Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 26, No. (1) : 2024 : 120-129

ISSN-0972-3005

DOI No.: http://doi.org/10.53550/AJMBES.2024.v26i01.022

IN VITRO ANTI-MICROBIAL AND PROBIOTIC CHARACTERIZATION OF KILLER YEAST ISOLATED FROM *CITRUS AURANTIFOLIA*

SRIVIDYA D., SEEMA J. PATEL[1]* AND ANIL H. SHYAM MOHAN[2]

[1]Department of Studies in Biotechnology, Davangere University, Shivagangothri, Davangere 577 007, Karnataka, India
[2]Department of Biotechnology, Dayananda Sagar College of Engineering, Kumaraswamy Layout, Shavige Malleswara Hills, Bengaluru, Karnataka, India

(Received 30 October, 2023; Accepted 17 December, 2023)

Key words: *Killer yeast, Kodamaea ohmeri, Citrus aurantifolia, Probiotic, Mycocin, Biocontrol.*

Abstract– Killer yeast possesses a wide range of anti-microbial virtues and secretes a glycoprotein called mycocin. They are commercially used as starters in fermentation, as anti-fungal in the agriculture sector and as therapeutic mycocin syrups for combating pathogenic microorganisms. A killer strain of *Kodamaea ohmeri* (C3 isolate) was isolated from *Citrus aurantifolia* and characterized by traditional and molecular means. The strain does not produce a germ tube and doesn't exhibit ureolytic activity. The C3 isolate demonstrated aggregation (45±5 % in 5 h), co-aggregation with *Candida albicans* (71.96±1.68 % in 24 h) and *Escherichia coli* (32.78±0.54 % in 24 h). The killer strain is tolerant to gastric and intestinal juices which are made of lytic enzymes at extreme pH environments. *K. ohmeri* also showed *in vitro* antagonism against the phytopathogen *P. citrinum.* Altogether the isolated killer yeast *Kodamaea ohmeri* is safe to use as a probiotic after the conduction of clinical trials.

INTRODUCTION

The break through innovation of toxin secretion from *Saccharomyces cerevisiae* in the early 1960s (Bevan and Makower, 1963) led to the uncovering of killer yeasts and their broad-spectrum biocontrol properties. Killer yeasts secrete a proteinaceous substance called 'killer toxin' or 'mycocin'. The killer yeasts and mycocins are generally used as food spoilage protectants, starter culture in winemaking, agricultural fungicides and biocontrol agents (Zagorc *et al.,* 2001; Mannazzu *et al.,* 2019; Schaffrath *et al.,* 2018). Unlike antibiotics, mycocins do not develop resistance, which makes them an attractive alternative.

In the present study, the killer yeast is isolated from *Citrus aurantifolia*, a tropical fruit grown in the Middle East and southern parts of Asia. Karnataka is the third largest exporter of fresh fruits of *C. aurantifolia* along with other species of lemon and their value-added products (Action Plan/Report for Lemon - Karnataka, 2019). The fruits are used in local cuisines to prepare pickles, juices, flavored rice etc and are known to possess therapeutic properties. Therefore, we aptly chose it to isolate killer yeasts.

This is the first report on the isolation of killer yeasts from *Citrus aurantifolia.* The isolated killer yeast was identified as *Kodamaea ohmeri,* an ascomycete belonging to *Debaryomycetaceae* (Lachance and Kurtzman, 2011). It is a commonly used yeast in wineries and distilleries for fermentation. In the present study, the killer yeast *Kodamaea ohmeri* was characterized with microscopic, morphologic, biochemical and molecular examinations. The important probiotic attributes such as aggregation, co-aggregation, gastrointestinal tolerance, stability at physiological temperature, and antimicrobial activity (Binda *et al.,* 2020) were explored.

MATERIALS AND METHODS

Media, chemicals and strains

The culture media ingredients and dextrose were

([1]*Associate Professor)
ORCID No. 000000025310153X

procured from Himedia Laboratories Pvt. Limited, India. The killer sensitive (MTCC 473) strain of *Saccharomyces cerevisiae* (MTCC 183) and *Penicillium citrinum* (MTCC 2547) was procured from Microbial type culture collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Primers were purchased from Integrated DNA Technologies (IDT), Singapore. The carbohydrate-impregnated circular rings were purchased from Micro Express, India. The Taq polymerase and DNA ladder were procured from Takara, India. All other media and chemicals were purchased from Himedia Laboratories Pvt. Limited, India unless specifically mentioned.

Isolation of yeasts from *Citrus aurantifolia*

The key lime fruit or *Citrus aurantifolia* locally called 'Heralekai/ Kanchigai' was purchased from the local bazaars at Davangere, Karnataka, India. The cut fruits of key lime (approx. 25g) were added into sterile flasks containing 200 ml of 5%, 10% and 15% sodium chloride solution respectively. The flasks were maintained for ten days at 28 °C without shaking. After incubation, the samples from the respective flasks were aliquoted and serially diluted for 10^4 and 10^5 times. Each of the serially diluted samples was plated on a YPD agar medium consisting of 1% Yeast extract, 2% peptone, 2% dextrose and 1.5% agar. The growth of yeast colonies on YPD plates was analyzed after two days of incubation at 28 °C.

Killer activity determination of the key lime isolates

The killer activity of the key lime isolates was checked using an agar diffusion assay as mentioned in the killer-zone experiment (Waema *et al*., 2009). Sterile plates of YPD agar containing 0.0025% methylene blue (pH 4.5 adjusted with citrate-phosphate buffer) were prepared. The plates were swabbed with killer-sensitive *S. cerevisiae* (MTCC 473) and under sterile conditions. Each of the key lime isolates was spotted on the plate, air-dried and incubated at 28 °C for 48-72 h. The plates were observed for the presence of a killer zone surrounding the key lime isolates.

Identification of the killer yeast

The selected killer yeast was characterized using microscopic, morphologic, biochemical and molecular analyses.

Morphological and microscopic attributes of the killer yeast

The selected killer yeast was inoculated to YPD broth and agar. The cultures were grown for 24 h at 28 °C. The morphological characteristics were observed and recorded. Microscopic characteristics of the selected isolate were recorded using a Zeiss Primo Star 3 bright field microscope attached to a camera. The isolate was examined for hyphae formation by Dalmau plate test (Beena, 2020) and ascosporeforations on Malt extract agar (MEA) plates (Suh *et al*., 2008).

The germ tube formation was assessed by the standard procedure provided in the Indian Council of Medical Research (ICMR) guidelines on fungal identification. The freshly grown C3 killer isolate was washed with sterile water and resuspended in sterile saline to achieve 0.5 OD/ ml. A freshly drawn human blood sample is centrifuged at 1500 rpm to separate serum. To 1 ml of the serum, 0.1 ml of the earlier prepared C3 killer yeast cells were mixed and placed in a water bath set to 37 °C for 3 h. A drop of this suspension was then observed through the microscope for germ tube formation.

Biochemical tests for the isolates

The biochemical tests were performed to verify the assimilation of different nitrogen and carbon sources. To test the nitrogen assimilation, yeast carbon base (YCB) media was blended with corresponding nitrogen sources. Briefly, the killer yeast was cultured in YPD broth for a day at 28 °C. An aliquot of cells (1 OD/ ml) was washed with sterile water resuspended in 1 mL of saline and incubated for 3 h at 28 °C. These starved cells (50 µl) were spotted on the agar plates of YCB containing the respective nitrogen source. The plates were incubated at 28 °C for 48 h and the results were recorded.

To test the assimilation and fermentation of carbon source, the 24 h grown killer yeast isolate was subjected to starvation for 3 h and later 0.1 OD of cells were inoculated to sterile peptone water plus 0.01 % phenol red containing respective carbohydrate discs (dextrose, galactose, maltose, lactose, trehalose, cellobiose and raffinose). The Durham tubes were inserted to check the fermentation ability of the yeast. A control was maintained with no carbohydrate discs added to it. The tubes were incubated at 28 °C for 7 days and the results were recorded.

The killer yeast isolate was also checked for the presence of urease enzyme. The test isolate was inoculated to Stuart's broth (Yeast extract - 0.01 %; Potassium phosphate, monobasic - 0.91 %; Potassium phosphate, dibasic - 0.95 %; urea - 2 %; phenol red - 0.01 %) and placed in the incubator for 4 h at 37 °C and the colour change was observed. *Candida albicans* (MTCC 183) was used as a positive control and sterile Stuart's broth was used as a negative control for the assay.

Molecular tests for the isolated killer yeast

The killer yeast was subjected to genomic DNA extraction and purification (Heintz and Gong, 2020). The commercially available NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') primers which amplify nuclear ribosomal small subunit 18s rDNA were used for PCR amplification with Ampliqon master mix. The PCR parameters were set at 94 °C for 8 min, followed by 30 repetitions of denaturation at 94 °C for 0.5 min, annealing at 56 °C for 0.5 min and extension at 72 °C for 1 min. The final extension was at 72 °C for 10 min. The amplified product was run on 1 % agarose gel, excised and subjected to Sanger sequencing (Men *et al.,* 2008; Malapelle *et al.,* 2012). The obtained results were aligned to the homologous sequences of the standard databases using the Basic Local Alignment Search Tool (BLAST) algorithm from the National Centre for Biotechnology Information (NCBI). The obtained result from BLAST was substantiated by multiple sequence alignment and phylogenetic tree reconstruction. The homologous sequences obtained from BLAST were collected and subjected to multiple sequence alignment using Clustal W hosted by Kyoto University Bioinformatics Center. The phylogenetic tree of the multiple sequence alignments by the neighbour-joining method was reconstructed using MEGA X software. The confirmed sequence of the killer isolate was then deposited into NCBI Genbank.

Extraction of mycocin and killer activity determination

The culture of isolated killer yeast from *Citrus aurantifolia* was added into 10 ml of YPD broth and incubated at 25 °C overnight with 160 rpm agitation. This pre-inoculum was inoculated into 100 ml of killer toxin production (KTP) media (Bajaj *et al*., 2013) consisting of 1% yeast extract, 1% peptone, 1% dextrose, 2% sodium chloride and 5% glycerol. The culture flask was placed in the shaker incubator set to 25 °C and 140 rpm agitation for 72h. The fully grown culture was centrifuged at 5000 rpm for 25 min and 5 °C. The separated culture broth was filtered using a 0.22-micron sterile filter and proceeded with ammonium sulphate precipitation (50%). The obtained precipitate was dialyzed against 0.05 M citrate–phosphate buffer (pH 4.4) containing 5% glycerol and used as a crude extract in the determination of killer activity as mentioned in the earlier section.

Characterization of probiotic traits in the isolated killer yeast

Growth assessment of C3 killer yeast at physiological temperature

The C3 killer yeast was streaked on YPD agar plates and incubated at 28 °C and 37 °C for 48 h. The growth at 37 °C, which is the physiological temperature of the human body was compared with the growth at 28 °C which is the ambient temperature for yeast growth.

Auto-aggregation and Co-aggregation capabilities of the killer yeast

Auto aggregation capabilities of the killer yeast were assayed using some modifications to the method mentioned by Wulan *et al.* (2021). The killer yeast isolate was grown in liquid YPD media at 25 °C in an incubator agitating at 100 rpm for 24 h. The freshly grown culture was pelleted at 4500 rpm for 10 min at 25 °C. The cell pellet was rinsed twice and resuspended in equal volume of Phosphate Buffered Saline (PBS), pH 7.4. The tubes were then placed in an incubator set to 37 °C for 24 h. The supernatant was carefully aliquoted at 0, 5, and 24 h of incubation. The absorbance of the supernatant was measured at A600 nm and the percentage of auto-aggregation was calculated as follows:

$$\%\ Aggregation = \left[1 - \left(\frac{A_t}{A_0}\right)\right] \times 100$$

Where A_t is the OD of 600nm at time t (5 and 24 h incubation), A_0 is the OD of 600 nm at time 0 h.

The co-aggregation assay was performedby co-culturing *Candida albicans* (MTCC 183) and *Escherichia coli* (MTCC 739) as per the protocol mentioned in Ladha and Jeevaratnam (2018). The killer yeast isolates C3 and *C. albicans* were cultured in liquid YPD media for 24 h at 25 ºC. *E. coli* was grown in a liquid nutrient broth medium for 24 h at

37 ºC. All the cultures were centrifuged in separate falcon tubesat 4500 rpm for 10 min at 25 °C. The pelleted cells were rinsed two times in PBS, pH 7.4 and resuspended in the same solution. Each culture was aliquoted (4 ml) into a fresh tube and incubated at 37 °C. To co-culture the pathogens with C3 killer yeast, 2 ml of pathogen (*C. albicans*/ *E. coli)* was mixed with 2 ml of C3 isolate suspension. All the cell suspensions were placed in the bench top incubator set to 37 °C. The absorbance value at 600 nm was measured at 0, 5 and 24 h for all the supernatants aliquoted from each tube. The co-aggregation percentage is calculated using the following formula.

$$\%\ Coaggregation = \left[\left(A_{pat} + A_{yeast}\right) - \frac{2(A_{mix})}{2(A_{pat} + A_{yeast})}\right] \times 100$$

where A_{pat} is the absorbance of the pathogen at 600 nm, A_{yeast} is the absorbance of the killer yeast isolate at 600 nm and A_{mix} is the absorbance of the mixed culture at 600 nm.

Gastrointestinal tolerance of the killer yeast

The foremost requirement for probiotic organisms for use in the health sector is their sustenance in the GI tract environment. To check this, the killer yeast was subjected to a sequential gastrointestinal tolerance study using an *in vitro* method (Lara-Hidalgo *et al.*, 2018). The killer yeast culture grown in YPD broth for 24 h was harvested by centrifugation. The cell pellet was washed with 0.9 % saline and mixed with simulated gastric juice (Pepsin 3g/l in PBS, pH adjusted to pH 2 with HCl). The suspension was incubated at 37 °C, 100 rpm for 1.5 h. An aliquot of this incubated cell suspension was serially diluted to 10^5 times and plated on YPD agar.

The remaining cell suspension treated with simulated gastric juice was again washed with 0.9% saline and resuspended in simulated intestinal juice (Pancreatin 1g/l and bile 3g/l were dissolved in PBS and adjusted to pH 8 with 1N NaOH). The incubation was continued at 37 °C, 100 rpm for 2.5 h. Cells were then serially diluted 10^5 times and plated on YPD agar. The percentage of cell viability was calculated as

$$Cell\ viability = \frac{CFU_f/mL}{CFU_i/mL} \times 100$$

Where CFU_f is the final colony-forming unit obtained after treatment with simulated gastric or simulated intestinal juice, and CFU_i is the colony-forming unit obtained initially before any treatment of cells.

Antifungal activity of the killer yeast *in vitro*

The antifungal activity of the isolated killer yeast was performed by dual culture assay. The fungus *Penicillium citrinum* (MTCC 2547) was revived on potato dextrose agar (PDA). The mycelia (1 sq. mm) from a freshly grown plate of *P. citrinum* were picked and placed on PDA plates marked as test and control. The killer yeast was grown for 72 h at 25 °C in KTP medium and 0.5 OD cells from this culture were streaked on either side, equidistant from the mycelial inoculation in the plates labeled as test. Both the plates were placed in the incubator set to 28 °C for 5-7 days and observed for antagonism.

RESULTS AND DISCUSSION

Citrus aurantifolia is a remunerative fruit grown in tropical and sub-tropical terrains of India. In this study, we have isolated a killer yeast from the whole fruit of *Citrus aurantifolia* available in local bazaars of Davangere district, Karnataka, India. The isolated killer yeast was characterized using morphological, biochemical and molecular tests. The killer yeast was also tested for *in vitro* antifungal activity. Further, the probiotic characteristic of the killer yeast was explored by verifying its aggregation – co-aggregation capabilities and gastrointestinal tolerance using *in vitro* assays. The details are described in the below paragraphs.

Isolation of yeasts from *Citrus aurantifolia*

Citrus aurantifolia is a sub-tropical fruit usually used in pickles and juices. It is found to possess anti-inflammatory, antibacterial, antifungal, anti-helminthic, and analgesic value (Chaudhari *et al.*, 2016). The fruits from the local bazaar were used to isolate the killer yeast. The saline suspensions of 5%, 10% and 15% with *C. aurantifolia* showed 25 × 10^6 CFU/ml colony counts altogether. The colonies having unique morphologies were identified and twenty-four different colonies were saved on the YPD agar plate to screen for the killer activity.

Killer zone assay for the determination of killer activity

The twenty-four isolates sourced from *C. aurantifolia* were subjected to killer zone assay. Of all the isolates, the C3 isolate was noted to inhibit the

sensitive *S. cerevisiae*. The inhibition was evident by an inhibition zone surrounding the C3 isolate and also the sensitive strain surrounding it was turned to blue because of the uptake of methylene blue dye present in the media (Fig. 1). The cationic dye methylene blue is reduced in the live cells, the dye colours only the dead cells (Kwolek-Mirek *et al*., 2014). Accordingly, the cells of the sensitive *S. cerevisiae* surrounding the C3 isolate were observed to be turned blue indicating the death of cells whereas in the control plate where no test isolate was spotted, the cells were seen to have a profuse growth. Therefore, C3 killer yeast was taken ahead for all further characterization.

Identification of the killer yeast

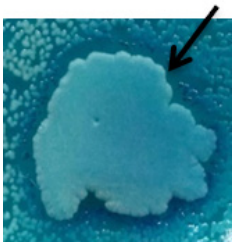
Morphological and microscopic attributes of the killer yeast

The colony characteristics of the C3 killer yeast on YPD agar were observed and documented (Fig. 2a). The cells appeared to be spherical, entire with raised elevation and butyrous texture. The microscopic observation of the C3 killer yeast culture showed oval-shaped cells through a 40 X objective lens (Fig. 2b). They sometimes appeared in aggregates. The aggregate formation capability helps yeasts in colonization, sexual reproduction and pathogenesis (Vallejo *et al*., 2013).

The killer yeast cells were streaked on MEA plates to allow the formation of ascospores. By definition, the phylum Ascomycota forms ascospores in the haploid phase, by meiotic cell division (Neiman, 2005). The microscopic observation demonstrates deliquescent ascospores seen 1-2 in number within the cells (Fig. 2d). The shapes are not recognized though, given the magnification and resolution of the bright field microscope are limited.

To test the formation of hyphae, the Dalmau method was followed wherein, the culture was inoculated on the YPD agar and a coverslip was introduced on the streak to create a local anaerobic environment (Mosch, 2002). The C3 isolate showed pseudo hyphae formation when observed through the microscope (Fig. 2e). This dimorphic nature of yeasts where it can form pseudo hypha or take yeast form is a key morphologic test in systematic yeast identification protocol. The dimorphic switching of the yeast also indicates the capability of invasiveness and adaptability to its surroundings (Mosch, 2002; Boyce and Andrianopoulos, 2015).

The formation of germ tube after incubation in human serum for 4 h at 37 °C indicates the presence of *Candida albicans*, a human pathogen. Germ tube test is an affordable presumptive assay for the distinction of *C. albicans* from other yeasts (Sheppard *et al*., 2008; Silva *et al*., 2012). Favourably, the C3



(a) (b)

Fig. 1. Killer-zone assay (a) C3 isolate showing killer zone around it (b) Control where the sensitive strain of *S. cerevisiae* has grown profusely.

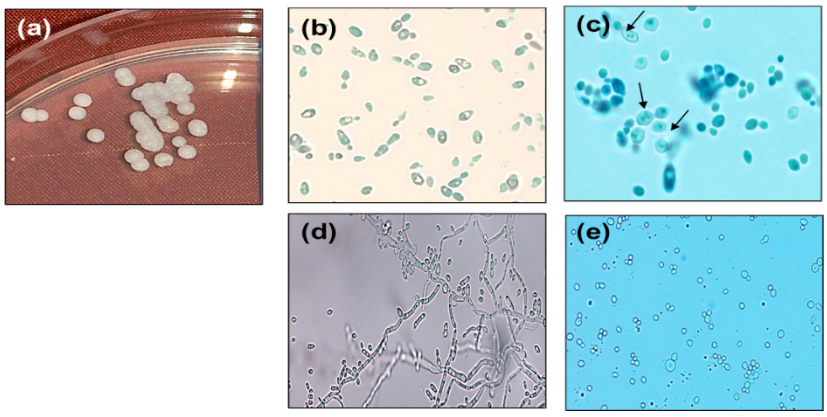


Fig. 2. Morphologic and microscopic observations (400x magnification) of C3 killer *yeast isolate* (a) Morphology of C3 on YPD plate (b) Microscopic field showing cells grown in YPD broth (c) Microscopic field showing ascospores (d) Microscopic field showing pseudohyphae formation (e) Microscopic field showing cells after treatment with human serum at 37 °C for 4 h.

killer yeast did not produce any germ tube (Fig. 2e) and therefore, it is *C. albicans* (a human pathogen) was ruled out. Nonetheless, necessary precautions were followed while handling the isolate.

Biochemical tests for the killer yeast

Biochemical tests provide a conventional means of yeast identification where the carbon and nitrogen assimilation are tested. In our study, we used three sources of nitrogen namely ammonium sulphate, potassium nitrate and sodium nitrite. The C3 killer yeast showed weak growth on media containing potassium nitrate and sodium nitrite. However, there was a luxurious growth on ammonium sulphate supplemented minimal media (Fig. 3a).

The carbohydrate assimilation and fermentation tests conducted for the C3 killer isolate showed both assimilation and fermentation capability for dextrose. The other carbohydrates could not be assimilated or fermented (Fig. 3b). The results of morphological and microscopic tests were correlated with molecular tests to identify the C3 killer yeast.

Urease is an important therapeutic drug target as there is no counterpart found in the human body. However, many pathogenic organisms contain urease enzyme, which functions to break down urea into ammonia and carbamic acid. This creates a local acidic environment which affects the host's physiological processes. Urease is also a known immune-elicitor and anti-urease antibodies are diagnosed in a few auto-immune diseases like rheumatoid arthritis, *Heliobacter pylori* infections, and atherosclerosis (Konieczna *et al.*, 2012; Rutherford, 2014). Therefore, it is an important test to exclude pathogenicity in probiotic microorganisms. In the present study, the C3 isolate did not demonstrate any colour change indicating there is no breakdown of urease and decrease in pH to cause acidity (Fig. 6b). Hene C3 killer yeast can be regarded as urease-negative and avirulent

Killer yeast identification by molecular analysis

The molecular tools available for organism identification have enhanced the magnitude of sequence databases to a large extent in recent years. In our study, Sanger sequencing of the conserved regions of 18s ribosomal DNA was performed for the killer yeast identification (Raja *et al.*, 2017). The extracted genomic DNA from the C3 isolate was run on 0.8% agarose gel. The band was intact indicating good-quality genomic DNA (Fig. 4a, Lane 1). The PCR amplification with NS1 and NS4 primers demonstrated a 1200 bp product (Fig. 4a, Lane 2). The PCR product was gel purified and sequenced by Sanger's protocol.

The obtained sequence of C3 was analysed using a blastn suite from NCBI, which demonstrated 100 % homology to partial sequences of 18s rDNA from *Kodamaea ohmeri* strains. A similar result was substantiated by the phylogenetic reconstruction using the neighbor-joining method (Fig. 4b). The neighbor-joining tree showed a nearer homology and a taxonomic relationship to the 18s rDNA sequences of *Kodamaea ohmeri* strains than to the uncultured marine fungus sequences deposited in the NCBI databank. The results correlated with morphologic and biochemical tests. They showcased that the obtained killer isolate C3 is *Kodamaea ohmeri.* Thus, the obtained sequence for the 18s rDNA region of *Kodamaea ohmeri* was deposited in the NCBI Genbank database with the accession number OP592257.1

Killer activity determination of the C3mycocin extracted from *Kodamaea ohmeri*

The killer zone assay performed with the crude extract of *Kodamaea ohmeri* mycocin (C3 mycocin)

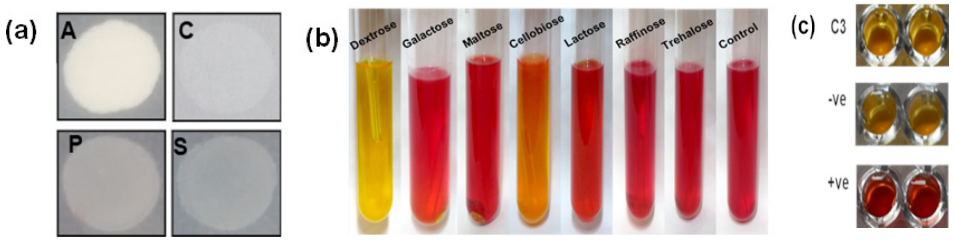


Fig. 3. Pictures showing results of biochemical tests (a) C3 killer yeast inoculated on minimal medium containing corresponding nitrogen source. A – Ammonium sulfate, P – Potassium nitrate, C – No nitrogen source, S – Sodium nitrite (b) C3 culture tubes showing assimilation and fermentation of the mentioned carbohydrate source (c) Results of urease test.

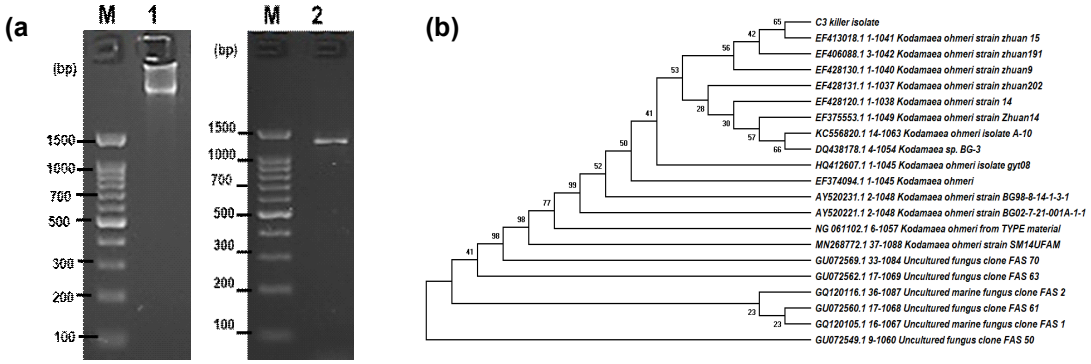


Fig. 4. Pictures showing results of molecular identification of C3 isolate (a) Agarose gel electrophoresis, Lane M – 100 bp DNA ladder, Lane 1 – Genomic DNA of C3 isolate, Lane 2 – PCR amplified product of NS1 and NS4 primers (b) Phylogenetic tree reconstructed for C3 isolate and its homologs by Neighbour-joining method using MEGA X.

demonstrated aninhibition zone of 8 mm (Fig. 5). The sterile media used as control did not show any activity. This is the first report of extraction of mycocinand killer activity from *Kodamaea* species. The killer yeasts *Pichia membranifaciens* (Santos *et al.*, 2009), *Pichia kudriavzevii* RY55 (Bajaj *et al.*, 2012), *Debaryomyces hansenii* DSMZ70238 (Al-Qaysi *et al.*, 2017), *Wickerhamomyces anomalus* (Comitini *et al.*, 2021) are some of the mentions of mycocin extraction and killer activity.

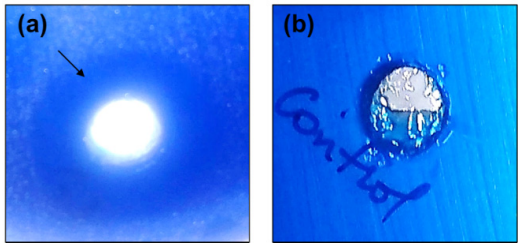


Fig. 5. Killer activity determination (a) Killer zone obtained from the activity of C3 mycocin (b) No inhibition zone seen in sterile media used as control.

Characterization of probiotic traits in the isolated killer yeast

Killer yeasts mainly find applications in biocontrol of food spoilage, starters in winemaking, antifungals and biotyping (Lowes *et al.*, 2000; Meinhardt and Klassen, 2009; Nascimento *et al.*, 2020). Though yeasts are heterogeneous and majorly dwell in fermented foods and fruits, only *Saccharomyces cerevisiae* is recognized as probiotic yeast (Gil-Rodríguez *et al.*, 2015). As killer yeasts are mostly used in food and agriculture applications, it is a need of the hour for the killer yeasts to be characterized for their probiotic properties.

One of the expected traits of a probiotic organism is the ability to survive at the physiological temperature (37 °C). The isolated *Kodamaea ohmeri* killer yeast showed profuse growth at both ambient (28 °C) and physiological temperatures (37 °C) (Fig. 6).

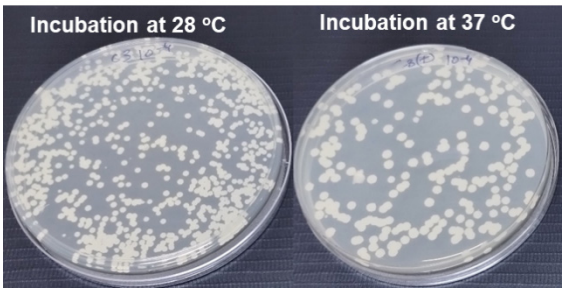


Fig. 6. Growth of C3 killer yeast plates after incubation at different temperatures for 48 h.

Aggregation and co-aggregation capabilities of the killer yeast

Adhesion and colonization are primary requirements in probiotic organisms. Auto-aggregation ability is suggestive of the adhering capacity of the organism to the epithelial cells in the physiological environment. The co-aggregation ability indicates that the probiotic organism will be able to de-colonize the pathogenic organism (Blagoeva *et al.*, 2014; Tuo *et al.*, 2013). Concerning the C3 killer isolate, the auto aggregation ability increased significantly with incubation time. However, after 24 h of evaluation, the aggregation ability was approximately 95-98% and no significant statistical difference was observed.

The co-aggregation of C3 killer yeast isolate against *C. albicans* and *E. coli* was studied. *C. albicans*

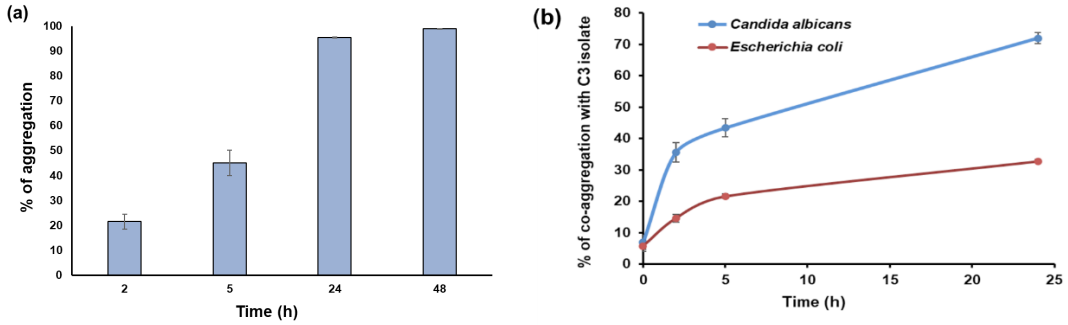


Fig. 7. Aggregation and co-aggregation capabilities of C3 killer yeast (a) Graph depicting the percentage of aggregation in C3 isolate over period of 48 h. (b) Graph depicting the co-aggregation ability of C3 isolate in mixed culture with *Candida albicans* and *Escherichia coli*.

is a commensal in the human gut, which acts as an opportunistic pathogen causing primary infection. The co-aggregation percentage was observed to be highest with *Candida albicans* compared to *Escherichia coli*. In *E. coli*, Type I fimbriae are a virulence factor responsible for adhesion to mucosal epithelia (Zalewska Piatek *et al.*, 2019). Since the cell wall of yeasts is rich in mannose, it helps in co-aggregation of pathogenic microorganisms and protect against colonization by pathogens.

Gastrointestinal tolerance assay

The bioactive compounds or probiotic organisms will be fit for human usage (orally as a drug) only when the molecule or the organism survives in the harsh gastrointestinal environment (Blagoeva *et al.*, 2014). So, we examined the gastrointestinal tolerance of the C3 killer isolate *in vitro* using simulated gastric and pancreatic juices. As evident from Table 1, C3 isolate was tolerant to the harsh pH and enzymatic environment of gastro-intestinal juices. Therefore, the killer isolate can be successfully used as a probiotic.

Anti-fungal activity of the killer isolate

Killer yeasts generally show an increased inhibitory activity against pathogenic fungi allowing them to be used as fungicides. The mechanism of killer activity is attributed to the presence of lytic enzymes such as ß-glucosidases or chitinases which can degrade the fungal cell wall components (Chanchaichaovivat *et al.*, 2007; da Cunha *et al.*, 2018; Díaz *et al.*, 2020).

Table 1. Cell viability percentage after incubation in simulated gastric and intestinal juices in vitro

% viability in simulated gastric juice	48.42 ± 0.38
% viability in the simulated intestinal juice	81.67 ± 0.52

Penicillium citrinum is a Phytopathogen which causes post-harvest decay in citrus fruits such as orange, lemon, etc. The C3 isolate was tested against *P. citrinum* using a dual culture assay. In the presence of C3 isolate, *P. citrinum* showed stunted growth with morphological differences. The radius of *P. citrinum* in the dual culture plate was estimated to be 12 mm whereas, in the control plate, the radius of growth was found to be approximately 26 mm (Fig. 8). This demonstrated the presence of a mycocin secreted to the media which acts upon cell wall degradation of *Penicillium citrinum.*

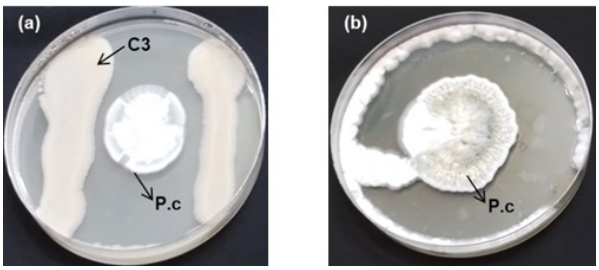


Fig. 8. Antagonism assay (a) Plate showing *Penicillium citrinum* (P.c) and C3 killer yeast isolate(b) Control plate where the luxurious growth of *Penicillium citrinum* can be visualized.

CONCLUSION

The rise of demand for natural antimicrobials in the food industry and therapeutics has led to the discovery of various biocontrol agents. Killer yeasts and their mycocins cater to a wide range of biological control from prokaryotes to eukaryotes, and pathogens to pests. Here we isolated a killer yeast, *Kodamaea ohmeri* from the tropical fruit *Citrus aurantifolia*. *K. ohmeri* was characterized using classical and molecular tools. The 18s rDNA partial sequence was deposited to the NCBI Genbank

database. The probiotic traits characterized revealed that the strain is avirulent, and has aggregating and co-aggregating properties. The killer yeast also possesses *in vitro* anti-fungal activity against the plant pathogen *P. citrinum*. Though *K. ohmeri* infections occur in immuno compromised patients, owing to the source of isolation, avirulent and probiotic properties, the C3 killer isolate can be regarded as a probiotic after human trials.

ACKNOWLEDGEMENTS

The authors acknowledge the facility given at the Institution of Excellence (IOE), Vijnana Bhavana, Manasagangothri, University of Mysore, Mysuru. Srividya D. is thankful to the Karnataka Science and Technology Promotion Society (KSTePs) for the award of the DST-Ph.D fellowship.

Conflict of interest

The authors declare that they have no conflict of interests.

REFERENCES

Al-Qaysi, S.A., Al-Haideri, H., Thabit, Z.A., Al-Kubaisy, W.H.A.A.R. and Ibrahim, J.A.A.R. 2017. Production, characterization, and antimicrobial activity of mycocin produced by *Debaryomyces hansenii* DSMZ70238. *International Journal of Microbiology.* 2017. doi:10.1155/2017/2605382

Bajaj, B.K., Raina, S. and Singh, S. 2013. Killer toxin from a novel killer yeast *Pichiakudriavzevii* RY55 with idiosyncratic antibacterial activity. *Journal of Basic Microbiology.* 53(8) : 645-656. doi: 10.1002/jobm.201200187

Beena, M.S. 2020. Characterization and virulence of *Candida* isolated from children with dental caries and its susceptibility to various antimicrobial agents. *In Dental Caries. Intech Open.* doi: 10.5772/intechopen.91912

Binda, S., Hill, C., Johansen, E., Obis, D., Pot, B., Sanders, M.E., Tremblay, A. and Ouwehand, A.C. 2020. Criteria to qualify microorganisms as "probiotic" in foods and dietary supplements. *Frontiers in Microbiology.* 11: 1662. https://doi.org/10.3389/fmicb.2020.01662

Blagoeva, G., Gotcheva, V. and Angelov, A. 2014. Aggregation and co-aggregation abilities of potentially probiotic amylolytic lactic acid bacteria strains. *In Proceedings of the ENGIHR Conference*.

Boyce, K.J. and Andrianopoulos, A. 2015. Fungal dimorphism: the switch from hyphae to yeast is a specialized morphogenetic adaptation allowing colonization of a host. *FEMS Microbiology Reviews.* 39(6): 797-811. https://doi.org/10.1093/femsre/fuv035

Chanchaichaovivat, A., Ruenwongsa, P. and Panijpan, B. 2007. Screening and identification of yeast strains from fruits and vegetables: Potential for biological control of postharvest chilli anthracnose (*Colletotrichum capsici*). *Biological Control.* 42: 326-335. https://doi.org/10.1016/j.biocontrol.2007.05.016

Chaudhari, S.Y., Ruknuddin, G. and Prajapati, P. 2016. Ethno medicinal values of Citrus genus: A review. *Medical Journal of Dr. DY Patil University.* 9(5): 560-565. doi: 10.4103/0975-2870.192146

Comitini, F., Agarbati, A., Canonico, L., Galli, E. and Ciani, M. 2020. Purification and characterization of WA18, a new mycocin produced by *Wickerhamomyces anomalus* active in wine against *Brettanomyces bruxellensis* spoilage yeasts. *Microorganisms*. 9(1): 56. https://doi.org/10.3390/microorganisms9010056

da Cunha, T., Ferraz, L.P., Wehr, P.P. and Kupper, K.C. 2018. Antifungal activity and action mechanisms of yeasts isolates from citrus against *Penicillium italicum*. *International Journal of Food Microbiology.* 276: 20-27.

Díaz, M.A., Pereyra, M.M., Picón-Montenegro, E., Meinhardt, F. and Dib, J.R. 2020. Killer yeasts for the biological control of postharvest fungal crop diseases. *Microorganisms.* 8(11): 1680. doi: 10.3390/microorganisms8111680

Gil-Rodríguez, A.M., Carrascosa, A.V. and Requena, T. 2015. Yeasts in foods and beverages: *In vitro* characterisation of probiotic traits. *LWT-Food Science and Technology*. 64(2): 1156-1162. doi: 10.1016/j.lwt.2015.07.042

Heintz, N. and Gong, S. 2020. Small-scale preparations of yeast DNA. *Cold Spring Harb Protoc*. 10: 430-432. doi: 10.1101/pdb.prot098152

Indian Council of Medical Research New Delhi, India, 2019. Standard operating procedures for fungal identification and detection of antifungal resistance, antimicrobial resistance surveillance and research network. New Delhi: Division of Publication and Information on behalf of the Secretary, 23-24.

Konieczna, I., Zarnowiec, P., Kwinkowski, M., Kolesinska, B., Fraczyk, J., Kaminski, Z. and Kaca, W. 2012. Bacterial urease and its role in long-lasting human diseases. *Current Protein and Peptide Science.* 13(8):789-806. doi: 10.2174/138920312804871094

Kwolek-Mirek, M. and Zadrag-Tecza, R. 2014. Comparison of methods used for assessing the viability and vitality of yeast cells. *FEMS Yeast Research*. 14(7): 1068-1079. doi: 10.1111/1567-1364.12202

Lachance, M.A., and Kurtzman, C.P. 2011. Kodamaea Y. Yamada, T. Suzuki, Matsuda and Mikata emend. Rosa, Lachance, Starmer, Barker, Bowles and Schlag-Edler (1999). In: *The Yeasts*: 483-490 Elsevier. https://doi.org/10.1016/B978-0-444-52149-1.00036-7

Lara-Hidalgo, C.E., Dorantes-Álvarez, L., Hernández-Sánchez, H., Santoyo-Tepole, F., Martínez-Torres, A., Villa-Tanaca, L. and Hernández-Rodríguez, C. 2019. Isolation of yeasts from guajillo pepper (*Capsicum*

annuum L.) fermentation and study of some probiotic characteristics. *Probiotics and Antimicrobial Proteins.* 11:748-764. doi: 10.1007/s12602-018-9415-x

Lowes, K.F., Shearman, C.A., Payne, J., MacKenzie, D., Archer, D.B., Merry, R.J. and Gasson, M.J. 2000. Prevention of yeast spoilage in feed and food by the yeast mycocin HMK. *Applied and Environmental Microbiology.* 66(3): 1066-1076. doi: 10.1128/aem.66.3.1066-1076.2000

Makower, M. and Bevan, E.A. 1963. The inheritance of a killer character in yeast (*Saccharomyces cerevisiae*).*In ProcIntCongr Genet* XI 1(202):235-44. doi:10.1017/S0016672300002743

Malapelle, U., Bellevicine, C., Salatiello, M., De Luca, C., Rispo, E., Riccio, P., Sparano, L., Stefano, A.D., Carlomagno, C., Maiello, F.M., Vita, G., Nappi, O. and Troncone, G. 2012. Sanger sequencing in routine KRAS testing: a review of 1720 cases from a pathologist's perspective. *Journal of Clinical Pathology.* 65(10): 940-944. doi: 10.1136/jclinpath-2012-200773

Mannazzu, I., Domizio, P., Carboni, G., Zara, S., Zara, G., Comitini, F., Budroni, M. and Ciani, M. 2019.Yeast killer toxins: From ecological significance to application. *Critical Reviews In Biotechnology.* 39(5): 603-617. doi: 10.1080/07388551.2019.1601679

Meinhardt, F. and Klassen, R. 2009. Yeast killer toxins: Fundamentals and applications. *Physiology and Genetics: Selected Basic and Applied Aspects.* 107-130. doi:10.1007/978-3-642-00286-1_6

Men, A.E., Wilson, P., Siemering, K. and Forrest, S. 2008. Sanger DNA sequencing. Next Generation Genome Sequencing: *Towards Personalized Medicine.* 1-11. doi:10.1002/9783527625130.ch1

Mösch, H.U. 2002. Pseudohyphal growth in yeast. *In Molecular Biology of Fungal Development*. 13-37. CRC Press.

Nascimento, B.L., Delabeneta, M.F., Rosseto, L.R.B., Junges, D.S., Paris, A.P., Persel, C. and Gandra, R.F. 2020. Yeast Mycocins: a great potential for application in health. *FEMS Yeast Research.* 20(3): foaa016. doi: 10.1093/femsyr/foaa016

Neiman, A.M. 2005. Ascospore formation in the yeast *Saccharomyces cerevisiae. Microbiology and Molecular Biology Reviews.* 69(4): 565-584. doi: 10.1128/MMBR.69.4.565-584.2005

Raja, H.A., Miller, A.N., Pearce, C.J. and Oberlies, N.H. 2017. Fungal identification using molecular tools: a primer for the natural products research community. *Journal of Natural Products.* 80(3): 756-770. https://doi.org/10.1021/acs.jnatprod.6b01085

Rutherford, J.C. 2014. The emerging role of urease as a general microbial virulence factor. *Plos Pathogens.* 10(5): e1004062. https://doi.org/10.1371/journal.ppat.1004062

Schaffrath, R., Meinhardt, F. and Klassen, R. 2018. Yeast killer toxins: Fundamentals and applications. Physiology and Genetics: *Selected Basic and Applied Aspects*. 87-118. doi:10.1007/978-3-319-71740-1_3

Sheppard, D.C., Locas, M.C., Restieri, C. and Laverdiere, M. 2008.Utility of the germ tube test for direct identification of *Candida albicans* from positive blood culture bottles. *Journal of Clinical Microbiology.* 46(10): 3508-3509. doi: 10.1128/JCM.01113-08

Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D.W. and Azeredo, J. 2012.*Candida glabrata, Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiology Reviews.* 36(2): 288-305. doi: 10.1111/j.1574-6976.2011.00278.x

Suh, S.O., Zhang, N., Nguyen, N., Gross, S. and Blackwell, M. 2008. *Lab Manual for Yeast Study.* Louisiana State University: Baton Rouge, LA, USA.

Enejoh, O.S., Ogunyemi, I.O., Bala, M.S., Oruene, I.S., Suleiman, M.M. and Ambali, S.F. 2015. Ethnomedical importance of *Citrus aurantifolia* (christm) swingle. *The Pharma Innovation.* 4(8, Part A):1.

Suntar, I., Khan, H., Patel, S., Celano, R. and Rastrelli, L. 2018. An overview on *Citrus aurantium* L.: Its functions as food ingredient and therapeutic agent. *Oxidative Medicine and Cellular Longevity.* 2018. doi: 10.1155/2018/7864269

Tuo, Y., Yu, H., Ai, L., Wu, Z., Guo, B. and Chen, W. 2013. Aggregation and adhesion properties of 22 *Lactobacillus* strains. *Journal of Dairy Science.* 96(7):4252-4257. doi: 10.3168/jds.2013-6547

Vallejo, J.A., Sánchez-Pérez, A., Martínez, J.P. and Villa, T.G. 2013. Cell aggregations in yeasts and their applications. *Applied Microbiology and Biotechnology.* 97:2305-2318. doi: 10.1007/s00253-013-4735-y

Visvesvaraya Trade Promotion Centre (VTPC), Karnataka. (n.d.). Action Plan/Report for Lemon - Karnataka.https://vtpc.karnataka.gov.in/storage/pdf-files/Lemon.pdf

Waema, S., Maneesri, J. and Masniyom, P. 2009. Isolation and identification of killer yeast from fermented vegetables. *Asian Journal of Food and Agro-Industry.* 2:126-134.

Wulan, R., Astuti, R.I., Rukayadi, Y. and Meryandini, A. 2021. Evaluation of Indigenous *Pichiakudriavzevii* from cocoa fermentation for a probiotic candidate. *Biodiversitas Journal of Biological Diversity.* 22(3).doi: https://doi.org/10.13057/biodiv/d220331

Zagorc, T.,Maráz, A., Cadez, N., Jemec, K.P., Péter, G., Resnik, M., Nemaniè, J. and Raspor, P. 2001. Indigenous wine killer yeasts and their application as a starter culture in wine fermentation. *Food Microbiology.* 18(4): 441-451. doi:10.1006/fmic.2001.0422

Zalewska-Piatek, B.M. and Piatek, R.J. 2019. Alternative treatment approaches of urinary tract infections caused by uropathogenic *Escherichia coli* strains-Review. *Acta Biochimica Polonica.* 66(2):129-138. doi: 10.18388/abp.2018_2787