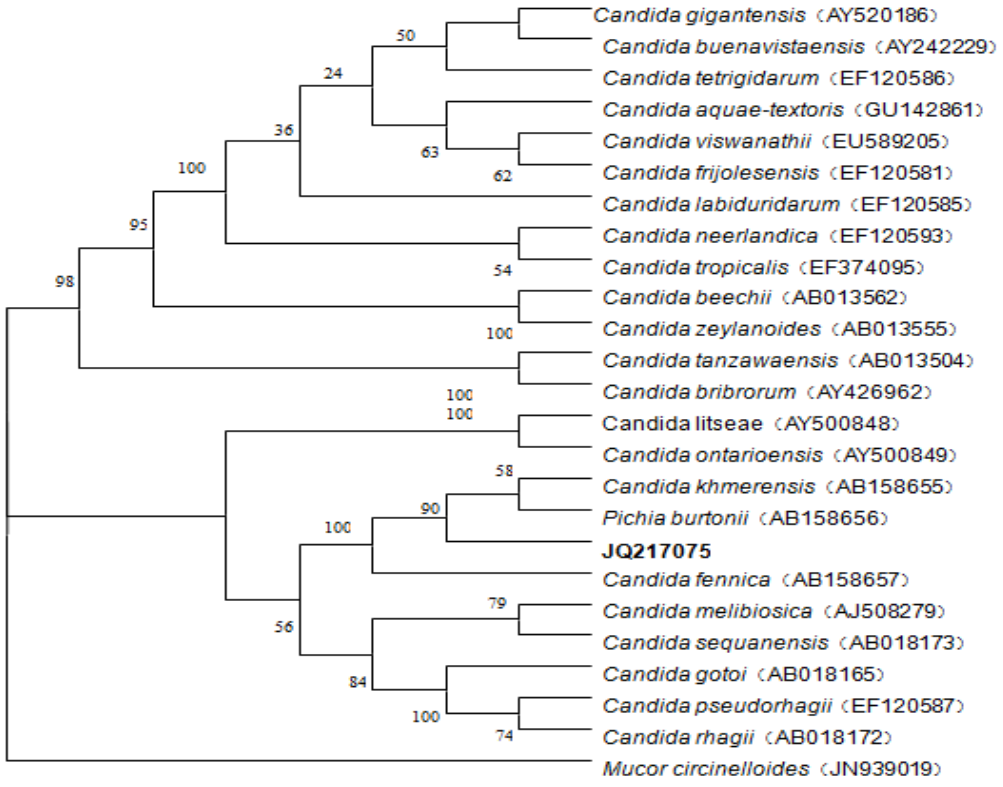

Fig. 5 Phylogenetic Tree of 18S rDNA from yeast species. JQ217075 is the accession number for the 18S rDNA sequence of the isolated yeast strain

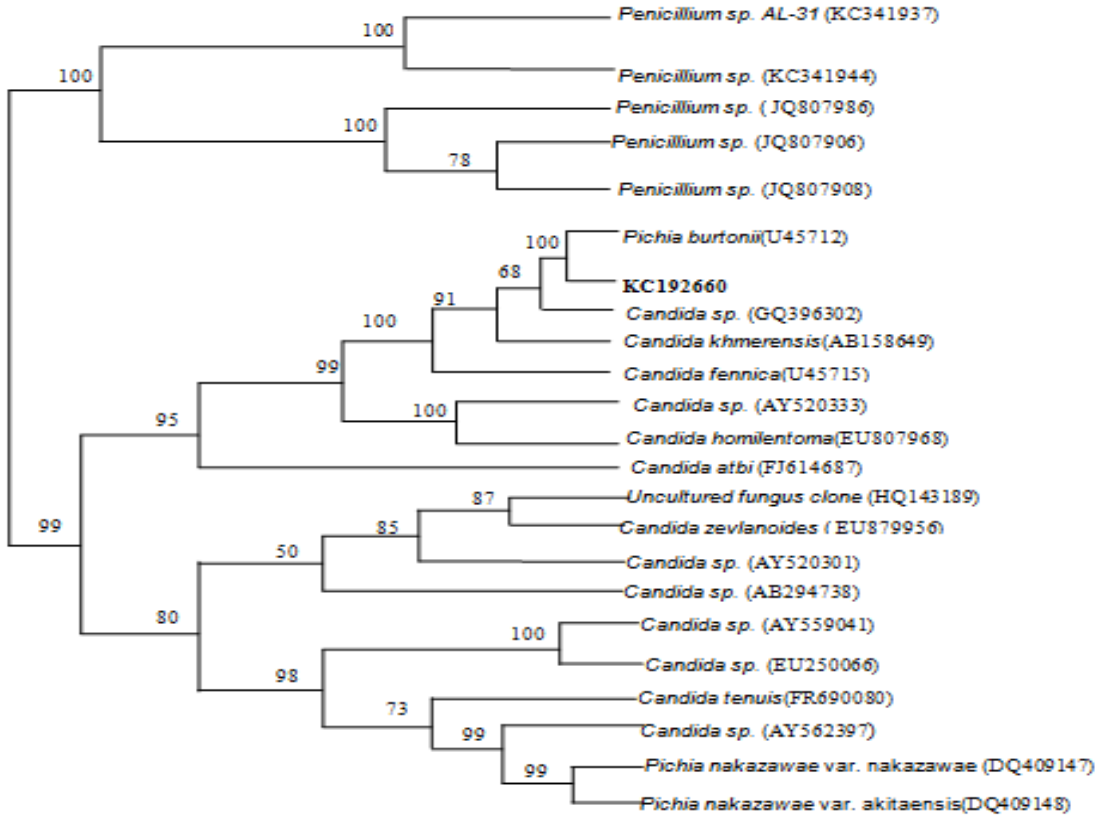

Fig. 6 Phylogenetic Tree of 26 Sr DNA from yeast strains. KC192660 is the accession number for the 26S rDNA sequence of the isolated yeast strain