

Full Length Research Paper

Probiotic attributes of a yeast-like fungus, *Geotrichum klebahnii*

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***Geotrichum klebahnii*, a filamentous yeast-like fungus, was isolated from a cheese sample. Several *in vitro* tests were carried out for its probiotic characterization. This isolate showed high survival rate of $100 \pm 1.8\%$ at low pH (pH 2) and $100 \pm 0.9\%$ at high oxbile concentration (1%) and also grew well at 37°C . It showed an auto-aggregation ability of $100.00 \pm 1.5\%$ after 20 h of incubation at 37°C , as well as $36.43 \pm 0.70\%$ and $52.13 \pm 1.50\%$ cell surface hydrophobicity with xylene and n-hexadecane, respectively. It had inhibitory activity against food-borne pathogens such as *Salmonella* sp., *Vibrio* sp. and *Staphylococcus aureus*. *G. klebahnii* produced the enzymes phytase and inulinase. It was a producer of vitamin B₁₂ and exopolysaccharides. It assimilated up to $29.42 \pm 2.1\%$ cholesterol after 48 h of incubation at 37°C . The organism did not produce gelatinase and DNase assay, indicating its safety as a probiotic microorganism. This is the first report of the probiotic potential of yeast like fungus, *G. klebahnii*.**

Key words: Probiotics, yeast, antimicrobial, phytase, inulinase.

INTRODUCTION

Yeasts contribute to the fermentation of a broad range of other commodities, where various species may work together with bacteria and/or filamentous fungi. Fermented milk products that are manufactured using starter cultures containing yeasts include acidophilus-yeast milk, kefir, Koumiss, Leban and cheese (Lang and Lang, 1975). *Saccharomyces* spp. For example *Saccharomyces burnetii*, *Saccharomyces kluyveri*, *Saccharomyces byanus*, *Saccharomyces rosinii*, *Saccharomyces cerevisiae* and *Saccharomyces boulardii* may be isolated from a variety of dairy products including milk, yogurt, cream, dahi, cheese and kefir. *Saccharomyces* spp. cannot ferment lactose so they develop in milk as a secondary flora, after bacterial growth. Lactic acid produced by lactic acid bacteria creates a high acid

environment, creates a selective environment for yeast growth (Fleet, 1990). *Saccharomyces cerevisiae*, which according to The European Food Safety Authority (EFSA) has a Qualified Presumption of Safety (QPS) status, is the most common yeast used in food fermentation where it has shown various technological properties.

Probiotics have been defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Fuller, 1989). *S. boulardii* is the only yeast with clinical effects and the only yeast preparation with proven probiotic efficiency in double-blind studies (Sazawal et al., 2006).

Geotrichum sp., filamentous yeast-like fungus, is ubiquitous and is found in a wide range of habitats such as plant tissues, silage and soil (Jacques and

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Caseregola, 2008). This genus belongs to the class Hemiascomycetaceae, order Saccharomycetales and family Dipodascaceae. It is also a component of the natural flora of human mouth, skin and gastrointestinal tract. Species belonging to this genus have been commonly isolated from dairy products such as cheese, fermented milk and also from juices. One of its species, *Geotrichum candidum* has been reported by the International Dairy Federation and European Food and Feed Culture Association as a microorganism with a documented history of use in dairy products (Mogensen et al., 2002). Sarkar et al. (1994) reported the population of *Geotrichum candidum* in 40-50% of market samples of Kinema (a soyabean fermented food) to be $0.8-4 \times 10^4$ cfu/g. Nago et al. (1998) isolated 54 strains of yeasts from Beninese ogi, out of which *Geotrichum* spp. accounted for 26% of the yeast isolates. *Geotrichum penicillatum* and *Geotrichum candidum* have been reported to be isolated from Boza, a beverage consumed in Bulgaria, Albania (Vasudha and Mishra, 2013; Gotcheva et al., 2000).

Geotrichum klebahnii ATCC 42397 (previously named *Trichosporon penicillatum* SNO-3) is a yeast-like fungus originally isolated from mandarin peel (Rojas et al., 2008). This study is the first report of probiotic properties of *G. klebahnii*, isolated from cheese sample.

MATERIALS AND METHODS

All the chemicals used were of AR grade and procured from Qualigens and Himedia (Mumbai, India).

Isolation procedure and Identification

A 25 g sample of the cheese sample was homogenized in 225 ml of sterile phosphate buffered saline (PBS; g/L: NaCl 8.0, KCl 0.2, disodium phosphate 1.44 and potassium phosphate 0.24), pH 7. 10-fold serial dilutions of the sample were prepared in sterile PBS, pH 7. The dilutions were spread plated on MYPG medium (g/L: malt extract 3.0, yeast extract 3.0, peptone 5.0, glucose 10.0 and agar 20.0, pH 5.6) and the plates were incubated at 30°C for 24-48 h and observed for microorganisms with yeast-like morphology under microscope.

The culture was identified at IMTECH, Chandigarh, India, using BioLog identification kits.

Acid tolerance test

The culture was inoculated (1%) into PBS maintained at low pH (2, 2.5 and 3) using 1 N HCl. Samples were taken after 5 h of incubation at 37°C and spread plated on MYPG. The survival rate was calculated as the percentage of colonies grown on MYPG medium after exposure to low pH as compared to the initial cell concentration.

Bile salt tolerance test

PBS tubes containing 0.3, 0.5 and 1% oxbile were inoculated with the yeast culture and incubated at 37°C for 5 h. The survival rate of each strain was expressed as the percentage of viable cells in the

presence of bile salt as compared to that without bile salt.

Comparison of growth at 30 and 37°C

Growth at both temperatures was compared by determining the number of viable cells by the plate count method after incubation for 48 h.

Auto-aggregation assay

G. klebahnii was grown for 48 h at 37°C in MYPG broth. The cells were harvested by centrifugation at 7000 rpm for 10 min, washed twice and resuspended in PBS. Cell suspensions (4 mL) were mixed by vortexing for 10 s and auto-aggregation was determined after 3 and 20 h of incubation at 37°C. An aliquot (0.1 mL) of the upper suspension of PBS after incubation was transferred to another tube with 3.9 mL of PBS and the absorbance (A) was measured at 600 nm (Del et al., 2000). The auto-aggregation percentage is expressed as: $1-(A_t/A_0) \times 100$, where A_t represents the absorbance at time $t = 3$, or 20 h and A_0 the absorbance at $t = 0$.

Cell surface hydrophobicity

One milliliter of the hydrocarbon, that is, n-hexadecane and xylene were added to tubes containing cell suspensions (3 ml). The cells were vortexed for 120 s. The suspension was then kept undisturbed at 37°C for 5 min to allow phase separate, and the hydrocarbon layer was allowed to rise completely. After 5 min, the aqueous phase was removed carefully and the absorbance (OD) was measured using a spectrophotometer at 600 nm (Rosenberg et al., 1980). The decrease in the absorbance was taken as a measure of the cell surface hydrophobicity (hydrophobicity (%)), calculated using the equation given below:

$$\text{Hydrophobicity (\%)} = (\text{OD}_{\text{initial}} - \text{OD}_{\text{final}}) / \text{OD}_{\text{initial}} \times 100$$

Where, $\text{OD}_{\text{initial}}$ and OD_{final} are the absorbances (at 600 nm) before and after extraction with the hydrocarbons.

Inhibitory action against enteric pathogens

Double layer method, was used to evaluate the antagonistic activity of *G. klebahnii* against enteric pathogens, that is, *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus*, *Vibrio cholerae* and *Pseudomonas* sp. obtained from the Departmental Culture Collection Centre. An overnight culture of *G. klebahnii* was prepared in MYPG broth at 30°C and inoculated onto plates by swabbing a 1 inch by 1.5-inch area in the center of each plate. The plates were incubated at 30°C for 48 h. The growth in each plate was then overlaid with 10 mL of molten nutrient agar (0.7% agar) previously inoculated with 1 mL of the prepared test pathogen cultures. The agar was allowed to solidify and the plates were incubated aerobically at 37°C for 24 h and examined for growth inhibition (Maia et al., 2001).

Enzyme based screening

Phytase

Phytase assay was performed according to the method described by Vohra and Satyanarayana (2001) using phytate minimal medium (g/L: glucose 15.0, sodium phytate 5.0, $(\text{NH}_4)_2\text{SO}_4$ 5.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 5.0, KCl 5.0, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.01 and agar 20.0).

The plate was incubated at 30°C for 48 h. After incubation, the plate was flooded with 2% cobalt chloride and then kept at room temperature for 5 min. After removing cobalt chloride solution, a freshly prepared solution containing equal volume of 6.25% ammonium molybdate and 0.42% ammonium vanadate was flooded onto the plates. They were incubated at room temperature for 5 min after which, the solution was removed. The plates were observed for zone of hydrolysis.

Inulinase

Inulin (1%) and phenol red (0.01%) was incorporated into medium with the following components, g/L: NH₄NO₃, 2.3; (NH₄)₂HPO₄, 3.7; KH₂PO₄, 1.0; MgSO₄, 0.5; yeast extract, 1.5. The pH of the medium was adjusted to 7.3-7.4 by using 1 N NaOH. Plate was point inoculated with a fresh culture of *G. klebahnii* and incubated at 37°C for 48 h. It was then observed for change in color from red to yellow around the colony of *G. klebahnii*.

Vitamin B₁₂ assay

To determine vitamin B₁₂ production, *G. klebahnii* was streaked on vitamin B₁₂ assay medium, containing all the vitamins except vitamin B₁₂, followed by incubation at 37°C. The growth of the organism on the assay plate was taken as positive for vitamin B₁₂ production.

Exopolysaccharide production

Exopolysaccharide production assay was performed according to the methods described by Mora et al. (2002). Freshly activated culture of *G. klebahnii* was streaked on the surface of plate containing ruthenium red milk agar (g/L: skim milk powder 100.0, sucrose 10.0 and ruthenium red 0.08 and agar 20.0). After incubation at 37°C for 24 h, the plate was observed for white (exopolysaccharide producing) or red (non-exopolysaccharide producing) colony.

Cholesterol reduction assay

The ability of *G. klebahnii* to assimilate cholesterol was determined according to the method described by Searcy and Bergquist (1960). The percentage assimilation was calculated using the formula:

$$\text{Assimilation (\%)} = \left[\frac{\text{Concentration of cholesterol in control} - \text{Concentration of cholesterol in sample}}{\text{Concentration of cholesterol in control}} \right] \times 100$$

Safety assessment

Gelatinase production

Gelatinase production was determined by point inoculating culture of *G. klebahnii* on plate containing tryptone-neopeptone-dextrose (TND) agar (g/L: tryptone 17.0, neopeptone 3.0, dextrose 2.5, NaCl 5.0, K₂HPO₄ 2.5 and agar 20.0), supplemented with 0.4% gelatin. The plate was incubated at 37°C for 48 h and was then flooded with saturated ammonium sulfate solution. Development of clear zones around the spots against the opaque background indicated a positive reaction (Gupta and Malik, 2007).

DNase production

DNase agar medium (HiMedia) was used to check production of

DNase enzyme and was streaked with *G. klebahnii* culture, followed by plate incubation at 30°C for 48 h. After incubation, a clear pinkish zone around the colonies against dark blue background was considered positive for DNase production (Gupta and Malik, 2007).

Statistical analysis

All the experiments were performed in triplicate. The data were assessed using analysis of variance (ANOVA) with a level of significance at $p < 0.05$. The results are presented as means \pm standard deviation. All statistical analysis was performed using 'Design Expert 6.0' software (Stat-Ease, Inc., Minneapolis, MN, USA).

RESULTS AND DISCUSSION

Morphological characteristics

Colonies on MYPG medium were white-cream, slightly raised, circular, fimbriate margins, with velvety appearance. Under microscope, septate hyphae and cylindrical individual cells were observed.

Acid and bile salt tolerance of *G. klebahnii*

Probiotics after ingestion are subjected to and need to survive the unfavourable physiological conditions of the gastrointestinal tract such as the extremely acidic environment and the detergent effect of bile secretions. The pH of the human stomach is around 1.5-3.5; whereas the physiological concentration of bile salts in the small intestine is between 0.2 and 2.0% (Gunn, 2000; Sahadeva et al., 2011). Sufficient survival of the probiotic microorganisms through the transit to GI tract is crucial to confer any health benefits on the host. In this investigation, *G. klebahnii* showed high acid tolerance with survival rate of $100 \pm 1.5\%$, $100 \pm 2.3\%$ and $100.37 \pm 1.8\%$ at pH 3, 2.5 and 2, respectively, after 5 h of incubation at 37°C. It was also highly tolerant to bile salt with a survival rate of $100 \pm 0.5\%$, $100 \pm 1.1\%$ and $100 \pm 0.9\%$, at 0.3, 0.5 and 1.0% oxbile, respectively, after 5 h of incubation at 37°C.

Comparison of growth at 30 and 37°C

For a probiotic organism, it is also necessary to grow at human body temperature of 37°C and *G. klebahnii* showed comparable growth at both temperatures (37 and 30°C). While most *S. cerevisiae* strains grow and metabolize at a temperature of 30°C, *S. boulardii* is a thermotolerant yeast that grows optimally at 37°C (Czerucka et al., 2007).

Autoaggregation and cell surface hydrophobicity of *G. klebahnii*

Adhering potency of probiotics to the intestinal tract of



Figure 1. Plates showing zones of inhibition against *Staphylococcus aureus*.

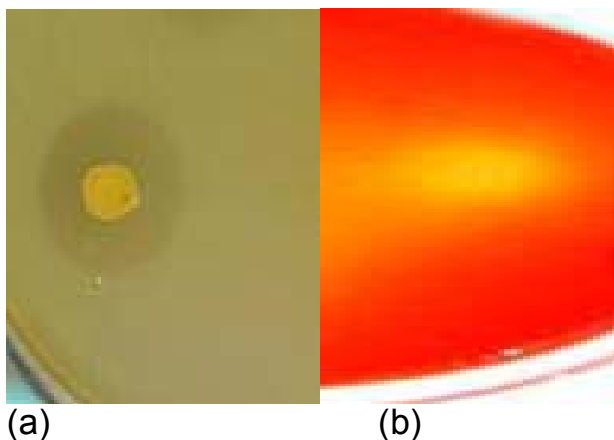


Figure 2. Plates showing enzyme production. (a) Phytase and (b) Inulinase.

host is believed to be crucial in order to ensure their maintenance in the intestinal tract for a longer period of time. Adhesion is initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesions (usually proteins) and complementary receptors. Aggregation ability is one of the factors related to cell adherence properties. It has been reported that auto aggregation ability above 80% is considered to be strong auto-aggregation (Radulovic et al., 2010). In this investigation, *G. klebahnii* showed $94.31 \pm 3.5\%$ autoaggregation ability, after 3 h of incubation at 37°C which further increased to $100.00 \pm 1.5\%$ after 20 h of incubation. Cell surface hydrophobicity is another factor responsible for adhesion to host tissues. In this study, in vitro cell surface hydrophobicity was observed to be $36.43 \pm 0.7\%$ and $52.13 \pm 1.5\%$, with

xylene and n-hexadecane, respectively. This method has been reported to be qualitatively valid to estimate the ability of a strain to adhere to epithelial cells (Pizzolitto et al., 2012; Keily and Olson, 2000). Isolates having good autoaggregation ability in conjunction with the good hydrophobicity values can strongly be related to the adhesion ability of these microorganisms. Though these traits are independent of each other; they are still related to adhesion property of a particular microbe (Rahman et al., 2008).

Antimicrobial activity

Probiotics might prevent infection by competing with pathogens for binding sites on epithelial cells (O'Sullivan et al., 1992). Probiotics might also inhibit the growth of pathogenic bacteria by producing bacteriocins such as nisin (Del Miraglia and De Luca, 2004). Antibacterial properties are one of the most important selection criteria for probiotics. In this study, *G. klebahnii* had antimicrobial activity against *Salmonella* sp., *Vibrio* sp. and *Staphylococcus aureus*. Antimicrobial activity was observed as a clear zone of inhibition (16-36 mm diameter) around the colony of *G. klebahnii* (Figure 1). *S. boulardii* have been shown to protect against various enteric pathogens and members of the family Enterobacteriaceae in animal studies (Czerucka and Rampal, 2002) such as *E. coli*, *Shigella*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *S. aureus* and *Entamoeba histolytica*.

Enzyme based screening

Probiotics improve host digestive processes by producing extracellular enzymes. Phytases are nonspecific phosphatase enzymes, which catalyze the dephosphorylation of phytate and release free inorganic phosphate (Pi) and inositol phosphate esters (Vucenik and Shamsuddin, 2006). Phytic acid or phytate, the primary storage form of phosphorus in mature seeds of plants, has antinutritional properties since it has a strong chelating capacity and lowers the bioavailability of divalent ions such as iron, zinc, calcium and magnesium by forming insoluble complexes and also can negatively influence the functional and nutritional properties of proteins such as digesting enzymes (Reddy and Pierson, 1994). Inulinases catalyze the hydrolysis of inulin, producing inulo-oligosaccharides, fructose and glucose as main products. Inulo-oligosaccharides are prebiotics and their positive effect on human health has been widely acknowledged (Vohra and Satyanarayana, 2003). In this study, *G. Klebahnii* was observed to produce phytase (Figure 2a) and inulinase (Figure 2b). Several fungi such as *F. verticillioides*, *Aspergillus* sp., *Penicillium* sp. and *Emericella* sp., have been reported to be phytase

Table 1. Probiotic properties of *G. klebahnii*.

Acid tolerance	100% tolerant at pH 2-3
Bile salt tolerance	100% tolerant at 0.3-1.0% oxbile concentration
Ability to grow at 37°C	Yes
Autoaggregation ability	100% after 20 h of incubation
Cell surface hydrophobicity	36.43 ± 0.7% with xylene and 52.13 ± 1.5% with n-hexadecane
Antimicrobial activity	Inhibited the growth of <i>Salmonella</i> sp., <i>Vibrio</i> sp. and <i>Staphylococcus aureus</i>
Enzymes produced	Phytase and inulinase
Vitamin B ₁₂ assay	Positive
Exopolysaccharide production	Positive
Ability to assimilate cholesterol	29.42 ± 2.1%
Gelatinase and DNase production	Negative

producers (Marlida et al., 2010). Mughal et al. (2009) reported 45.65 IU/mL inulinase production from *G. candidum*.

Vitamin B₁₂ assay

Another benefit to human health is the ability to provide growth factors such as vitamin B₁₂ (Abramov et al., 2003), which play a key role in the normal functioning of the brain and nervous system and for formation of the blood. Most bacteria and yeasts produce vitamin B₁₂. There are few fungi with the ability to synthesize vitamin B₁₂, *Agaricusbisporus* being one of them. In this investigation, *G. klebahnii* was observed to produce vitamin B₁₂, which has not been reported till now. Whole yeast cells of *S. cerevisiae* have nutritive value since they are an excellent source of amino acids, good source of mineral and vitamin B complex (Yamada and Sgarbieri, 2005)

Exopolysaccharide production

Exopolysaccharides (EPSs) are exocellular polymers are thought to play a role in protection against desiccation, toxic compounds, bacteriophages, osmotic stress and to permit adhesion to solid surfaces and biofilm formation (Vuyst and Degeest, 1999). Another physiological benefit is that EPS is retained longer in the gastrointestinal tract, so that colonization by the probiotic microorganisms can be enhanced. EPS has immunostimulatory and anti-tumoral activities, and phosphate groups in EPS play an important role in the activation of macrophages and lymphocytes (Uemura et al., 2003). In this study, *G. klebahnii* was observed to be positive as its colony appeared white against red background. EPS production by fungi including *Alternaria* sp., *Aspergillus* sp., *Botrytis* sp., *Cladosporium* sp., *Mucor* sp., *Ganoderma alucidum*, *Agaricus blazi*, *Cordyceps* sp., *Lentinus edodes*, *G. frondosa* and many others have been reported (Mahapatra and Banerjee, 2013).

Cholesterol reduction

High levels of serum cholesterol have been associated with the risk of coronary heart disease and also in inducing colon cancer. It has been recorded that even 1% reduction in cholesterol can reduce the risk of cardiovascular diseases by 2-3% (Manson et al., 1992). In this study, *G. klebahnii* was found to assimilate up to 29.42 ± 2.1% cholesterol thus can be considered as a promising probiotic agent. One study in hypercholesterolemic mice showed that administration of low levels of the probiotic bacteria *Lactobacillus reuteri* for 7 days decreased total cholesterol and triglyceride levels by 38 and 40%, respectively, and increased the high-density lipid : low density lipid ratio by 20% (Taranto et al., 1998). The cholesterol-lowering potential of *L. acidophilus* has been widely studied (Lin et al., 1989).

Gelatinase activity and DNase production test

According to FAO/WHO (2002) every probiotic strain needs to be assessed for safety to be used as a food or feed supplement. Gelatinases (MMP-2 and MMP-9) are MMPs capable of degrading almost all ECM and basement membrane components and might provide suitable substrate for further activity of human gelatinases or other bacterial proteinases (Zhao et al., 2011). Extracellular DNase provides a growth advantage to the pathogen by enlarging the pool of available nucleotides by DNA hydrolysis helping in the dissemination and spread of the pathogen by liquifying pus and also aids the evasion of the innate immune response by degrading neutrophil extracellular traps (NETs) (Hasegawa et al., 2010). A microorganism should not produce these enzymes so as to be used as a probiotic in food and feed. In this investigation, *G. klebahnii* was found to be negative for gelatinase and DNase activity even after the longest incubation period, validating their relative safety as probiotic candidates. A summary of the probiotic properties of *G. klebahnii* is presented in Table 1.

In conclusion, *G. klebahnii* isolated from cheese has been observed to have excellent qualities of a probiotic microorganism, such as its ability to survive conditions similar to the human gastrointestinal tract (acidic pH, high bile salt concentration, growth at 37°C, high auto-aggregation ability and cell surface hydrophobicity). With its great antimicrobial activity, it can be helpful in curing infections and reducing antibiotic use. It produces phytase enzyme that degrades phytate (an antinutrient), improves nutritional status of food and aids digestion. It also produces inulinase, an enzyme that produces inulo-oligosaccharides which have anti-cancerous effect. It also has the ability to produce vitamin B₁₂, exopolysaccharides (immunostimulants) and to assimilate cholesterol reducing chances of hypercholesterolemia and heart diseases. Being a non-gelatinase and non-DNase producer, it proves to be safe as a probiotic and possesses the ability to confer health benefits on the host.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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