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CHARACTERIZATION OF PROBIOTIC POTENTIAL OF *PICHIA FERMENTANS* AND *PICHIA KUDRIAVZEVII* OBTAINED FROM *SURA*, A FERMENTED BEVERAGE OF NORTH WESTERN HIMALAYAN

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ABSTRACT

Traditional fermented beverages, such as *sura* from the northwestern Himalayas, are rich in microbial diversity and have potential probiotic properties. However, their probiotic potential remains largely unexplored. This study aims to isolate, characterize, and evaluate the probiotic potential of microbial strains from *sura*. Fifteen yeast isolates were obtained from *sura* and screened based on their morphological and biochemical properties. The two most promising isolates, identified through molecular techniques as *Pichia fermentans* Y77 and *Pichia kudriavzevii* Y136, were further analyzed for probiotic characteristics, including gastrointestinal (GI) tract tolerance, antibiotic susceptibility, hydrophobicity, autoaggregation, antibacterial activity, cholesterol assimilation, enzyme production, and safety assessment. Both isolates exhibited high survival rates under simulated GI conditions and demonstrated antibacterial activity against tested pathogens. They were resistant to vancomycin, penicillin, clindamycin, gentamicin, and ampicillin but susceptible to antifungal agents. *P. fermentans* Y77 displayed higher hydrophobicity and cholesterol assimilation (80.93 % with taurocholate) compared to *P. kudriavzevii* Y136. Both isolates produced exopolysaccharides and extracellular enzymes (amylase, protease, lipase, and β-galactosidase) while exhibiting no hemolytic activity, indicating their safety for consumption. The findings suggest that *P. fermentans* Y77 and *P. kudriavzevii* Y136 possess strong probiotic potential and could be utilized as starter cultures or adjuncts for the development of probiotic-based functional foods.

Keywords: North western Himalaya, *P. fermentans, P. kudriavzevii*, *sura*, yeast

INTRODUCTION

Due to the geographic and climatic characteristics of the northwestern Himalaya, fermented beverages have become an integral part of the culture and diet of the local communities. A wide variety of traditional alcoholic drinks—such as *daru, sura, chhang, lugri, chakti, angoori, chulli, aara,* and *chiang*—are commonly prepared and consumed by the tribal and rural populations of this region **(Joshi and Sandhu, 2000; Thakur *et al.*, 2004; Kanwar *et al.*, 2011; Joshi *et al.*, 2012)**. The quality and characteristics of these beverages differ across regions, depending on the type of raw materials, fermentation practices, containers used, fermentation duration, and the composition of chemical constituents and microflora in the final product **(Bassapa, 2002)**.These fermented beverages are prepared by locally available raw material like barley, rice, millet and fruits by using traditional inoculum. Such beverages are traditionally produced and consumed, holding significant cultural and religious value.

Sura, in particular, is a millet-based alcoholic beverage traditionally consumed during rituals and ceremonies including marriages, funerals, and seasonal festivals in regions like Barot, Chuwar, Lag, and Chota Bhangal in Mandi, Kullu, and Kangra districts of Himachal Pradesh **(Thakur *et al.*, 2004)**. It is prepared from finger millet (*Eleusine coracana*) through a two-stage fermentation process—initially through spontaneous fermentation, followed by the addition of *dheli*, a traditional starter culture made from a mixture of 36 herbs and roasted barley flour (*sattu*) that imparts flavor and enhances fermentation **(Joshi *et al.*, 2015; Savitri *et al.*, 2019)**. *Lactobacillus plantarum, Pediococcus pentosaceus, Enterococcus* spp., *Saccharomyces fibuligera, Pichia kudriavzevii* and *S. cerevisiae* are involved in *sura* fermentation **(Thakur *et al.*, 2015).**

Studies have shown that *sura* is rich in water-soluble B-complex vitamins, essential amino acids, and probiotic lactic acid bacteria, contributing to its nutritional and functional properties **(Thakur *et al.*, 2015; Asrani *et al.*, 2019)**. Furthermore, it has been claimed that the methanolic extract of *dhaeli* or *dheli*, starter culture of *sura* possesses antioxidant qualities, which enhance its overall functional properties and promote the metabolic health of the natives. During *sura* fermentation, **Thakur *et al.* (2015)** discovered that protein content increased, total carbohydrate and starch decreased, and reducing sugars, amylase and protease activity, and B vitamin levels increased as fermentation progressed.

Live microorganisms that when consumed provide positive effects to the consumers are referred as probiotics. Probiotics have a number of beneficial effects such as enhancing the nutritional value of product, lowering cholesterol levels, preventing gut infections and increasing immunity, preventing urinary tract infection and reducing cardiovascular diseases **(Zendeboodi *et al.*, 2020)**. Probiotics produce extracellular carbohydrates (exopolysaccharide) and different enzymes which can offer protection against the harsh environment of the gastrointestinal tract. For a microbial strain to qualify as probiotic, key criteria include tolerance to acidic and bile environments, the ability to adhere to and colonize the gastrointestinal tract, viability during storage and delivery, safety for human use, and the production of antimicrobial substances **(Goktas *et al.*, 2021)**.

Yeasts offer several advantages over bacteria as probiotics, including their natural resistance to antibiotics and the absence of antibiotic resistance gene transfer to pathogens. *Pichia fermentans* and *Pichia kudriavzevii* were associated with different fermented foods and beverages **(Beyene *et al.*, 2020; Lata *et al.*, 2022)**. Traditionally fermented beverages of the northwestern Himalayas, such as *sura*, have remained largely unexplored. Moreover, the ethnomedicinal knowledge and health-promoting attributes of such traditional beverages are under-documented in scientific literature.

In this context, the present study was undertaken to isolate and characterize yeast strains from *sura*, with the objective of evaluating their probiotic and technological properties. This work aims to fill the existing research gap by providing scientific validation of the probiotic potential of microbes found in *sura*, a culturally and nutritionally important beverage. The novelty of this study lies in the first-time reporting of the probiotic properties and functional enzyme production of *P. fermentans* Y77 and *P. kudriavzevii* Y136 isolated from *sura*. These isolates may be used as starter cultures or adjuncts in the development of functional, probiotic-rich foods rooted in traditional fermentation practices.

MATERIALS AND METHODS

Sample collection and yeast isolation

Traditional *sura* samples were procured from the Pangi region of Chamba district, Himachal Pradesh. Sterile vials were used for collection, and samples were maintained at 4 °C until analysis. For yeast isolation, 10 g of each sample was homogenized with 90 ml of sterile physiological saline (0.9% NaCl). The yeast isolation was done on Yeast Malt Agar medium using serial dilutions as discussed earlier **(Lata *et al.*, 2022)**. Plates were incubated at 30 °C for 24–48 h.

Physiological and technological characteristics of yeast isolates

Yeast isolates were cultured in YPD broth (HiMedia, India) to evaluate growth under various stress conditions. Tests included catalase and urease activity, growth at different temperatures (15, 37, and 45 °C), tolerance to different pH levels (2.5, 3.5, 8.5, and 9.5), and growth in the presence of ethanol (5%). Sugar assimilation patterns were determined using the API 20C AUX kit.

Molecular identification

Molecular characterization was carried out by Biologia Research India Pvt. Ltd. (New Delhi, India). The internal transcribed spacer (ITS) region was amplified using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). Sequences obtained were submitted to the GenBank database (Accession No. OL454637 and OL658841). Phylogenetic analysis was performed with MEGA 11 software using the neighbor-joining method.

Probiotic characterization

Survival under stressed gastrointestinal conditions

Tolerance to acidic conditions, bile salts, and lysozyme was evaluated following **Maragkoudakis *et al.* (2006)**. Survival was assessed at pH 2, 3, and 7; bile concentrations of 0.5%, 1%, and 2%; and lysozyme concentrations of 50, 100, and 150 µg/ml. Results were expressed as log cfu/ml.

Susceptibility of yeast to antibiotic and antifungal agents

The disc diffusion technique **(Turchi *et al*., 2013)** was used to examine sensitivity to various agents. Antifungals included ketoconazole (30 µg), clotrimazole (10 µg), itraconazole (30 µg), amphotericin B (50 µg), nystatin (50 µg), and fluconazole (10 µg). Antibiotics tested were vancomycin (30 µg), penicillin (10 U), erythromycin (15 µg), clindamycin (2 µg), and ampicillin (10 µg). Zones of inhibition were recorded after 24 h incubation at 30 °C.

Determination of cell surface characteristics

Hydrophobicity

Adhesion to hydrocarbons (n-hexadecane, xylene, and toluene) was determined according to **Rosenberg *et al.* (1980)**. Cultures grown in YPD broth were harvested, washed, and adjusted to OD600 = 0.8. Equal volumes of yeast suspension and hydrocarbon were mixed, vortexed for 5 min, and incubated at 37 °C for 1 h to allow phase separation. The aqueous phase was collected, OD measured, and hydrophobicity (%) calculated as:
[(A0−A)/A0]×100[(A0 - A)/A0] \times 100[(A0−A)/A0]×100
Where, A0 = absorbance before mixing, A = absorbance after mixing.

Autoaggregation

Autoaggregation was analyzed following **Collado *et al.* (2008)**. Absorbance was measured at 0, 3, and 24 h. Percentage aggregation was calculated as:
[1−(At/A0)]×100[1 - (At/A0)] \times 100[1−(At/A0)]×100
Where, At = absorbance at time t, A0 = absorbance at t = 0.

In vitro beneficial effects of probiotic yeast isolates

Antimicrobial activity

The antagonistic potential against different foodborne pathogens **(Lata *et al*., 2023)** was assessed by agar well diffusion **(Mishra and Prasad, 2005)**. Zones of inhibition were measured.

In-vitro cholesterol assimilation ability

According to **Liong and Shah (2005)**, o-phthalaldehyde was used to assimilate cholesterol by yeast isolates. A filter-sterilized cholesterol solution (70 µg/ml final concentration) prepared in 96% ethanol was added to YPD broth supplemented with 0.2% (w/v) bile salts (ox bile, sodium taurocholate, or cholic acid). Yeast inoculum (1% v/v) was introduced, and cultures were incubated for 20 h at 30 °C. Uninoculated broth served as the control.

Exopolysaccharide (EPS) production

EPS production was screened by streaking overnight cultures on ruthenium red milk agar (10% skim milk, 1% sucrose, 0.08 g/L ruthenium red, 1.5% agar) and incubating at 30 °C for 24 h **(Mora *et al*., 2002)**.

Safety assessment

Haemolytic activity

Cultures were streaked on Columbia blood agar plates (Oxoid) supplemented with 5% sheep blood and incubated at 30 °C for 48 h. Hemolysis was recorded as clear zones around colonies **(Lombardi *et al*., 2002)**.

Enzyme Production

The production of enzymes having potential applications in food industry namely α-amylase, protease, lipase and β-galactosidase were evaluated in the yeast isolates.
Amylase production was assessed using a modified starch agar well diffusion method. Wells were cut into the medium composed of peptone (5 g/L), yeast extract (2 g/L), soluble starch (10 g/L), $MgSO_4 \cdot 7H_2O$ (0.1 g/L), $CaCl_2 \cdot 7H_2O$ (0.1 g/L), KH_2PO_4 (0.5 g/L), and agar (15 g/L). Each well was loaded with 80 µl of cell-free supernatant from 24 h-old yeast cultures. The plates were incubated at 30 °C for 72 h **(Sato *et al*., 2011)**. Following incubation, they were flooded with Lugol's iodine solution (0.1% iodine and 1% potassium iodide). The presence of a clear halo around the wells indicated starch hydrolysis.
Protease production was determined using skim milk agar as the substrate medium. Wells prepared in skim milk agar (10% w/v skimmed milk powder) were filled with 50 µl of cell-free supernatant from 24 h-old yeast cultures. After diffusion of the supernatant for 2 h at room temperature, plates were incubated at 30 °C for 24 h **(Larsen *et al*., 1998)**. The appearance of a clear zone around the wells confirmed proteolytic activity.
Lipase production was tested on tributyrin agar plates containing 0.5% peptone, 0.3% yeast extract, 2% agar, and 0.1% tributyrin. Yeast isolates were inoculated on the plates and incubated at 30 °C for 48 h **(Kumar *et al*., 2012)**. Formation of a zone of clearance around colonies signified lipase activity.
For β-galactosidase detection, yeast isolates were streaked onto YM agar supplemented with 60 µl of 2% X-gal and 10 µl of 1% IPTG as inducer. The plates were incubated at 30 °C for 48 h, and colonies that produced β-galactosidase appeared blue **(Vinderola and Reinheimer, 2003)**.

Statistical analysis

All statistical analyses were carried out using SPSS software version 21.0 (SPSS Inc., Chicago, IL). Data were expressed as mean of two independent experiments performed in triplicates. One-way ANOVA with Tukey's HSD test was applied for comparisons, and results were considered significant at $p < 0.05$.

RESULTS

Isolation of yeast

A total of 15 yeast isolates were obtained from *sura,* traditional fermented beverages of Pangi region, Himachal Pradesh. The isolates were subsequently characterized based on their morphological and biochemical traits, and their ability to grow under specific conditions was regarded as an indicator of positive probiotic potential. In the current investigation, all the isolates showed good growth at 37 °C and 5 isolates grew at 15 °C, 2 isolates grew at low pH i.e. 2.5 and 3.5, all isolates showed growth at 5 % v/v concentration of ethanol, 8 showed catalase activity and none showed urease activity. Most of the tested yeast isolates fermented D-glucose, D-galactose, D-maltose, D-saccharose and D-raffinose. Finally, two best isolates were chosen for further studies.

Molecular identification of yeast isolates

Molecular sequencing identified the two selected isolates as *Pichia fermentans* (Y77) and *Pichia kudriavzevii* (Y136). Their sequences were submitted to the GenBank database under accession numbers OL454637 and OL658841, respectively. Phylogenetic relationships were established using the neighbor-joining method in MEGA 11 software (figure 1).

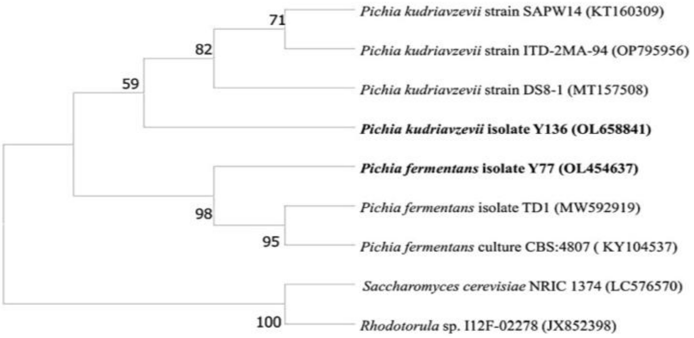

Figure 1 A neighbor joining phylogenetic tree based on ITS DNA sequences of *P. fermentans* Y77 and *P. kudriavzevii* Y136

Probiotic characterization

Survival under stressed gastrointestinal conditions

The survival of *Pichia fermentans* Y77 and *Pichia kudriavzevii* Y136 under gastrointestinal stress is summarized in Table 1. Both isolates exhibited strong tolerance to acidic conditions, maintaining viability at pH 2 and pH 3 for 3 hours. No significant difference in survival was observed when compared to the neutral pH 7 control. Additionally, both yeasts showed considerable resilience to bile salts, with only a minimal reduction in viable counts. The isolates also displayed robust resistance to lysozyme, tolerating concentrations ranging from 50 to 150 µg/ml without significant loss of viability.

Table 1 Survival of yeast isolates in acidic, bile and lysozyme conditions

Isolates	Survival rate (%)										
	Acid tolerance			Bile tolerance				Lysozyme tolerance			
	pH 7 (control)**	pH 3**	pH 2**	Control**	0.5 %**	1 %**	2 %**	Control**	50 µg/ml**	100 µg/ml**	150 µg/ml**
P. fermentans Y77	100±0.02	100±0.06	96±0.11	100±0.14	99±0.07	98±0.23	98±0.02	100±0.01	98±0.15	98±0.04	96±0.03
P. kudriavzevii Y136	100±0.52	96±0.21	95±0.03	100±0.04	98±0.07	97±0.09	97±0.13	100±0.02	99±0.08	98±0.03	98±0.05

Values represented as mean ± standard deviation (SD) of triplicate analysis
** Significant at p<0.05 measured by 2 sided Tukey's post hoc range test between replications

Susceptibility of yeast to antibiotic and antifungal agents

The isolates were found to be sensitive to the antifungal agents used in the study, with the exception of isolates *P. kudriavzevii* Y136, which was resistant to fluconazole. Both the yeast isolates were resistant to vancomycin, penicillin, clindamycin, gentamicin and ampicillin and *P. fermentans* Y77 was sensitive to erythromycin.

Table 2 Antibiotic susceptibility profile (zone diameter in mm) of yeast isolates

Isolates	KT	CC	IT	AP	NS	FLC	VA	P	E	CD	GEN	A
P. fermentans Y77	25	20	25	18	22	22	R	R	10	R	R	R
P. kudriavzevii Y136	17	22	24	15	21	R	R	R	R	R	R	R

Ketoconazole (KT), clotrimazole (CC), itraconazole (IT), amphotericin-B (AP), nystatin (NS), fluconazole (FLC), vancomycin (VA), penicillin (P), erythromycin (E), clindamycin (CD), ampicillin (A), R-resistant

Determination of cell surface characteristics

Cell surface hydrophobicity and autoaggregation ability

The findings of the current study revealed significant differences in hydrophobicity among the isolates as well as same isolate for different hydrocarbons. *P. fermentans* Y77 showed significantly (p<0.05) higher percent hydrophobicity as compared to *P. kudriavzevii* Y136 (figure 2a). It was found that autoaggregation increased over time and the autoaggregation percentage was measured for 3 h and 24 h. Both the isolates exhibited excellent autoaggregation ability (figure 2b).

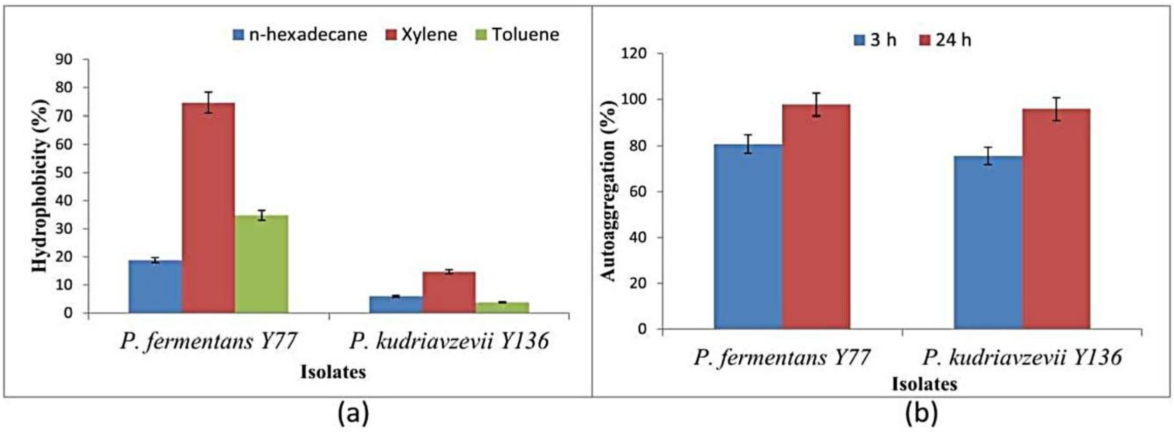

Figure 2 (a) Hydrophobicity (%) of *P. fermentans* Y77 and *P. kudriavzevii* Y136 towards n-hexadecane, xylene and toluene (b) autoaggregation percentage of *P. fermentans* Y77 and *P. kudriavzevii* Y136 at 3 h and 24 h of time interval

In vitro beneficial effects of probiotic yeast isolates

Antimicrobial activity

The antimicrobial potential of *Pichia fermentans* Y77 and *Pichia kudriavzevii* Y136 was evaluated against eight food spoilage and pathogenic bacteria, including *Listeria monocytogenes* MTCC 657, *Bacillus cereus* MTCC 1272, *Staphylococcus aureus* subsp. *aureus* MTCC 96, *Pseudomonas aeruginosa* MTCC 424, *Escherichia coli* MTCC 118, *Shigella* spp., *Salmonella typhi*, and *Aeromonas hydrophila*. Both yeast isolates exhibited broad-spectrum antimicrobial activity, effectively inhibiting all tested bacterial strains (Table 3).

Table 3 Antimicrobial activity (mm) of yeast isolates against pathogens

Isolates	*S. typhi*	*E. coli*	*Shigella*	*P. aeruginosa*	*B. cereus*	*S. aureus*	*A. hydrophilla*	*L. monoctyogenes*
P. fermentans Y77	9.50±0.50	11.50±0.50	10.00±1.00	9.00±1.00	10.00±1.00	10.00±1.00	23.50±1.50	10.50±0.50
P. kudriavzevii Y136	11.50±0.50	10.50±0.50	12.00±0.00	11.00±1.00	10.00±1.00	11.50±0.50	16.00±1.00	9.00±1.00

Values represented as mean ± standard deviation (SD) of triplicate analysis

***In-vitro* cholesterol assimilation**

Nowadays, an increase in blood cholesterol is a serious health issue and probiotics are found to be useful in lowering the cholesterol level in blood. Figure 3 depicts the assimilation of cholesterol by yeast isolates during 20 h of growth. Significantly ($p<0.05$) higher cholesterol assimilation was observed in case of *P. fermentans* Y77 with taurocholate i.e. 80.93 %. In *P. fermentans* Y77 and *P. kudriavzevii* Y136, the percentage of cholesterol assimilation was 37-81 % and 28-64 %, respectively.

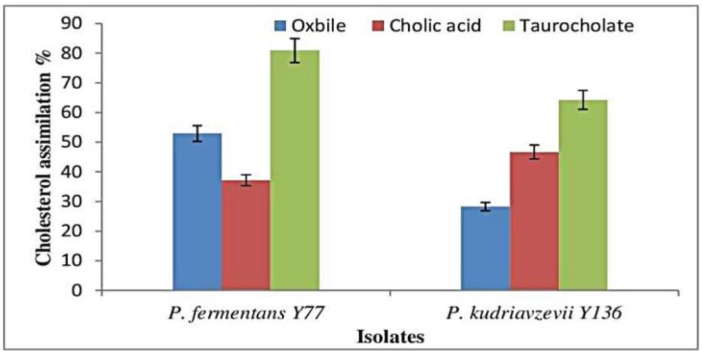

Figure 3 Cholesterol assimilation of *P. fermentans* Y77 and *P. kudriavzevii* Y136 in different bile salts

Exopolysaccharide production

Both yeast isolates showed production of exopolysaccharide when streaked on ruthenium red milk plates (figure 4a).

Safety Assessment

Haemolytic activity was used to assess the pathogenicity of yeast isolates. Both yeast isolates showed no haemolysis after 48 h of incubation (figure 4b).

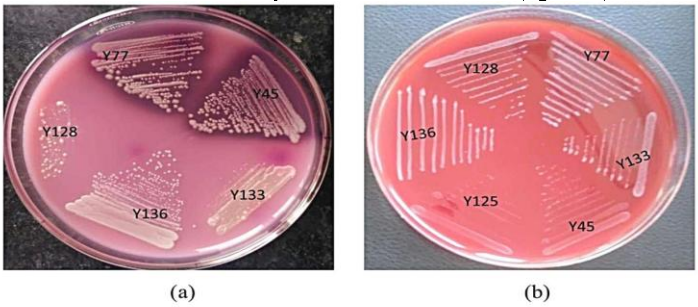

Figure 4 a) Exopolysaccharide production on ruthenium red agar plates b) γ-haemolytic activity on Columbia blood agar plate of *P. fermentans* Y77 and *P. kudriavzevii* Y136

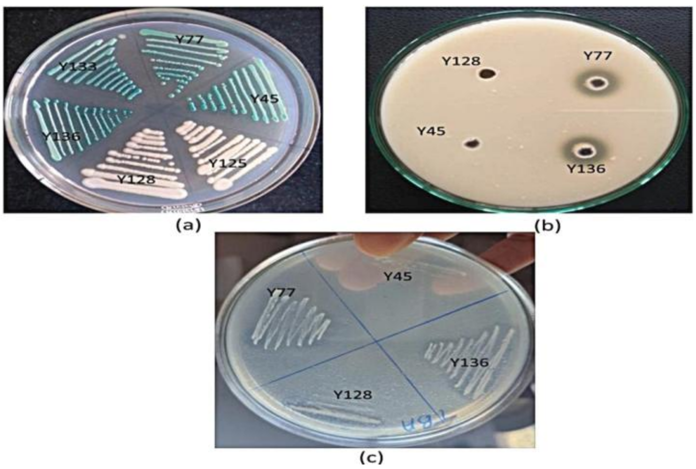

Figure 5 a) β-galactosidase activity b) protease activity and (c) lipase activity of *P. fermentans* Y77 and *P. kudriavzevii* Y136

Enzyme Production

The yeast isolates were assessed for their ability to produce extracellular enzymes, including amylase, protease, lipase, and β-galactosidase**.** Both isolates demonstrated positive activity for all the enzymes tested (figure 5).

DISCUSSION

The climatic conditions of northwestern Himalayas are harsh and remains snow covered for approximately 6–7 months annually. Due to these climatic conditions, the local population traditionally relies on a variety of fermented foods and beverages, as they are easily digestible. *Sura*, a traditional alcoholic beverage made from *kodra* (millet) using *'dheli'* a starter culture and is commonly prepared in the rural areas of Himachal Pradesh. Other millet-based fermented alcoholic drinks, such as *thumba* and *jaanr*, are also produced and consumed in various Himalayan regions **(Tamang, 1998)**. These beverages not only have cultural and religious importance but are also associated with local festivities. For example, in the Kullu district of Himachal Pradesh, *sura* is prepared for occasions such as ***Seori Sajja*** and marriage ceremonies. *Sura* is enriched with *Dheli*, a herbal additive composed of over 36 traditional medicinal herbs, which contributes bioactive compounds and exhibits stimulatory effects. Studies have shown that *sura* is not only an alcoholic beverage but also possesses high nutritional value, including significant levels of essential amino acids and B vitamins **(Thakur *et al*., 2004; Thakur *et al*., 2015)**. Considering the beneficial effects of *sura*, this study focused on isolating and characterizing probiotic yeast strains from *sura* samples collected in the Pangi region of Himachal Pradesh. A total of fifteen yeast strains were obtained, out of which two were selected based on various morphological and technological characteristics. Molecular identification of selected isolates revealed the presence of *P. fermentans* Y77 and *P. kudriavzevii* Y136 in *sura*. Similarly, **Thakur *et al.* (2015)** observed the presence of *P. kudriavzevii* and *Saccharomyces cerevisiae* in *sura* along with lactic acid bacteria.

The current study emphasized the probiotic properties of *P. fermentans* Y77 and *P. kudriavzevii* Y136. Both isolates demonstrated strong tolerance to acidic conditions, bile salts, and lysozyme, suggesting their potential to survive the stressful conditions of the gastrointestinal tract. They were resistant to the acidic conditions and showed a significant growth at different bile and lysozyme concentration. Several studies suggested that yeasts obtained from fermented products showed good tolerance to the gastrointestinal conditions (**Bajwa and Sharma, 2018; Hsiung *et al*., 2020; Wulan *et al*., 2021**). Similarly, *P. kudriavzevii* Y33 also showed significant growth in acidic and bile conditions **(Lata *et al*., 2022)**. **Merchán *et al.* (2020)** revealed that *P. fermentans* and *P. kudriavzevii* isolated form fermented cheese showed better growth in these stressed conditions. **Noroul Asyikeen *et al.* (2020)** during study found that out of 13 *Saccharomyces* strains, four strains viz. SD6, SMK9, SS12 and SK14 (>30 % at 25 ppm) showed higher growth in the presence of lysozyme and other strains were sensitive to lysozyme.

A key requirement for a microorganism to be considered probiotic is its safety. The current study showed that the both strains were sensitive to the antifungal agents and were resistant to vancomycin, penicillin, clindamycin and ampicillin. *P. fermentans* Y77 was found to be sensitive to erythromycin. These results are in accordance with other works that reported the resistance of yeast isolates towards antibiotics **(Syal and Vohra, 2013; Fakruddin *et al*., 2017)**. These yeast isolates may be beneficial for patients undergoing antibiotic therapy, as they exhibit resistance to commonly used antibiotics.

Another important probiotic characteristic is cell surface hydrophobicity**,** which plays a key role in the ability of the strains to adhere to gastrointestinal epithelial cells and thereby inhibit the colonization of pathogenic microorganisms. This adherence is primarily influenced by surface properties such as hydrophobicity and autoaggregation **(Somashekaraiah *et al*., 2019)**. During the present study, *P. fermentans* Y77 showed higher percent hydrophobicity towards xylene i. e. 74.60 %. Both the yeast isolates demonstrated excellent autoaggregation ability and their autoaggregation ability increased with increase in time. In a study, *P. fermentans* 1859 obtained from traditional soft cheese showed 90.35 % auto-aggregation and 34.83 % of cell surface hydrophobicity (**Merchán *et al*., 2020)**. A similar result was observed in *P. kudriavzevii* Y33, where isolate showed 27 % of hydrophobicity and 87 % of autoaggregation after 24 h of incubation **(Lata *et al*., 2022)**.

In addition, the ability to inhibit pathogenic microorganisms is considered a crucial probiotic trait, highlighting the significance of antimicrobial activity in evaluating potential probiotic strains. Yeasts may exert antimicrobial effects through competitive exclusion, degradation of enterotoxins, immune response activation**, or** receptor binding inhibition **(Buts and Bernasconi, 2005)**. It was observed that both *P. fermentans* Y77 and *P. kudriavzevii* Y136 showed inhibitory action against pathogens used during study. **Silva *et al.* (2011)** demonstrated that *P. fermentans* isolated from brine olives showed inhibitory action against *Staphylococcus enteritidis* and *Staphylococcus aureus. P. fermentans* and *P. kudriavzevii* isolated from different fermented products exhibited antimicrobial activity against pathogens (**Alakeji and Oloke, 2020; Kathade *et al*., 2020; Merchán *et al*., 2020**). These isolates were further examined for cholesterol assimilation and it was observed that both the yeast isolates exhibited good cholesterol assimilation rate. Elevated levels of both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol are recognized as significant risk factors for cardiovascular disorders. Individuals with increased blood cholesterol are reported to have nearly threefold higher chances of suffering a heart attack compared to those with normal lipid profiles **(Ghosh, 2012)**. In a study by **Chen *et al.* (2010)**, *Pichia fermentans* BY5 and *Pichia kudriavzevii* BY10, isolated from raw milk, demonstrated cholesterol assimilation capacities of 43.2% and 40.3%, respectively. Similarly, cholesterol-reducing ability has also been reported in *P. kudriavzevii* strains obtained from traditional homemade mango pickle and from the gastrointestinal tract of the freshwater snail *Pila globosa* **(Kathade *et al.*, 2020; Lata *et al.*, 2022)**. EPS production by probiotic can modify the flora in the gut by enhancing their colonization and promote human health **(Darilmaz *et al*., 2011)**. Both *P. fermentans* Y77 and *P. kudriavzevii* Y136 produce the exopolysaccharide in the present study. Previously, **Lata *et al*. (2022)** observed the exopolysaccharide production in *P. kudriavzevii* Y33 obtained from fermented pickle. As safety is the main concern of probiotics, so the absence of haemolytic activity is necessary that allow their acceptance and use as starter culture. Both the yeast isolates were non haemolytic in nature that assure the safety of isolates.

Yeasts are known to produce a variety of functional enzymes that enhance the digestibility of complex carbohydrates, including indigestible fibers, thereby contributing positively to human health. These enzymes facilitate nutrient absorption by hydrolyzing lipids, proteins, and carbohydrates. In the present study, both isolates were found to produce extracellular amylase, protease, β-galactosidase, and lipase. Earlier, **Syal and Vohra (2013)** reported that seven yeast strains isolated from Indian fermented foods were capable of producing protease, phytase, β-galactosidase and lipase, although none exhibited amylase activity. Similarly, *Pichia kudriavzevii* Y33 was shown to synthesize multiple extracellular enzymes, including amylase, protease, phytase, β-galactosidase and lipase **(Lata *et al.*, 2022)**. In another study, *Saccharomyces cerevisiae* DABRP5, isolated from bollo batter—a traditional fermented food of Goa—demonstrated the production of β-galactosidase, α-amylase and lipase **(Pereira *et al.*, 2021)**.

CONCLUSION

In recent years, the demand for probiotics has grown significantly, and numerous studies are currently exploring this field. Fermented products serve as an important reservoir of beneficial microorganisms with potential health-promoting properties. In this study, an effort was made to isolate and characterize probiotic yeast strains from a traditional fermented beverage. This is the first report highlighting the probiotic characteristics and enzyme production of *Pichia fermentans* Y77 and *Pichia kudriavzevii* Y136, both isolated from sura beverage. These yeast strains demonstrated notable tolerance to harsh gastrointestinal conditions. They also exhibited resistance to antibiotics, suggesting their potential use in adjuvant therapies to help maintain normal gut microflora during antibiotic treatment. Both strains showed favorable cell surface properties, with *P. fermentans* Y77 displaying a hydrophobicity percentage of 74.60 %. Additional beneficial traits observed included antimicrobial activity, cholesterol assimilation, and exopolysaccharide (EPS) production. Notably, both yeasts exhibited broad-spectrum inhibitory activity against pathogens, indicating their potential use in food preservation to prevent spoilage. *P. fermentans* Y77 demonstrated the highest cholesterol assimilation capacity. Furthermore, the isolates were capable of producing extracellular enzymes, which could offer added health benefits to consumers. In summary, *Pichia fermentans* Y77 and *Pichia kudriavzevii* Y136 demonstrated noteworthy probiotic attributes, including tolerance to gastrointestinal stress conditions, antimicrobial activity, cholesterol assimilation, and production of functional enzymes. These characteristics highlight their potential as promising starter cultures for the formulation of novel probiotic foods. Future investigations should focus on validating their health benefits through in vivo studies, assessing their safety for human consumption, and exploring their applicability in functional food development, clinical use, and large-scale industrial fermentation.

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