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IN VITRO STUDY OF POTENTIAL PROBIOTIC PEDIOCOCCUS PENTOSACEUS ISOLATED FROM IDLI BATTER AND BIOMASS PRODUCTION USING WHEY

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Probiotic strain Pediococcus pentosaceus ID9 was isolated from idli batter, identification was confirmed by 16s rRNA and evaluated through invitro studies. This strain shows maximum probiotic potential efficiency. It exhibited tolerance to pH 2.5, survived and proliferated in 2% bile salts, exhibited bile salt hydrolysis activity, nonhaemolytic activity and invitro cell adhesion ability on intestinal epithelial cells INT 407. This organism shows antimicrobial activity against Staphylococcus aureus, E.coli, Salmonella typhii, Enterobacter aerogens, and sensitive to antibiotics. A cost effective medium was formulated using whey, half strength MRS supplemented with 1% jaggery and 1% tryptone was formulated for biomass production. In this combination biomass was significantly enhanced from 0.218 g/L to 1.042 g/L.

Keywords: *Pediococcus pentosaceus*, *probiotics*, Lactic acid bacteria, Biomass, Whey

INTRODUCTION

Consumers demand for functional foods is encouraging food industry to develop new food products. Various probiotic food products with health benefits have emerged in market (Abadia-Gracia, 2013; Ying *et al.*, 2013; and Joana Barbosa *et al.*, 2015). Probiotics are defined as "microorganisms which when consumed in adequate amount are able to confer health benefits in the host" (WHO/ FAO, 2002). All microorganisms cannot be used as probiotic organisms, there are set of *invitro* evaluation criteria which enables microorganism to be marketed as probiotic. In India ICMR (Indian Council of Medical Research) and DBT (Department of Biotechnology) has set guidelines for assessment of probiotics. They are a) microbes should be non-pathogenic, non-toxic, b) resistant to gastric acidity and organic acids, c) Tolerance to bile, d) should be viable for long periods under storage conditions, e) ability to colonize in gastrointestinal tract, f) GRAS status (generally recognized as safe), g) production of antimicrobial substances, h) molecular identification of strain (genus and species level).

Generas of lactic acid bacteria marketed extensively as probiotics are *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus* (Champange *et al.*, 2011). Lactic acid bacteria group is generally considered as safe (GRAS) and used in fermentation as starter culture. Apart from these genera *Pediococci* are also emerging probiotics. *Pediococci* are a group of coccus shaped gram positive, homofermentative lactic acid bacteria (Semjonovs and Zikimanis, 2009). A few *Pediococcus* strains used as probiotic are *P. damnosus, P. parvulus, P. inopinatus, P. cellicola, P. ethanolidurans, P. claussenii, P. stilesii, P. acidilactici, P. pentosaceus, and P. dextrinicus* (Dobson *et al.*, 2002). *Pediococci* have immense economic

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significance in food industry and are used as starter culture in preparation of various fermented-foods (Spicka *et al.*, 2002; and O'Connor *et al.*, 2007). These strains exhibit antimicrobial property through production of lactic acid and pediocins. Lactic acid is used in food, pharmaceutical, cosmetic and chemical industries. In food industry it serves as flavour enhancer, pH regulator, preservative. (Wee *et al.*, 2006). European Food Safety Authority (EFSA 2009) approved *P.acidilactici* as feed supplement to shrimps, pigs and chickens. *P.acidilactici* along with other lactic acid bacteria reduced concentrations of coliforms in ceacum of intestine (Mountzouris *et al.*, 2010), has increased weight gain in piglets (Di Giancamillo *et al.*, 2008).

Lactic acid bacteria are wide spread in nature. They are present in dairy (Wassie and Wassie, 2016) fermented (Satish Kumar *et al.*, 2013; and Abegaz, 2014) non-fermented food products, intestinal tract of animals. As abundant lactic acid bacteria are present in non-dairy fermented cereal batters (idli, appam batter), it was included in our study for isolation of potential probiotic bacteria. Idli is a south Indian fermented food, it is prepared from black gram (*Phaseolous mungo L*) and parboiled rice. Fermentation of idli is carried out by lactic acid bacteria. These include *Leuconostoc mesenteroides, Lactobacillus coryneformis, L. delbrueckii, L. fermentum, L. lactis, Streptococcus faecalis,* and *Pediococcus cerevisiae* (Yajurvedi, 1980).

Design of cost effective medium for biomass production of probiotic bacteria is a great challenge. Numerous physical and nutritional factors influence biomass production. Nutritional components like whey can be considered as an excellent medium for mass production of lactic acid bacteria. Whey is a by-product obtained from dairy industry during manufacture of cheese or paneer. It contains about 93.1% water, 5.1% lactose, 0.9% protein, 0.6% ash and 0.3% fat (Shendurse *et al.*, 2009). Whey protein consists of α-lactalbumin, β-lactoglobulin and glycomacropeptides (GMP) bovine serum albumin, lactoferrin, immunoglobulins (Pouliot *et al.*, 1999).

This study was aimed to isolate and evaluate the probiotic properties of *Pediococcus pentoseaus* and to formulate cost effective whey based medium for biomass production.

MATERIAL AND METHODS

Microorganisms and Culture Conditions

Probiotic LAB was isolated from dairy (milk, homemade curd, butter, cheese) and non dairy products (cabbage, cauliflower) and fermented batters (idli and appam batter). Table salt was added to these samples and incubated for 18 h. At certain salt concentration, lactic acid bacteria grow more quickly than other microbe. All samples were serially diluted, 10^{-5} and 10^{-7} dilutes were plated on MRS agar and incubated for 24-48 h at 37 °C. Isolated colonies were selected based on gram characteristic and catalase activity and purified using pure culture techniques. Isolates were preserved on MRS agar slants with regular sub-culturing for every 15 days. Inoculum size of $4° 10^7$ (A600 nm = 2.0 ± 2.2) lactic acid bacterial isolates was used for all below described screening tests.

Five indicator strains were procured from IMTECH Chandigarh and two from ARS (Agriculture Research Service, USA) culture collection centers. They are MTCC 657 *Listeria monocytogenes*, MTCC 1680 *Escherichia coli*, MTCC 98 *Salmonella typhi*, MTCC 736 *Bacillus subtilis*, MTCC 3160 *Staphylococcus aureus*, B-14144 *Enterobacter aerogenes* and B-2617 *Pseudomonas aerogenes* respectively. These cultures were grown aerobically in brain heart infusion broth at 37 °C for 24 hours and maintained on brain heart infusion agar slant with sub culturing for every 15 days.

Antimicrobial Activity

Isolated lactic acid bacteria were screened for antimicrobial activity against indicator organisms (*E.coli, Listeria monocytogenes, Staphylococcus aureus, Bacillus subtilus, Salmonella typhii, Enterobacter aerogenes, Pseudomonas aerogenes*). Antimicrobial activity was determined by agar spot test method. Lactic acid bacteria cultures incubated for 16 h were centrifuged at 10000 rpm for 10 min at 4 °C, supernatant obtained was heated at 80 °C for 10 minutes and filter sterilized through 0.45 µm pore size filter. Indicator organisms (100 µl, $OD_{600\ nm}$ - 0.3) were plated on brain heart infusion soft agar (0.7% agar). 10 µl of cell free supernatant was spotted on brain heart infusion soft agar and incubated at 37 °C for 24 h to obtain inhibition zones.

Characterization of Antimicrobial Substance

Filter sterilized cell free supernatant was treated with protease (1 mg/ml), catalase (1 mg/ml) and supernatant pH was adjusted to 6 using 1N NaOH. All three treatments were done separately and incubated for 1h. After incubation for an hour antimicrobial activity was determined by spot test method as described above.

Lactic Acid Estimation by Titration Method

In this method, MRS broth (10 ml) containing two drops of phenolphthalein indicator was titrated against 0.1 N NaOH until the colour of the sample changed to light pink and stayed for 30 seconds.

%Lactic acid = (Consumed volume of 1 N NaOH x 0.09/ amount of sample) x 100

Survivability in Simulating Conditions of Gastrointestinal Tract

Survival in Different pH

Overnight isolates of lactic acid bacteria grown in 10 ml MRS broth were harvested by centrifugation (10000 rpm/10 min), washed with phosphate buffer (pH 7) and suspended in MRS broth adjusted to pH 2.5, 3, 4, 5, 6, 7, 8 and 9 with 1 N HCl, NaOH and incubated for 24 h. After 24 h, samples were drawn and growth was measured at 600 nm. For pH 2.5 along with absorbance at 600 nm, colony forming units were also determined on agar plates.

Survival in Bile Salts

Survivability of isolated lactic acid bacteria were determined in the presence of bile salt at concentration 0.3, 1 and 2%. MRS broth was supplemented with 0.3%, 1% and 2% bile salts separately and inoculated with lactic acid bacteria cultures and incubated at 37 °C for 24 h. After incubation growth was measured at $O.D_{600\ nm}$. MRS broth without bile salts served as control.

Bile Salt Hydrolysis

Isolated lactic acid bacteria were streaked on MRS agar containing 0.5% (w/v) sodium salt of taurodeoxycholate acid. In parallel controls agar plates were setup without bile salts. The hydrolysis effect was indicated by different colony morphology (partial hydrolysis) from the control MRS plates, after 48 h of incubation at 37 °C (Argyri *et al*., 2013).

Haemolytic Activity

Lactic acid bacteria isolates were streaked on blood agar supplemented with 5% (v/v) human blood, incubated for 24 to 72 h at 37 °C. Blood agar plates were examined for signs of hemolysis (α haemolysis, β haemolysis, ã haemolysis).

Antibiotic Sensitivity Tests

Susceptibility to antibiotics was studied by disc diffusion method. A total of 16 antibiotics (Pencillin, Amphicillin, Chloramphenicol, Streptomycin, Tetracyclin, Erythromycin, Gentamycin, Kanamycin, Vancomycin, Polymyxin, Cefoxitin, Amoxyclav, Cefalaxin, Ciprofloxacin, Clindamycin and Co-trimoxazole) were used for antibiotic susceptibility test. 100 µl cultures were inoculated on MRS agar. Antibiotics discs were placed on surface of MRS agar. These plates were incubated at 37 ºC for 24 h. Zone of inhibition around discs were measured.

16s rRNA Sequencing of Lactic Acid Bacteria

Isolated lactic acid bacteria ID9 was found to possess all the probiotic characteristics. Therefore culture ID9 was identified through 16s rRNA gene sequencing. The primer 785F with sequence GGATTAGATACCCTGGTA and primer 907R with sequence CCGTCAATTCMTTTRAGTTT used as 16s rRNA universal primers.

Adhesion Assay

The adhesion capacity of *P. pentosaceus* ID9 was explored using intestinal epithelial cell lines INT 407 procured from National Centre for Cell Science (NCCS), Pune. The cell lines were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum, 1% non essential aminoacids, 50 µg/ml streptomycin, and 50 µg/ml of pencillin at 37 ºC. Assay was performed in standard six well tissue culture plates. Monolayers were washed twice with phosphate buffer saline (pH 7). Approximately 10^6 cells of *Pediococcus pentosaceus* ID9 was added to each well containing monolayer of cell lines and incubated for 1h at 37 ºC. Afterward unattached bacteria was removed by washing three times with PBS, fixed with methanol and finally stained with giemsa stain. Adhered bacteria were counted in 20 random microscopic fields under oil immersion microscope. Adhesion capacity of bacteria was enumerated based on number of bacterial cells attached on INT 407. When <5 bacterial cells were attached those bacteria were considered as non-adhesive, when 6-40 bacteria were adhered to epithelial cells are adhesive and when >40 bacterial cells were found attached on INT 407 cells were considered as strong adhesive.

Media Formulation for Enhanced Biomass

Effects of carbon and nitrogen sources were studied for biomass production. MRS medium was enriched with 1% carbon (glucose, fructose, mannose, maltose, xylose, sucrose, lactose, sorbitol, jaggery) and 1% nitrogen sources (peptone, beef extract, yeast extract, tryptone) separately.

These combinations were inoculated with *Pediococcus pentosaceus* ID9 and incubated for 24 h. Afterwards broth was centrifuged at 10000 rpm for 10 min and dry weight of pellet was measured.

Synthetic media employed for biomass production (MRS media) is not economically feasible due to high cost of nutrients such as yeast extract, peptone and salts (Hofvendahl and Hahn-Hagerdal, 2000). Numerous inexpensive raw materials (industrial and agro waste) are promising substrates for biomass production. One of the potential substrate is whey for lactic acid bacterial biomass production. In this study a cost effective food grade medium was developed using whey as a major component for *Pediococcus pentosaceus* ID9 for biomass production. Whey is highly nutritive and majority of whey is unutilized. This wastage can be prevented if whey can be used as a substrate for value added products. The combinations of full strength and half strength preparation of MRS media along with whey is given in Tables 1 and 2.

Table 1: Combination of Whey and MRS

Combination No	MRS Concentration (W)	Whey Concentration (V)	Distilled Water (V)
I	Half strength	1000 ml	-
II	Full (Single) strength	1000 ml	-
III	Half strength	500 ml	500 ml
IV	Full (Single) strength	500 ml	500 ml
V	Full (Single) strength	-	1000 ml

Table 2: MRS Composition

S. No.	Constituents	MRS Half Strength g/L	MRS Full Strength g/L
1	Proteose peptone	5	10
2	Beef extract	5	10
3	Yeast extract	2.5	5
4	Dextrose	10	20
5	Polysorbate	0.5	1
6	Ammonium citrate	2	2
7	Sodium acetate	2.5	5
8	Magnesium sulphate	0.01	0.1
9	Manganese sulphate	0.025	0.05
10	Dipotassium phosphate	1	2

RESULT AND DISCUSSION

Isolation of Lactic Acid Bacteria and Antimicrobial Activity of Probiotic Organism

Lactic acid bacteria are rod or coccus shaped gram positive, catalase negative, facultative anerobe and ubiquitous heterogeneous microbe. In this study a total forty seven strains were isolated from dairy and non dairy products. These strains were initially screened for gram positive and catalase negative characteristic. Among these only twenty two were gram positive and catalase negative. These isolates were selected for further screening. Shehata *et al.* (2016) reported LAB were isolated from various sources such as raw milk, Saraniya and Jeevaratnam (2014) and Abegaz (2014) reported isolation of probiotic organisms from cheese fermented beverage, traditional fermented foods. In this study isolated potential probiotic *Pediococcus pentosaceus* was isolated from fermented idli batter.

Screening for Antimicrobial Activity

Cell free supernatants from these twenty two isolated strains were screened for their antimicrobial activity against indicator strains mentioned in materials and methods. The production of antimicrobial compounds (organic acids, hydrogen peroxide short chain fatty acids and bacteriocins) is one of the characteristic properties of probiotics (Fuller, 1989). These antimicrobial compounds inhibit the growth of microorganism causing food spoilage and used as natural preservatives in foods and also provides protection against harmful pathogens in intestinal tract (Suskovic *et al.*, 2010). Among twenty two isolates, eight isolates commonly inhibited *E.coli* and *Salmonella typhii*. Among these eight, *Pediococcus pentosaceus* ID9 inhibited *Enterobacter aerogens* and *Staphylococcus aureus* along with *E.coli* and *Salmonella typhii.* The details of the results are shown in Table 3. All the indicator organisms are food borne pathogens. *Salmonella* and *E.coli* are the most common reported foodborne pathogen causing intestinal infection. Many out breaks are associated with contaminated egg, meat and poultry products. The *Staphylococcus aureus* is

Table 3: Antimicrobial Activity of Isolated Lactic Acid Bacteria Against Indicator Organisms

Isolates	*Enterobacter aerogens*	*Staphylococcus aureus*	*E.coli*	*Salmonella typhii*	*Listeria monocytogenes*	*Bacillus subtilis*
Lactobacillus CA1	+	-	+	+	-	-
Lactobacillus CF4	-	-	+	+	-	-
Lactobacillus ID7	+	-	+	+	-	-
Lactobacillus C8	-	-	+	+	-	-
P.pediococcus ID9	+	+	+	+	-	-
Lactobacillus C10	-	-	+	+	-	-
Lactobacillus D11	-	-	+	+	-	-
Lactobacillus CA6	-	-	+	+	-	-

an opportunistic pathogen capable of producing thermostable enterotoxins. It is a common environmental microbe found in raw milk (Jackson *et al.*, 2012) and dairy products. In preparation of fermented foods, starter culture activity was poor at temperature above 10 ºC, in this situation contamination of staphylococcal intoxications (Cretenet *et al.*, 2011) are reported.

Staphylococcus aureus infections are treated with pencillin, but recent years pencillin resistant *Staphylococcus aureus* has increased (Shafigi *et al.*, 2012). Researchers are in search for new effective alternative to control *Staphylococcus aureus* infection. The isolated Probiotic lactic acid bacteria ID 9 can control the growth of *Staphylococcus aureus* along with other food born infection causing organisms such as *E.coli, Enterobacter aerogens and Salmonella typhii.*

Characterization of Antimicrobial Substances

Cell free supernatant from these eight isolates when treated with protease and catalase did not affect inhibitory activity. This indicates isolates antagonism was not due to bacteriocin or hydrogen peroxide production. Supernatant adjusted to pH 6 also did not exhibit any inhibition on indicator strains. This proves inhibition was due to acidity that is production of organic acids. Shokryazdab *et al.* (2014) reported isolated *Lactobacillus* strains produced organic acids which inhibited *E.coli*. Neal-Mckinney *et al.* (2012) also found that inhibitory effects of *L.acidophilus, L.crispatus, L.gallinarum* and *L.helviticus* against *Campylobacter jejuni* were due to production of organic acids. Quantification of lactic acid was determined by 10.71% by titration method.

Survivability of P.pentosaceus ID9 in Simulating Conditions in Gastrointestinal Tract

Survival at Different pH

Acid tolerance and bile salt tolerance are most important criteria in probiotic selection (Shin *et al.*, 2008). To exert beneficial effects on host, probiotics must reach the distal ileum and colon in large quantities for adhesion and colonization. But there are hurdles that prevent probiotics from establishing in large intestine. They are low pH (2.5) maintained in stomach, pH 8 in intestinal tract, food vehicle used for probiotics (such as yoghurt and fermented milk) are also acidic (pH 4-5). Probiotics must survive in wide pH range. Therefore tolerance of probiotic lactic acid bacteria in wide pH range is a critical criterion for selection of probiotics.

In this study we investigated survivability of the *eight* isolates from pH 2.5 to 9. At pH 2.5 no significant absorbance was measured in any isolates. But in MRS agar viable colonies were observed with *P.pentosaceus* ID9 at pH 2.5. All strains survived and proliferated from pH 3 to 9. (Table 4).

Yuksekdag and Aslim (2010) studied the survivability of various *Pediococcus* strains at pH 1 to 3. Only two *Pediococcus pentosaceus* strains Z12P and Z13P were able to survive at pH 1- 3. The probiotic *Pediococcus acidilactici* P2, *Lactobacillus curvatus* RM10, *Pediococcus pentosaceus* FF (Erkkila and Petaja 2000) and *Leuconostoc mesentroides* (Allameh *et al.*, 2012) observed growth at pH 3. In this present study, all the isolates were able to proliferate from pH 3 to 9 but *P.pentosaceus* ID9 was able to survive at pH 2.5 to 9.

Table 4: Acid Tolerance of Probiotic Isolates

Isolates	pH							
	2.5	3	4	5	6	7	8	9
Lactobacillus CA1	-	+	++	+++	+++	+++	+++	++
Lactobacillus CF4	-	+	++	+++	+++	+++	+++	++
Lactobacillus ID7	-	+	++	+	+++	+++	+++	++
Lactobacillus C8	-	+	++	+++	+++	+++	+++	+++
P.pediococcus ID9	+	+	++	+++	+++	+++	+++	+++
Lactobacillus C10	-	+	++	+++	+++	+++	+++	++
Lactobacillus D11	-	+	++	++	+++	+++	+++	++
Lactobacillus CA6	-	+	++	++	+++	+++	+++	++

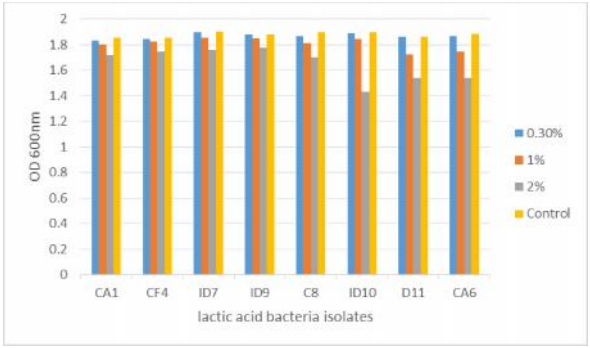
Note: *optical density 0.5-1.0 "+"; 1.0-2.0 "++"; >2.0 "+++" from pH 3 to 9. pH 2.5 survivability on agar plates.

Survival in Bile Salts

Bile salts are synthesized in liver and secreted in duodenum. Bile salts acts as detergents and reduce the survivability of probiotics by disrupting their cell-membrane lipids and fatty acids. At normal hours the bile salt concentration is in between 0.1%-0.5%. During first hour of digestion bile salts reaches to 2% in animal or human intestine (Gotcheva *et al.*, 2002; and Subrato Hati *et al.*, 2014)

In this study, effect of bile salts on survivability of probiotics was examined with 0.3%, 1%, 2% concentrations. After 24h of incubation visible growth was observed in all concentrations of bile salt (Figure 1). This result indicates that *P.pentosaceus* ID 9 and other isolates can effectively survive in 2% bile salt concentration. Many authors have studied the tolerance of probiotic on various concentrations of bile salts. Ajitha and Aparna *et al.* (2016) reported that isolated *Bacillus* spp were able to survive and proliferate in 2% bile salt. *Pediococcus acidilactici* and *Pediococcus pentosaceus* showed tolerance in 0.4% bile salt (Noohi *et al.*, 2016) *Lactobacillus plantarum* C6 effectively survived in 2% bile salts (Subrato Hati *et al.*, 2014), *Lactobacillus* Spp. *Lactococcus* spp. *Leuconostoc* Spp. (Balamurugan *et al.*, 2014) displayed tolerance to 0.3% bile salts. Decreased in absorbance when compared with control was measured with *Pediococcus pentosaceus* KCC 23 in presence of 0.5% bile salts (Ilavenil *et al.*, 2015). In this study all isolates survived and proliferated at all bile salt concentration. Based on the results the isolated strain *Pediococcus pentosaceus* ID 9 was able to survive and proliferate in intestinal conditions.

Figure 1: Bile Salt Tolerance of Isolates at Diffferent Concentration



Bile Salt Hydrolysis

The ability of probiotic strains to hydrolyse bile salt is often considered as important criteria for probiotic strains selection. Cholesterol plays a crucial role in human heart health (Hongbao Ma, 2004). High-Density Lipoprotein (HDL) is good cholesterol and Low-Density Lipoprotein (LDL) is bad cholesterol. LDLs have little protein and high levels of cholesterol and HDL has a lot of protein and very little cholesterol. High cholesterol in serum leads to cardiovascular disease such as coronary heart disease and stroke (Tabas, 2002). Bile salt hydrolysis is partially related to cholesterol lowering (Begley *et al.*, 2006). Bile salt hydrolysis is part of the bile salt metabolism in mammals and is dependent on the intestinal microûora (Tanaka *et al.*, 1999). Some lactic acid bacteria secrete bile salt hydrolase enzyme which hydrolyses conjugated bile acids to deconjugated bile acids and also release amino acids. When these salts are secreted from the gastrointestinal tract, the demand for cholesterol is increased, thus lowering cholesterol levels (De Rodas *et al.*, 1996). Microbial bile salt hydrolysis aids in integration of cholesterol into bacterial cell wall, assimilation of cholesterol by bacteria (Liong and Shah, 2005) and thus provides a better chance for probiotics to survive and colonise in gut. (Taranto *et al.*, 2003) and also reduction in serum cholesterol.

P.pentosaceus ID9 along with other isolates exhibited bile salt hydrolysis activity which was observed as different colony morphology when compared with colonies on control plates. Ilavenil *et al.* (2015) isolated *Pediococcus pentosaceus* from Italian rye exhibited bile salt hydrolysis activity. Argyri *et al.* (2013) also reported bile salt hydrolase activity in lactic acid bacteria. Therefore *P.pentosaceus* ID9 may be effective in lowering cholesterol in human.

Haemolytic Activity

Haemolysis is the ability of bacteria to lyse the blood cells. Haemolysin plays an important role in virulence and it augments the chance of infection (Morandi, 2006). Probiotics should be non-virulent non-haemolysis. All eight isolates exhibited non haemolytic activity in blood agar. Similar results were reported by Saraniya and Jeevaratnam (2014) with *Lactobacillus* isolated from uttappam batter. Ryu and Chang (2013) reported isolated *Lactobacillus plantarum* NO1, *Pediococcus pentosaceus* MP1 and *Lactobacillus plantarum* AF1 from kimchi were exhibit non haemolytic activity.

Antibiotic Sensitivity

Antibiotic susceptibility influence probiotic survivability in human gut. Resistance to antibiotics is the major concern worldwide. It restricts the application of lactic acid bacteria as probiotics (Tsaia *et al.*, 2004). According to EFSA bacteria with antibiotic resistant genes are undesirable for food industry. It rises the chance of transfer of resistant genes to pathogens. As per the European Union (EU) Scientific Committee on Animal Nutrition (SCAN) guidelines, probiotic used in feeds should be free of any acquired antibiotic resistances (SCAN, 2002). Results of 16 antibiotics against *P.pentosaceus* and other isolates are tabulated in Table 5. In the present study *P.pentosaceus* ID9 was sensitive to antibiotics namely pencillin, amphicillin, chloramphenicol, erythromycin, amoxyclav, clindamycin, co-trimoxazole. And moderately sensitive to ciprofloxacin, gentamycin whereas resistance to streptomycin, kanamycin, vancomycin, polymyxin, cephalexin, cloxacillin, cefoxitin. Antibiotic susceptibility results are referred with reported strains of *Pediococcus pentosaceus* KCC-23 (Ilavenil *et al.*, 2015) *P. pentosaceus* CRAG3 (Shukla and Goyal, 2014) with minor variations.

16srRNA Gene Sequencing

Among eight lactic acid bacteria strains, isolated lactic acid bacteria ID9 displayed maximum tolerance to acid and bile salts, exhibit non haemolytic activity, antimicrobial activity against *Staphylococcus aureus, E. coli, Salmonella typhii, Enterobacter aerogens and* sensitive

Table 5: Antibiotic Sensitivity of Isolated Pediococcus Pentosaceus ID9

Antibiotics	Concentration µg	*Pediococcus pentosaceus* ID9	*P.pentosaceus* CRAG3	*P.pentosaceus* KCC-23
Pencillin	10	S	M	NA
Amphicillin	10	S	M	S
Amoxyclav	10	S	M	NA
Chloramphenicol	25	S	S	S
Kanamycin	30	R	R	S
Tetracyclin	30	S	S	S
Streptomycin	25	M	NA	S
Gentamycin	25	M	M	R
Cefoxitin	30	R	R	S
Cephalexin	10	R	NA	R
Co-trimoxazole	25	M	R	R
Ciprofloxacin	10	S	R	NA
Vancomycin	30	M	R	NA
Erythromycin	15	S	S	NA
Polymyxin	30	R	NA	NA
Clindamycin	2	S	S	NA

Note: * Depending on zones isolates were categorized into resistance (displaying inhibition zone of 0-2 mm) moderate (3-6 mm) and sensitive (7-13 mm).

Table 6: Biomass Obtained in Combination of MRS and Whey

Combination No	MRS Concentration (W)	Whey Concentration (V)	Distilled Water (V)	Biomass Dry wt G/L
I	Half strength	1000 ml	-	0.601
II	Full (Single) strength	1000 ml	-	0.641
III	Half strength	500 ml	500 ml	0.598
IV	Full (Single) strength	500 ml	500 ml	0.574
V	Full (Single) strength	-	1000 ml	0.218

to maximum antibiotics. This strain was identified as *Pediococcus pentosaceus* based on 16srRNA gene sequencing. Table 6 represents BLAST report of *Pediococcus pentosaceus* ID 9.

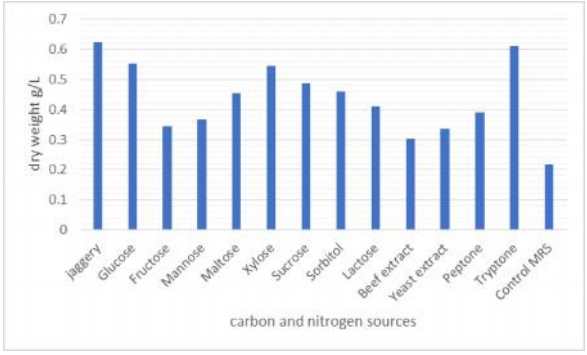
Adhesion on Cell Lines

Adhesion property is the key for probiotics to colonize and compete with pathogens in gastrointestinal tract (Shukla and Goyal, 2014). Isolate *P.pentosaceus* ID9 showed good adherence to intestinal epithelial cell lines, i.e., above 40 *P.pentosaceus* ID9 were attached to intestinal cells of INT 407. The isolated probiotic *Pediococcus pentosaceus* adhered to INT 407 are depicted in SEM photograph 1 and 2.

Media Formulation for Enhanced Biomass Production

In this study, initially the effect of carbon and nitrogen compounds in MRS medium was investigated. Among the carbon sources jaggery and glucose produced maximum biomass of 0.624 g/L and 0.584 g/L respectively. Among nitrogen sources tryptone produced maximum biomass 0.612 g/L (Figure 2). A large amount of probiotic biomass is required for industrial food applications. There is a need to develop cost effective food grade media for probiotic biomass production.

Figure 2: Biomass (Dry Weight g/L) of *Pediococcus pentosaceus* ID 9 in Various Carbon and Nitrogen Sources



Whey is a major by-product of dairy industry. World whey production is over 160 million tons per year and more that 30% of the whey is still unutilised and dumped into river and seas (Shreyansh Jain *et al.*, 2013) whey could cause high environmental damage equivalent to that caused by faecal waste produced by 1900 humans (Bozanic, 2014). This whey is a serious pollutant, as the biological oxygen demand of whey is in between 30,000-50,000 mg/lit (Sushim Chaudhary and Balasubramanyam, 2015). Whey is rich source of carbohydrate and proteins, hence when supplemented with extra nutritional sources act as an efficient growth media for probiotics. Different concentration of MRS (full strength and half strength) in combination with varying proportion of whey was further studied for biomass production. When compared with control (full strength MRS in distilled water), elevated biomass was measured in presence of whey. Maximum biomass was obtained with both half strength and full strength MRS constituents dissolved in whey, i.e., combinations I and II (table no1 (0.601g/L and 0.641g/L of dry weight) respectively (Table 7). Hence these combinations were further studied by supplementing with 1% Jaggery and 1% Tryptone. A significant improvement of biomass was observed in half strength of MRS dissolved in whey (1.042 g/L) compared to full strength of MRS components dissolved in whey (1.010 g/L). MRS is composed of nutritive compounds which are not cost effective. Finally a whey based media was formulated with half strength MRS, 1% tryptone and 1% jaggery dissolved in whey was used as an effective growth media for *Pediococcus pentosaceus.* In this combination biomass production was enhanced from 0.218 g/L to 1.042 g/L (dry weight).

CONCLUSION

The probiotic *Pediococcus pentosaceus* ID9 was isolated from idli batter and exhibited acid tolerance, resistance to bile salt, shows bile salt hydrolase activity, antibiotic sensitivity, non haemolytic activity and invitro cell adhesion ability. This strain shows antimicrobial activity against potential food pathogens such as *E.coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Enterobacter aerogens*. An efficient and economically feasible media was formulated using whey and jaggery. Therefore the isolated strain *Pediococcus pentosaceus* ID9 fulfils the invitro tests stated jointly by ICMR and DBT for selection of potential probiotic strain for human and animal consumption.

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