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Research Article

Probiotic effects of Metschnikowia isolated from diary products aquatic environments

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ABSTRACT

The aim of this study was to characterize the probiotic yeasts isolated from both aquatic environment and dairy products by sanger sequencing method and drawing phylogenetic tree for the identified probiotic yeasts. Sampling of dairy and non-dairy products was done randomly. Characterization of yeasts was constructed by molecular strategies based on the amplification and sequencing of the ribosomal DNA internal transcribed spacer region. MEGA7 software was also applied for alignment (Muscle algorithm) and to create an agreement neighbour-joining analysis to determine the phylogenetic relationship of isolated species. For an in vitro selection of the probiotic candidates, survival of isolates at different temperatures, pH and bile salts was assessed. Based on biochemical assays and gene sequencing, the isolates were detected as *Candida albicans* (Wb), *Clavispora lusitaniae* (WC), *Metschnikowia* (KC) and *Saccharomyces cerevisiae* (Vc) strains. We indicated that Wb and KC isolates could significantly grow at 37°C after 3 hours. Wb, KC and Vc species also were proliferated at pH~1.5. We concluded that Wb and KC strains isolated from the marine environment and dairy products have great potential for use as probiotics in the food industry based on their resistance in human body at temperature equal to 37°C and gastric PH equal to 1.5.

1. Introduction

Currently, considerable investigations have been allocated to the expansion of beneficial foods that contain probiotic strains in charge of health-promoting effects. Probiotics are clearly characterized as live microorganisms and when consumed in proportional quantities, admits a profitable result on the health of the host''(Spacova et al., 2020). When probiotic microorganisms (bacteria, yeasts and etc.) are inserted into the meals as dietary supplements, they can ameliorate human health. Various dairy yields and fermented milk are frequently applied as probiotic fountainheads for transferring probiotics to the human digestive tract (Tripathi and Giri, 2014; Tabanelli et al., 2016). In spite of the fact that lactic-acid bacteria and bifidobacteria are chiefly probiotic microorganisms (De Llanos et al., 2006), several yeasts suchlike Saccharomyces and Kluyveromyces strains are also thinking about

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as probiotic because of their impressive characteristics (Diosma et al., 2014).

Yeasts, as an unavoidable portion of the microflora of different fermented meals and liquors, discover in a variety of meals from plant or animal sources and aquatic environment and have a remarkable effect on meals protection and food safety (Erten et al., 2014). Marine yeasts omnipresent in the aquatic environment. They are regularly recognized within the digestive system of marine mammals and in sea water and seashore sand (Chen et al., 2009; Zaky et al., 2014). The ideal development condition for marine yeasts is at shortest lag phase at acidic pH values (Krause et al., 2013). Yeasts undertake a pivotal function in the segregation of plants are resistant to deterioration by heat, pressure, or chemical attack, and cycling of nutrients and mostly detected on rotting algae(Caspeta and Nielsen, 2015; de Jong and Hagen, 2019).

The examination of yeasts in different marine situations has incredibly progressed in the last years with the accessibility of molecular taxonomic strategies (Fell, 2012; Sarkar and Rao, 2016). For yeast distinguishing proof, most of surveys have given special attention to sequencing of the 600–650-bp D1/D2 locus of the large subunit (LSU) rRNA gene, for which sufficient sequence data are accessible (Raja et al., 2017). Engaged pre-screening or stratification techniques has been used, to confinine time-consuming and expensive sequencing nearly in all investigations. Parallel to the classification of phenotypes, particularly PCR-based processes and restriction fragment length polymorphism (RFLP) analysis have been utilized so far (Pham et al., 2011).

Yeasts are suggested for the curbing and remedy of human gastrointestinal disorders, in particular IBD (inflammatory bowel disorders) and ADD (antibiotic associated diarrhea), and the managing of serum cholesterol and acute diarrhea in adults and children. It also appeared to be useful in the cure of infections with Clostridium difficile and Helicobacter pylori (Czerucka et al., 2007; Rima et al., 2012; Sharif et al., 2016). The yeasts also exhibit a competent exchangeable with probiotic bacteria due to it is resistant to the antibiotic outcomes, may steer clear of the antibiotic-associated human intestinal disorders (Rima et al., 2012), can decline the consumption of antibiotics, and, thus, restrict the progress of antibiotic resistance. These explanations propel researchers to concentrate their consideration on the exploration of further yeast species with probiotic features. The main objective of this study was to identify and characterize the probiotic yeasts isolated from both aquatic environment and dairy products by Sanger sequencing method and then phylogenetic tree was thrown for the identified probiotic yeasts.

2. Materials and Methods

2.1. Yeast isolation and identification

The first sampling from dairy and non-dairy products was done randomly in October 2017 and thirty samples were isolated. Dairy products were included yogurt, milk, buttermilk, cheese, curd and non-dairy products were collected from aquatic environments such as a river, spring and lake located in Tehran, Iran.

Each sample was stirred in three states in the 50 ml Falcons, and then suspension and sediment were removed. Then, 1 ml of each of the three states was used and poured into a sterile plate. After that, 20 ml of SDA was added to the plate. Finally, the medium was incubated at 30 ° C for 3-5 days in aerobic conditions. The net cultures of samples were maintained on the broth seaboard via freezing at -70°C. The morphology and biochemical properties of yeasts were detected using various procedures, including staining, Chrom Agar for *Candida* species detection, sugar test (glucose, sucrose, maltose, galactose and lactose), urease and nitrate reduction tests. Based on the colony characteristics (white and creamy texture) ovoid microscope shape, ascospores formed by the yeast isolate was detected for indication of the ascomycetous yeast (Freydiere et al., 2001; Larypoor and Frsad, 2011).

2.2. PCR amplification and sequencing analysis

The Characterization of yeasts was conducted by molecular strategies based on the amplification and sequencing of the ribosomal DNA internal transcribed spacer region (ITS). The specific primers (Sinaclon, Tehran, Iran) including ITS1 (5′-CGG GAT CCG TAG GTG AAC CTG CGG-3′) and ITS4 (5′-CGGGAT CCT CCG CTT ATT GAT ATG C-3′) (6) were applied for amplifying the ITS/5.8S rDNA

region. The PCR analysis was carried out in an Eppendorf thermal cycler PCR system (Roche, Mannheim, Germany) with the following program: 95°C/ 3 min, 35 cycles of 95°C C /30 s, 55°C /30 s and 72°C /1 min; and final extension 72°C/5 in a final volume of 25 ml. Then electrophoresis in a 1.5% (w/v) agarose gel were used to check the quality and purity of extracting DNA. Afterwards, the purified PCR products were forwarded to the Iranian Biological Research Center (Tehran, IRAN) for DNA sequencing according to Sanger sequencing method. We downloaded the .abi files and also visualized DNA chromatograms via Chromatogram Explorer V.2.0.0 software. Resulting obtained sequences were explored versus sequences in the Genbank non-redundant (nr) nucleotide database via Standard Nucleotide BLAST (blastn), and the top-scoring hits were noted for each isolate (de Jong and Hagen, 2019).

2.3. Phylogenetic trees realization

The alignment of the established sequences was examined manually and modified, and homology values were assigned using Chromas software V2.5. MEGA7 software was also applied for alignment (Muscle algorithm) and to create an agreement Neighbour-Joining analysis to determine the phylogenetic relationship of isolated species. Gaps were denied and the strength of tree branches was evaluated with 1,000 replicates. The analysis of phylogenetic and molecular evolutionary was performed in agreement with Felsenstein (Felsenstein, 1985), Tamura and Nei (Tamura and Nei, 1993) and Kumar et al. (Kumar et al., 2018) investigations.

2.4. In vitro selection of the probiotic candidates

2.4.1. Survival at different temperatures

The efficacy of temperature on the development of strains was assessed by adding 100 ml of the active yeast suspensions (~10^9 CFU/ml of each isolate) into sabouraud dextrose broth and incubated at 25, 30, 37 and 42°C.

2.4.2. Survival at low pH

The development of isolation at acidic pH (<7) was determined by adding 100 ml of activated strains suspensions (~10^9 CFU/ml) into sabouraud dextrose broth by a primary pH of 1.5, 2.0 and 3.5 and was incubated at 30°C.

2.4.3. Bile tolerance

We inoculated 100 ml of yeast suspensions (~10^9 CFU/ml of each isolate) into sabouraud dextrose broth and then10 cc of Bile solution (0.3%) was added to the solution. The light absorption of this solution was read by spectrometry at the same moment. Then the solution was incubated at 30 ° C for 8 hours and the light absorption was read after 8 hours again. The obtained number was placed in the following formula. If the obtained number was less than 0.4 indicates that our isolates are tolerance to bile salts.

$$\text{Cnih} = \frac{(\Delta T8 - T0 \text{ control} - \Delta T8 - T0 \text{ treatment})}{\Delta T8 - T0 \text{ control}}$$

3. Results

3.1. Characterization and sequencing of isolates

From 30 samples isolated from the diary and non-dairy products, a total of four isolates, named VC, KC, Wb and Wed, were isolated from vinegar C, kefir C, water B and water D samples and originally detected as yeast strains according to standard and differential biochemical assays (Table 1).

In this study, DNA was extracted from all 4 isolates and the isolates were sequenced using Chromatogram explorer software and Sanger sequencing technique. Sequencing results showed that Wb, Wc, Kc and Vc isolates were *Candida albicans*, *Clavispora lusitaniae*, *Metschnikowia* and *Saccharomyces cerevisiae* strains, respectively (Table 2).

3.2. Phylogenetic tree analysis

The phylogenetic trees have been drawn out to assess the taxonomic dependency the isolates discovered in the present survey with source strains. They were created via the technique of distances neighbour-Joining using the Blast program. Figure 1 indicates the communication between yeasts. According Figure 1, the species of *Metschnikowia* sp (S1), *Clavispora lusitaniae* (S4) and *Candida albicans* (S2) were in the same branch and in terms of phylogenetic characteristics, also were close to each other. However, *Saccharomyces cerevisiae* (S3) was observed in a separate branch from other isolates.

Table 1. Results of differential biochemical assays

Ascospore	Kno3	Growth on SDA	Growth on CMA	Sugar fermentation									Sabriose	Dextrose	Urea	Strain name
				Glucose	Maltose	Sucrose	Lactose	Galactose	Mono-Inositol	Xylose	Raffinose	Trehalose				
0	0	+	+	+	+	+	0	+	0	+	0	+	0	+	0	**Wb**
0	0	+	+	+	+	+	0	+	0	+	+	+	0	+	+	**Wd**
0	0	+	+	+	+	+	0	+	0	+	0	0	+	+	+	**Kc**
+	0	+	+	+	+	+	0	+	0	0	+	+	0	+	0	**Vc**

3.3. Probiotic Aptitudes

Following detecting the 4 yeast species, the assessing of possible probiotic features was carried out. Attain this objective, a series of essays, including growth at 37 ° C, the capability to remain alive at low pH (acidic) and in high doses of bilea, were performed.

According Figure 2, Candida albicans species have significant resistance to pH~5 and pH~ 1.5 and can grow at 30 and 37°C. Figure 3 also indicates, the best growth of Clavispora lusitaniae was at PH~ 5 and PH~1.5, respectively. This isolates also was not sufficiently compatible with pH~ 2. We can see at PH~ 2, the number of microorganisms reached almost zero after 3 hours.

The best growth of these species was shown at 37 ° C and the lowest growth was indicated at 25°C. The significant growth was observed after 1 hour at 30°C, but after 3hours, the growth of this isolate reached near to zero.

According to Figure 4, at pH 5, growth and adaptation of Metschinikowi specie was higher than other PH. We also observed little growth at pH~3 after 3 hours. This specie showed remarkable development at 30 ° C after 2 hours, but this resistance gradually decreased. The growth of Metschinikowi isolates also was ascending at 42 and 37°C, however, we see lower growth rates at 42°C in comparison with 37 ° C. As stated in Figure 5, after 2 hours, the highest growth of *Saccharomyces cerevisiae* was at pH 2. However, the maximum pH resistance was observed at pH 1.5 in 3 hours' time. Also, the lowest development rate was at pH 3 after 3 hours. This yeast also had the greatest growth rate at 30°C overtime and had the same development in other temperature with the passage of time. Considering that after placing the numbers in the Cin h formula, all 4 isolates were equal to 0.4 or less, which showed that all 4 isolates were resistant to bile environment and were able to grow in different bile salt concentration. With respect to Figure 6, spore number of three isolates, including *Candida albicans, Clavispora lusitaniae* and *Saccharomyces cerevisiae* strains increased at 25°C, however *Metschnikowia* strain spores had no change at different temperatures. We also see that the spore population gradually had reduced with increasing temperature, in a manner that all the 4 selected yeasts almost had no spores at 42°C. The highest spore population was presented for *Saccharomyces cerevisiae* at 25°C. According Figure 7, a greater capacity for blastospore growth at pH~ 5 was observed for *Clavispora lusitaniae* strains. Data also indicated spores number were almost zero at pH 1.5 and 2 for the strains belonging *Clavispora lusitaniae*, *Saccharomyces cerevisiae* and *Metschnikowia* genus. However, the spore population of *Candida albicans* isolates gradually increased at pH~1.5, pH~ 2 and 3 but reduced at pH~ 5.

Table 2. Sequencing outcomes for Wb, Wc, Kc and Vc isolates

Isolates name	Sequence	Species name
Wb	AGGAATTTGGCTTAATTGCGCCACATGTGTTTTTCTTTGAACAAACTTGCTTTGG CGGTGGGCCCACCTGCCGCCAGAGGTCTAAACTTACAACCAATTTTTTATCAAC TTGTCACACCAGATTATTACTTAATAGTCAAAACTTTCAAGAACGGATCTCTTGG TTCTCCCATCAGGGAAAAACGCGCGAAATGCGATACTTAATATGAATTGCAGAA ATTCAGAAATCATCAAATCTTTGAACGCGATAGGCGCCCTCTGGTATTCCGGAG GCATGCCTGTTTAAAGGACTTTTCCCCTGAAC	*Candida albicans*
Wd	CTAAGATTATACACTTTGCATTTGCGACAAAAAAATAAATTTTTTTATTCGAATC ATTTAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA CGCAGCGAATTGCGATACGTAGTATGACTTGCAGACGTGAATCATCGAATCTTT GAACCGCATTGGGCCCTCGAGGCATTCCTCGAGGCATGCCTGTTGGGCGTCCCT CCCCCTCAAACCCCGAAAGGCGTGGTCCGAATATCATTCGCGCTGTCAACACGA AAATTATTTTTTTTTCCCCTCGGGAAGGGAGAATTCTTTTTAAGGTCCTCACGGG GGAAGAAACCAGGAG	*Clavispora lusitaniae*
Kc	AAGTTATAGGACGTCCACTTAACTTGGAGTCCGAACTCTCACTTTCTAACCCTGT GCACTTGTTTGGGATAGTAACTCTCGCAAGAGAGCGAACTCCTATTCACTTATA AACACAAAGTCTATGAATGTATTAAATTTTATAACAAAATAAAACTTTCAACAA CGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCA TGGTATTCCGTGGAGCATGCCTGTTTGAGTGTCATGAATACTTCAACCCTCCTCT TTCTAATGATTGAAGAGGTGTTTGGTTTCTGAGCGCTGCTGGCCTTTACGGTCTA GCTCGTTCGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTTGGCGTA ATAGACTATTCGCTGAGGAATTCTAGTCTTCGGACTAGAGCCGGGTTGGGTTAA AGGAAGCTTCTAATCAGAATGTCTACATTTTAAGATTAGATCCTCAAATCAGGT AGGACTACCCGCTGAACTTAGCATATCCAATTTAGA GCCGGGAGGA	*Metschnikowia sp.*
Vc	CAAAATTTAATATTTTGAAAATGGATTTTTTTGTTTTGGCAAGAGCATGAGAGCT TTTACTGGGCAAGAAGACAAGAGATGGAGAGTCCAGCCGGGCCTGCGCTTAAG TGCGCGGTCTTGCTAGGCTTGTAAGTTTCTTTCTTGCTATTCCAAACGGTGAGAG ATTTCTGTGCTTTTGTTATAGGACAATTAAAACCGTTTCAATACAACACACTGTG GAGTTTTCATATCTTTGCAACTTTTTCTTTGG	*Saccharomyces cervisiae*

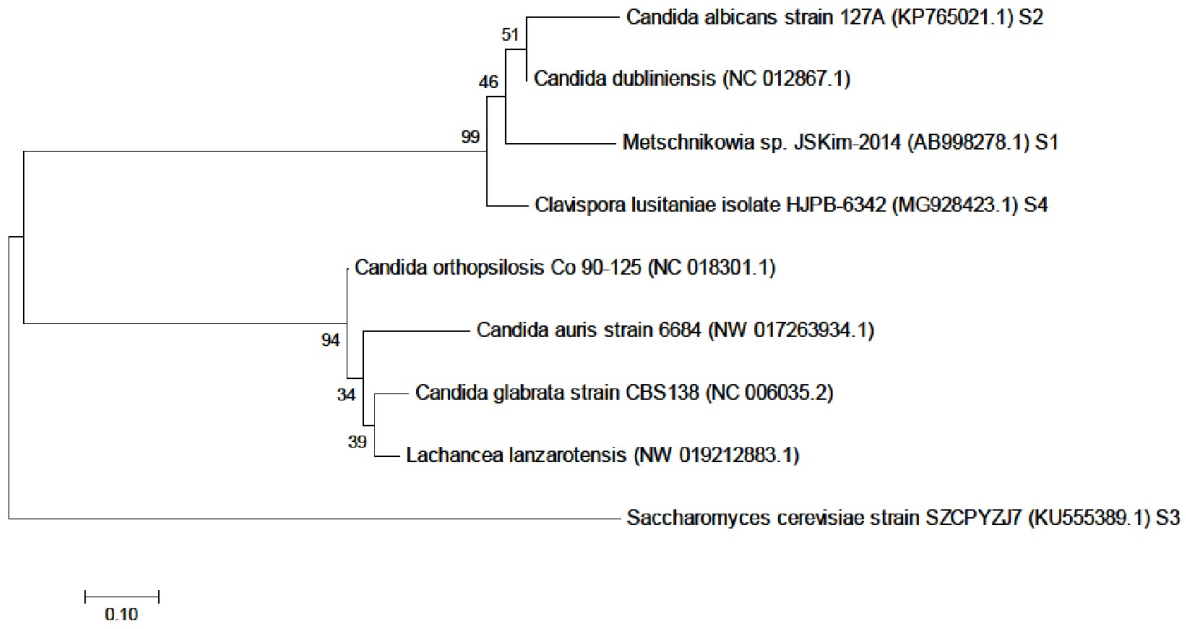

Figure 1

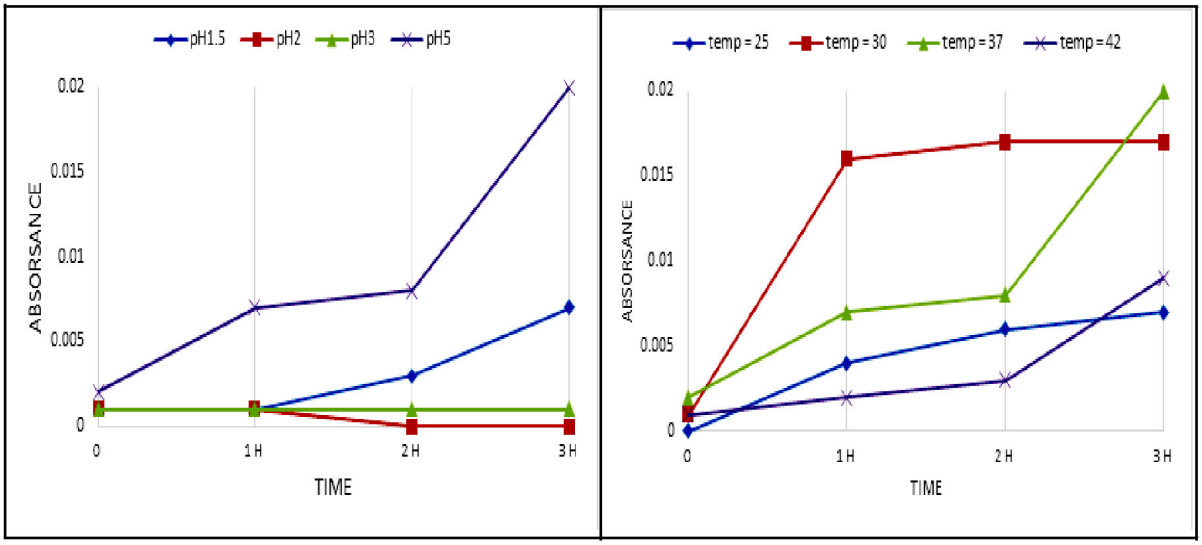

Figure 2

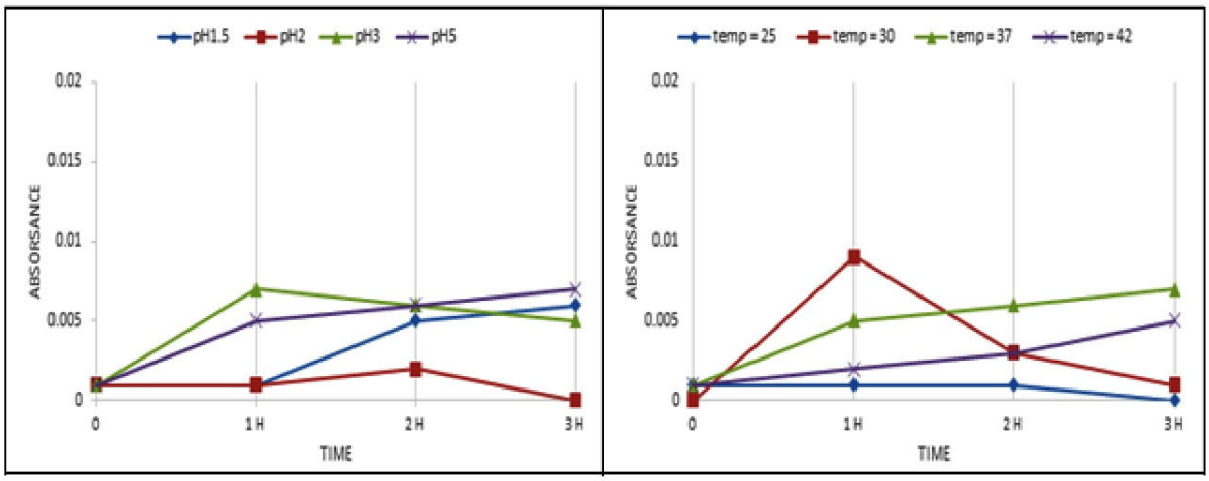

Figure 3

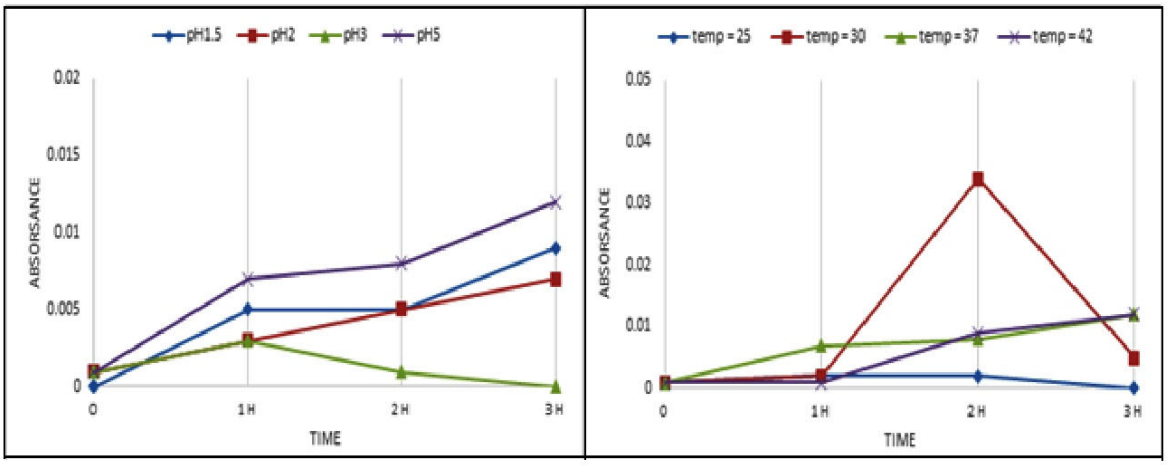

Figure 4

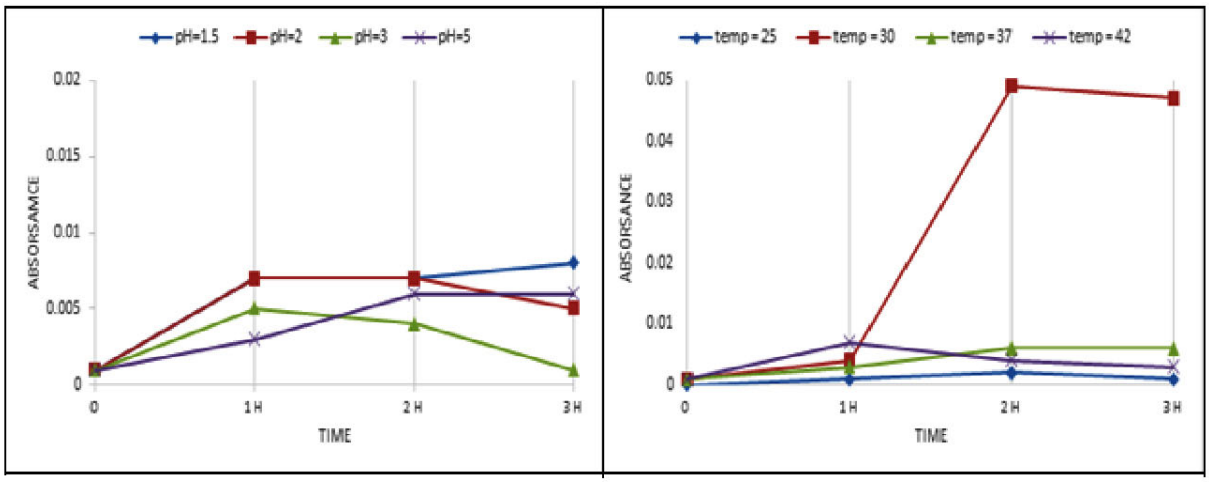

Figure 5

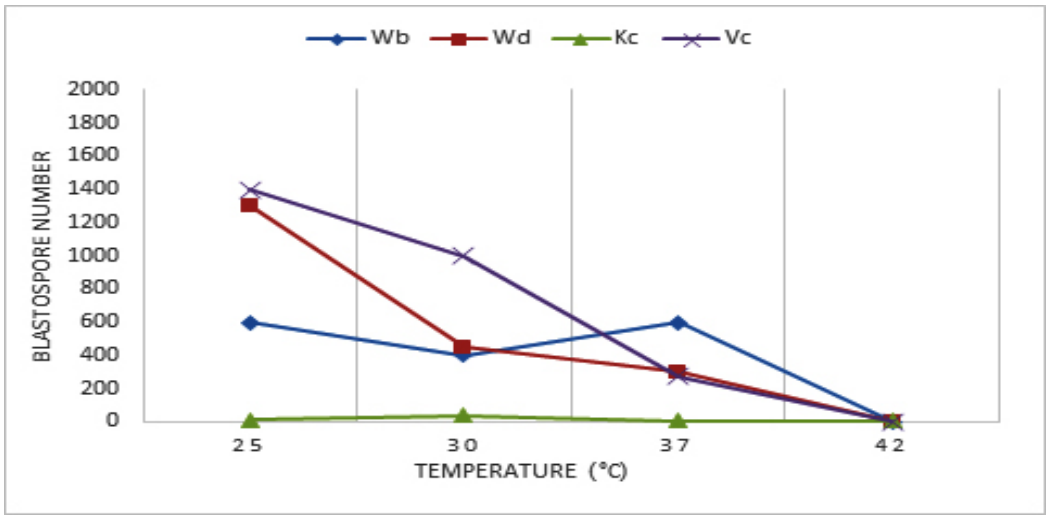

Figure 6

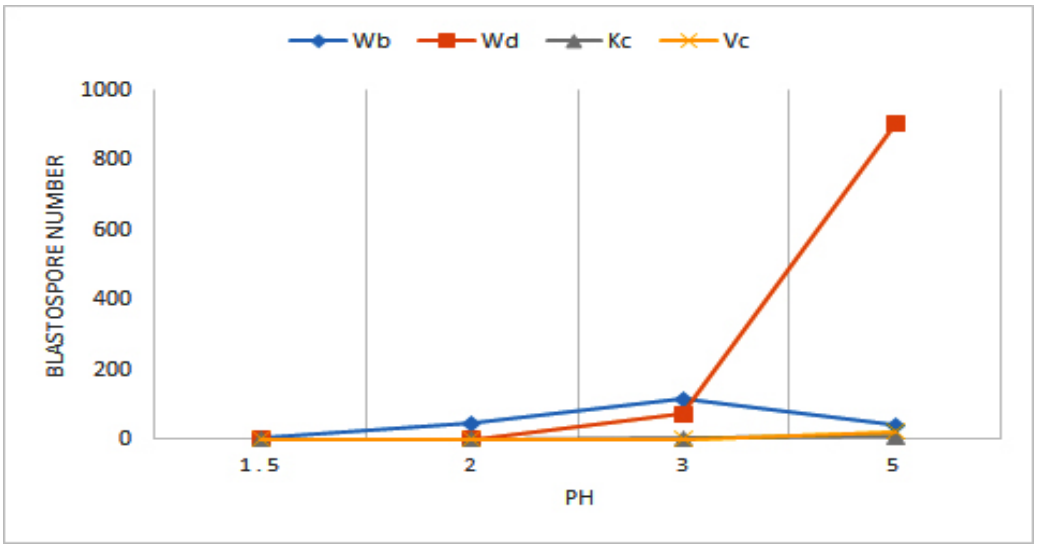

Figure 7

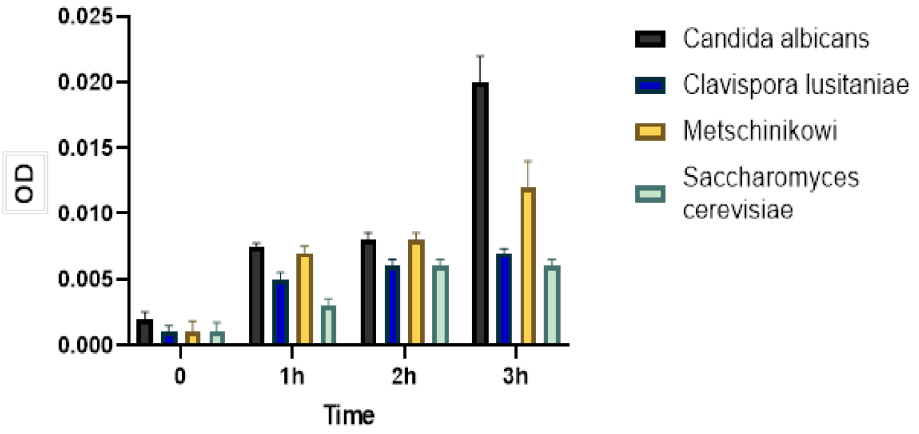

Figure 8

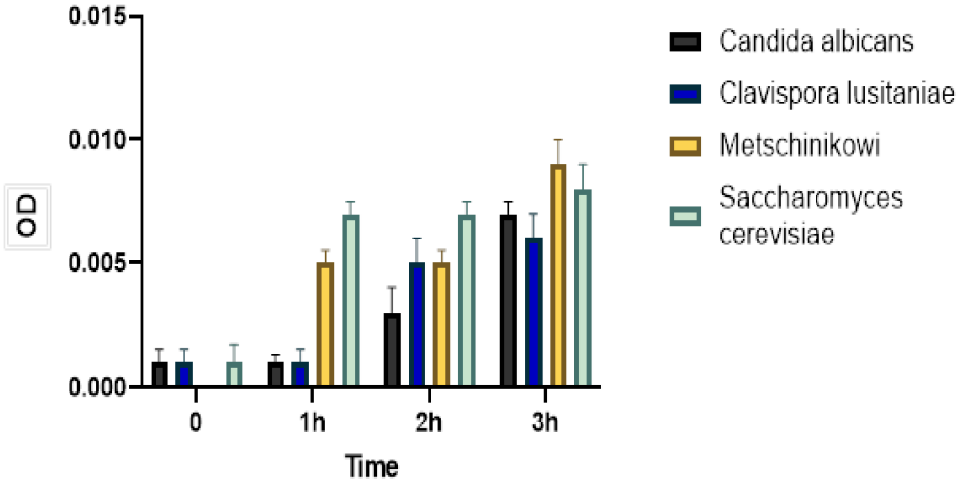

Figure 9

Figures 8 and 9 indicate, the growth of *Candida albicans*, *clavispora lusitaniae*, *Metschinikowi* and Saccharomyces cerevisiae isolate at 37°C and PH~1. 5 (human body condition, respectively.

According Figure 8, the growth of *Candida albicans* strains had significantly increased at 37°C after 3 hours compared to development of *clavispora lusitaniae* and *Metschinikowi* species. After both 1 and 3hours incubation of *Candida albicans* and *Metschinikowi* isolates at 37°C, an increase in growth was detected in comparison with *Saccharomyces cerevisiae* strains after same times ($P<0.05$). There was also no significant difference between viability of *Metschinikowi* and *Saccharomyces cerevisiae* strains versus viability of *clavispora lusitaniae* after both 1 and 2 hours at 37° C. However, after the incubation period of *Metschinikowi* isolates at 37°C for 3 hours, the growth of mentioned strains was remarkably increased compared to *Clavispora lusitaniae* strains ($P<0.05$).

According Figure 9, after 1, 2 and 3 hours, there was an increase in the growth of *Saccharomyces cerevisiae* strains at pH~1.5 compared to *Candida albicans* strains. The development of *Metschinikowi* strains at pH~1.5 was observed to be enhanced in comparison with candida species after 1 and 3 hours ($P<0.05$). However, analyses of variance showed no significant change in Metschinikowi strains growth at pH~1.5 compared to Candida group after 2 hours. The growth of *clavispora lusitaniae* isolates did not show differences after

1 and 3 hours compared to Candida strains, which it can considered significant at pH~1.5 after 2 hours ($P<0.05$). Exposure of *Saccharomyces cerevisiae* strains to acidic pH (pH ~1.5) after 1 and 2 hours led to significant increase in viability of Saccharomyces cerevisiae isolates in comparison with *Metschinikowi* strains ($P<0.05$).

4. Discussion

Over the past few decades, yeasts have been suggested as a useful microorganism which could be applied to probiotic production. This has made it possible that scientists look for the yeasts from dairy and non-dairy products to find procedures for screening these isolations and following up assays for particular probiotic specifications (Chaucheyras-Durand and Durand, 2010; Sen and Mansell, 2020). Variety of probiotic products from different sources is growing in the markets of Iran. Therefore, it should be noted that the use of Iranian native microorganisms in products and diets has been several advantages for Iranian population, because these microorganisms have adapted to the digestive system over time and can show better effects in improving gastrointestinal system efficacy (Chaucheyras-Durand and Durand, 2010; de Vries et al., 2020; Larypoor et al., 2020; Sen and Mansell, 2020).

In this regard, a huge amount of data indicated diversity of yeasts has been applied in food industry and healthy products due to their beneficial effects such as declining risk of lactic acid acidosis, boosting fiber digestibility, secretion of the digestive enzymes and modifying animal efficiency by boosting their growth rate and enhancing diary and meat production(Chaucheyras-Durand et al., 2012; Elghandour et al., 2020). In the current survey, we detected yeast strains with probiotic capability through biochemical and molecular techniques. In view of the fact that yeast detection methods, which are established on morphological, biochemical and physiological properties may cause unconfirmed classification, these procedures are rarely replicable (Orbera-Ratón, 2004). Therefore, molecular techniques are almost regarded as more trustworthy ones. In the present study, we proved our biochemical-based detection of the yeast isolates with DNA sequencing and phylogenetic tree. Among 30 isolate with different origins, four yeast isolates Wb, Wd, Kc and Vc were detected, followed by biochemical tests, phylogenetic tree analysis and DNA sequencing as *Candida albicans, Clavispora lusitaniae, Metschnikowia* and *Saccharomyces cerevisiae,* respectively. Various Analysis of ascomycetous taxonomic using maximum likelihood indicated the monophyletic origin of the Hemiascomycetes (Saccharomycetales) which including *Candida albicans*, *Clavispora lusitaniae*, *Metschnikowia* and *Saccharomyces cerevisiae* species (Diezmann et al., 2004).

To amplify the potential usage of yeast species as probiotics, we surveyed the principal probiotic properties of yeast strains originated from marine environments and dairy products. Modifying the characteristic states of the gastrointestinal tract is a pivotal feature for viewing a microorganism as probiotic (Menezes et al., 2020). For that reason, the isolates were investigated for tolerance against dissimilar temperatures, high dose of bile salt and acidic pH. Our result indicated that *Candida albicans* and *Metschnikowia* isolates could significantly grow at 37°C after three hours and tolerate bile salts. *Candida albicans*, *Metschnikowia* and *Saccharomyces cerevisiae* species also could proliferate at pH~1. 5, however the best pH for growth of *Clavispora lusitaniae* was a 5. This examination focus attention on that *Candida albicans* and *Metschnikowia* isolates could carry out duty as effective probiotic candidates. Several reports showed marvelous probiotic features of few yeast strains such as *Metschnikowia ziziphicola* and *Saccharomyces cerevisiae* species, albeit these probiotic talents were severely strained-dependent (Agarbati et al., 2020). In contrast with our findings which show *Saccharomyces cerevisiae* could not serve as appropriate prebiotic, various studies revealed that *Saccharomyces cerevisiae* var. boulardii is the only yeast usable for human application as probiotics (Kunyeit et al., 2020). According to DNA sequencing analysis in YILDIRAN and et al study, all isolates from commercial yields and the natural environment were characterized as Saccharomyces cerevisiae, *Candida coppola*, *Candida guidance, Clavispora lusitaniae, Hanseniaspora appointee, Hanseniaspora uvarum, Kazakhstan Bovina, Kluyveromyces marxianus, Metschnikowia. pulcherrima, Metschnikowia* sp, *Meyerozyma carribbica*,

Pichia kluyveri and *Wickerhamomyces anomalus* and were discovered to be resistant to simulated gastric juice at pH~ 2.5 for 2 h and could proliferate at both 30 and 37 °C (Yildiran et al., 2019). In this regard, a few studies have been performed on the probiotic properties of Candida strains and this is the first study that presents the probiotic features of the pathogenic *Candida albicans* strain.

Several yeasts including *Saccharomyces cerevisiae* and *Metschnikowia* have been applied as probiotics to inhibit or cure various infectious and inflammatory diseases (Saber et al., 2017). The favorable efficacies of yeasts as probiotics are due to concurrent operation of some mechanisms such as compilation of various features of systemic immune responses, catching pathogenic factors of bacterial cells on yeast surface, and preservation of intestinal epithelium totality. The mentioned mechanisms are considered to be in charge for a decline of inflammatory procedure, bacterial translocation and intestinal permeability noticed during infectious and inflammatory diseases (Angrand et al., 2019).

The desirable temperature and PH for the growth of yeasts as probiotics in human body and survive in stomach conditions are in the range of 37–43°C and 1.5-3, respectively (Hossain et al., 2020). We demonstrated candida albicans and Metschnikowia isolates were the most resistant strains in human body at temperature equal to 37°C and gastric PH equal to 1.5 between other isolates. These isolates are Iranian native strains, economical and easily accessible, which these features add further importance to the use of our isolates as pprobiotics.

In coclusion, considering that *Candida albicans* is the most common human fungal pathogen. It is recommended that only Metschnikowia, which were the most resistant strains in human body at temperature equal to 37°C and gastric pH equal to 1.5 , should be used in the food industry and food supplements. *Metschnikowia* isolates may consider as probiotics due to their capability to grow at 37 ° C and relative resistance at pH~1.5 (similar to stomach pH) as well as resistance to bile salts. Subsequent survey is required to perspicuously define the yeasts, their human health protection and the concentrations, following the WHO criteria and EFSA recommendations.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Authors Contribution

M.Larypoor developed the original idea and the protocol, abstracted and analyzed data, Study concept and design, edit the manuscript, and critical revision of the manuscript for important intellectual content and is guarantor. Hedieh Abolghasemi and Farzaneh Hosseini contributed to the development of the protocol, abstracted data, and prepared the manuscript.

Refereces

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