ISSN 2321-743X
International Journal of Research in
Engineering and Bioscience
Volume 3 (Issue 2) Pages: 19-29
IJREB
Journal home page: www.ijreb.org

EXOPOLYSACCHARIDE PRODUCING PROBIOTICS FROM EMU

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ABSTRACT

6 strains were isolated from EMU based on their probiotic activity. The strains were named as EM 1 to EM 6. The strains were gram positive rods, catalase negative, absence of ammonia from Arginine, forms acid curd from Litmus test and gas from glucose. 60% of the strains show their ability to grow at pH3. They are resistant to bile and develop bile salt hydrolase activity. All strains were positive in Exopolysaccharide production, 4 strains are fast acidifying isolates in lactic acid production, the strain EM 5 revealed the highest auto aggregation property, EM 1showse highest hydrophobic ability. We conclude that the strains could belong to lactobacilli and they can be used as a potential probiotic.

KEYWORDS: Probiotics, Auto aggregation, Hydrophobicity, Exopolysaccharide and DPPH

ISSN 2321-743X
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INTRODUCTION

Probiotics are cultures of beneficial bacteria that improve the balance of the intestinal milieu by modifying the intestinal Microflora and suppressing enhanced inflammatory responses. Recent scientific investigation has supported a role for probiotics as a part of a healthy diet for humans and animals and may be an avenue to provide a safe, cost effective, barrier against microbial infection (Parvez *et.al*., 2006).Probiotics have been considered to have potential health promoting benefits as biotherapeutic agents (Begley *et.al*., 2006).

Several work have shown that probiotic bacteria can be isolated from a variety of habitats including dairy products, meat products, sewage, humans, plants and arnimals (Kandler & Weiss, 1986). They are non-pathogenic, technologically suitable for industrial processes, acid fast, bile tolerant, adhere to the epithelial tissue and produce antimicrobial substances, including organic acids, hydrogen peroxide & bacterio cins (Dunne *et. al.*, 1999).

Lactobacillus sp, has been associated with several probiotic effects in humans and animals (Park *et.al*., 2007).Lactic acid bacteria are classified in the group of gram positive bacteria. They usually non sporulating bacteria that produce lactic acid. Lactic acid bacteria produce a number of anti microbial substances such as lactic acid, H_2O_2, Diacetyl and Bacteriocins (Klaenhammer, 1988).

MATERIALS AND METHODS

Sampling

In the present study, fecal matter samples of emu collected from the poultry farm located in and around erode. The samples were collected in sterile small bottles and stored in laboratory under refrigeration at 4°C until they were used for experiments.

Isolation and Enumeration of Probiotic Strains

The Samples were homogenized and serially diluted from 10^{-1} to $10^{-9.}$ From the dilution factor 10^{-6}, 10^{-7}, 10^{-8} was selected and 0.1 ml of solution from the above dilution was spread onto Rogosa and Sharpe (MRS) supplemented with 0.5% L-cystine. Plates were incubated in Anaerobic Jar (Himedia) provided with disposable gas generating pack (Himedia) at 37°C for 48 hr. The conventional pour plate method for the enumeration of microbe was employed. According to the growth habits and

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characteristics [Cell Sharpe, Cell Size, Gram and Colony Morphology] the colonies were randomly picked, purified thrice by streaking on the corresponding isolation medium plates.

The preliminary identification were done essentially according to Sharpe (1979) as follows: Gram's staining, catalase test, ammonia from Arginine, Litmus Test, gas production from Glucose, cell motility was detected by cultivation in a semi-solid medium (Gerhardt *et.al*., 1981). Sugar fermentation patterns were determined using API to CHL system (Himedia, India) according to the instructions of the manufacturer. The growth effects of temperature were studied in MRS broth at 40°C and 70°C. The growth effects of pH were studied in MRS broth at pH 3 .The growth effect of NaCl were studied in MRS broth at 1%, 3% and 5 % NaCl.

Bile salt Tolerance

The Bile salt tolerance test was adapted from the method of Sirilun *et.al*. (2010). MRS agar with and without bile salt were prepared. The pH of the agar was adjusted to 6.5 Overnight culture of each strain was streaked on MRS agar with 0.15 % and 0.30% (W/V) bile salt (Sigma, USA). The culture plates were incubated anaerobically at 37°C for 48 h. The growth strain was mentioned as bile salt tolerant strain.

Bile Salt Hydrolase activity (BSH)

The BSH activity assay was measured using a modified method of Sirilun *et.al*., (2010).Stationary phase growth at each isolate was investigated for the bile salt hydrolase activity by streaking on MRS agar plate supplemental with 0.5 (W/V) sodium salt of Taurodeoxycholic acid (Sigma, USA) and 0.37 g/l of $CaCl_2$ (Himedia, India). Plates were incubated anaerobically at 37°C for 72 h. The precipitation zone surrounding colonies indicated the bile salt hydrolose activity of bacteria.

Auto Aggregation Assay

Strains were grown as described above in 3 ml MRS broth pH 6.0 with cystine and they were harvested at 2400 X g. Supernatant was retained in a different tube. The pellet was washed twice with phosphate buffered saline (PBS) 0.02 M pH 7.4 and resuspended in same buffer until an optical density (O.D) of 0.9 units at 600 nm was reached. From this suspension 3 ml were harvested at 2400 X g. Supernatant was eliminated and cells were resuspended in their original broth. They were incubated for 2h at 37ºC and then, 1 ml was

ISSN 2321-743X
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IJREB
Journal home page: www.ijreb.org

taken from the superior part of the culture and the O.D was measured. Finally culture was shaken and total O.D was measured. The auto aggregation (%A) is expressed in the following equation 1- (O.D superior culture / O.D Total) x 100 (Del. Re *et.al*. 2000). The experiment was done in triplicate.

Hydrophobicity

Strains were grown as described above in 3 ml of MRS broth pH 6.0 with cystine cultures were washed with PBS buffer and resuspended as described previously. 2 ml of bacterial suspension were transferred in to another tube and 0.4 ml of xylene was added. Tubes were shaken for 2 min and reposed for 15 min. After that O.D of aqueous phase at 600 nm was measured. O.D. decrease in aqueous phase was considered as a measurement of cells surface Hydrophobicity (% H), %H was calculated according to the following equation $[(A_0 – A) / A_0] \times 100$. Where A_0 & A, are the absorbance before and after Xylene extraction respectively (Del Re *et.al*., 1998; Gusils *et.al*., 2002).

Estimation of Lactic Acid

The production of Lactic acid was determined by titrating 100 ml of the supernatant against 1 M NaOH using 1 ml of Phenolphthalein indicator (0.5% in alcohol). The titratable acidity was calculated as Lactic acid (%V/W) each ml of 1 N NaOH is equivalent to 90.08 mg of lactic acid (A.O.A.C 1990).

Exopolysaccharide Production

Exopolysaccharide production was evaluated as reported by Mora *et.al*. (2002). Overnight cultures were streaked on the surface of plates containing ruthenium red Milk (10% W/V., Skim milk Powder, 1% W/V, sucrose and 0.08 g/l ruthenium red, 1.5% W/V agar) (Sigma). After incubation at 37ºC for 24 h, non-ropy strains gave red colonies due to the staining of the bacterial cell wall, while ropy stairs appeared as white colonies.

Resistance to Antibiotics

Bacterial antibiotic resistance was determined on solid MRS medium by the use of 6 different antibiotics (Himedia). The result was expressed as sensitive (S) or resistant (R) .Thanks to the standard disc diffusion method (National Committee for clinical Laboratory standards, 1999). Two strains with known antibiotic resistance were used as the control strains.

ISSN 2321-743X
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Antimicrobial Activity

The antimicrobial activity was screened by agar well diffusion method (Schillinger *et.al*., 1989). Overnight culture of strains was isolated in MRS broth and incubated for 24 hours at 37ºC. Cells were removed by centrifugation at 10,000 x g for 15 minutes at 4ºC. The supernatant fluid was adjusted to pH 7.0 with 4M NaOH. Prepoured MRS agar plates were overlaid with 5 ml MRS soft agar containing 70 ml of indicator culture .Wells of 5 mm in diameter were cut into the agar plate by using a cork borer and 100 ml of the culture supernatant fluid was placed in to each well. The plates were incubated over night at 37ºC. After 24 h of incubation time, the diameter of the zone of inhibition was measured and scored. The inhibition zone was scored as follows, large than 10 mm equals strong inhibition (+++), between 5 and 10 mm equals moderate inhibition (++) and less than 5 mm equals weak inhibition (+).

RESULTS

A total of 6 probiotic strains have been isolated from the Sample and they designated as EM1, EM2, EM3, EM4, EM5, and EM6.

Morphological, physiological and biochemical examination

All isolates were Gram positive, non-motile, catalase negative rods. They are observed as single but occasionally in pairs and rarely in short chains. The isolates were mesophilic and facultative anaerobes, the optimum temperature and pH for the growth lies around 37^{o}C and 6.8 respectively. The isolates were not grown at 4^{o}C and all most all the isolates grow at 70^{o}C (Table 1). The isolates showed a considerable growth in 1% NaCl and strains 4 and 5 exhibit growth at 3% NaCl. Except the strain EM 4 all other isolates were inhibited by the presence of 5% NaCl.

At pH 3, isolates EM1, EM2, EM5 and EM6, were showed a good survival rate. In Litmus test, all the isolates were developing a Acid curd by producing a Lactic acid (Table – I). All the isolates showed negative results in ammonia from arginine test. All the 6 strains did not produce gas from glucose. The patterns of carbohydrate fermentation were obtained. The more significance difference between the fermentative patterns was presented in Table 1.

Bile salt tolerance test

The strains were tolerable to 0.15% and 0.30% bile salt (Table 1).

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Engineering and Bioscience
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IJREB
Journal home page: www.ijreb.org

Bile salt hydrolose activity

All the 6 strains were displayed BSH activity by developing the precipitation zone around colonies on plate assay (Table 1)

Exopolysaccharide production

The colonies are ropy and they appeared as white, it shows all the 6 strains were positive in exopolysaccharide production (Table 1).

Table 1. Biochemical and physiological characters

S.No	Strain Name	Catalase Test	SUGAR FERMENTATION TEST								Gas from Glucose	Determination of Growth of Different pH		Growth of Different Temp		Growth of Different Nacl %			Litmus Test	R to Bile	NH from Arginine	Bile salt Hydrolyte (BSHA)
			F	L	G	M	M	S	Go	I	-	pH3	pH5	4°C	70°C	1%	3%	5%				
1	EM1	-	A	A	A	-	A	-	-	A	-	+	+	-	-	+	-	-	AC	R	-	+
2	EM2	-	A	A	A	A	-	-	-	A	-	+	+	-	+	+	-	-	AC	R	-	+
3	EM3	-	A	A	A	-	A	-	A	A	-	-	+	-	+	+	-	-	AC	R	-	+
4	EM4	-	A	A	A	-	A	A	-	A	-	-	+	-	+	+	+	+	AC	R	-	+
5	EM5	-	A	A	A	-	-	-	-	A	-	+	+	-	+	+	+	-	AC	R	-	+
6	EM6	-	A	-	A	A	-	-	-	A	-	+	+	-	+	+	-	-	AC	R	-	+

F – Fructose, L – Lactose, G – Glucose, M – Maltose, M – Mannitol, S – Sucrose, G – Galactose, I – Inluin A – Acid, AC – Acid Curd, R – Resistance, "+" - Positive, "-" - Negative

Resistance to antibiotices

Table 2 shows the results obtained for antibiotic susceptibility of the 6 strains tested. All strains have showed a multi resistance to 4 different antibiotics Penicillin, Vancomycin, Amphicillin and Methicillin. Most of the strains show intermediate to Erythromycin. (Strains EM1, EM2, EM3, and EM6.) The strains EM3 and EM 4 showed resistance to chloromphenicol. The strains EM6 susceptible to chloromphenicol. Except the strains EM 3, all other strains were showing a resistance to the antibiotics tetracycline (Table 2).

Estimation of lactic acid

Among the 6 strains tested for lactic acid production, 2 strains (EM1 and EM 6) are fast acidifying isolates and produced 4040.38 ± 0.97 and 2224.4 ± 0.04 mg/ml of lactic acid, 2 strains (EM2 & EM3) showed a medium acidification activity (1538.71 ± 0.1, 1445.89 ± 0.7 mg/ml lactic acid respectively) and strains EM 4 AND EM 5 showed a slow acidification

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activity, 358.5 mg. ml of lactic acid. The results are shown in Table 3.

Auto aggregation assay

Auto Aggregation abilities increase the chance of bacterial maintenance in the gastrointestinal tract. Among the investigated strains EM5 revealed the highest auto aggregating properties and EM 3 the lowest ones. The results are shown in Table 3.

Hydrophobic assay

The highest hydrophobic properties were revealed by EM 1 and EM 4, whereas the lowest ones 2 (Table 3).

Table 2. Antibiotic sensitivity test

Sl.No	Methicillin (30 mcg)	Erythromycin (15 mcg)	Penicillin (10 units)	Vancomycin (30 mcg)	Amphicillin (75 mcg)	Chlorom-phenicol (30 mcg)	Tetra cyclin (30 mcg)
EM 1	R	I	R	R	R	I	R
EM 2	R	I	R	R	R	I	R
EM 3	R	I	R	R	R	R	S
EM 4	R	I	R	R	R	R	R
EM 5	R	R	R	R	R	I	R
EM 6	R	R	R	R	R	S	R

R – Resistance, I – Intermediate, S – Sensitive

Table 3. Lactic acid estimation, Hydrophobicity and autoaggrigation

Strain No.	Lactic Acid Estimation mg/ ml	Hydrophobicity $A_H = \frac{A_o - A}{A} x100$	Auto Aggrigation %A = 1-(ODSC) / OD Total x 100
EM 1	4040.38 ± 0.97	65.82 ± 0.27	48.47 ± 0.50
EM 2	1538.71 ± 0.1	35.09 ± 0.10	52.45 ± 0.44
EM 3	1445.89 ± 0.7	46.14 ± 0.21	42.65 ± 0.31
EM 4	358.5 ± 1.34	62.36 ± 0.72	53.67 ± 0.24
EM 5	358.5 ± 0.36	38.03 ± 0.05	62.69 ± 0.30
EM 6	3224.4 ± 0.04	40.68 ± 0.15	56.00 ± 0.18

Mean ± SD

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Antimicrobial activity

A good probiotic should present the antimicrobial actions particularly to the pathogens in the GI systems. All the 6 strains displayed antimicrobial activity against all target microorganisms such as *E .coli, Bacillus, Klebsiella, Pseudomonas & Staphylococcus*. The results revealed that the antibacterial activity of the six strains could inhabit all test pathogenic bacteria at different inhabitation levels as shown in Table 4.

Table 4. Antibacterial activity

Strain No	*E.coli*	*Bacillus sp*	*Klebsiella sp*	*Pseudomonas sp*	*Staphylococcus sp*
EM 1	+	++	++	++	++
EM 2	+	++	++	+	+
EM 3	+	++	++	+	+
EM 4	++	++	++	++	++
EM 5	+	+++	++	++	++

DISCUSSION

Identification and characterization of a strain are important criteria for the selection of probiotic (Vander Aa Kuhle *et.al*. 2005). All the 6 isolates were catalase negative, gram positive rods and their sugar fermentation pattern could support the characterization of lactobacilli. Most of the strains grow at pH3 and none of the strain was grown at 70^0 C.

In our study, the strains showed resistant to 0.30% and 0.15% bile salt. Resistant against Bile salt are important factor for the selection for Probiotics, because the upper intestine contain High concentration of bile. (Corzo *et.al*., 1999). In this study, the isolated probiotic strains were evaluated for BSH enzyme activity. BSH activity was observed in all the 6 strains. The ability of strains to auto aggregation seems to be as essential prerequisite for the adhesion of bacterial cells to intestinal epithelium. (Del Re *et.al*. 2000). The screening of 6 strains of probiotic bacteria isolated from emu showed a significant auto aggregation properties. The percentage of auto aggregation lies between 50% to 66%.

Hydrophobicity of bacterial cell wall was determined on the basis of their adhesion to the hydrocarbon phase of selection (Perez *et.al*., 1998. Vinderola *et.al*., 2004). The results

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Engineering and Bioscience
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IJREB
Journal home page: www.ijreb.org

revealed that the highest value of 65 % hydrophobicity was found in strain no. EM1. All the 6 strains have significant production of lactic acid. Lactic acid inhibits the growth of other micro organisms allow them to be established in the intestinal tract. All the strains were able to grow as white or pink colonies in ruthenium red skim milk indicating their ability to produce exopolysaccharide.

Results indicated that most of the probiotic strains were resistance to the antibiotics tested. This is in accordance with various reports indicating that probiotics are normally resistant to the principle antibiotics such as penicillin, Vancomycin, Amphicillin, chloromphenicol, erythromycin and Tetracycline (Halami *et.al*., 2000 and Coppola *et.al*., 2005)

As a functional probiotic, antimicrobial activity is one of the most important properties. 6 strains had ability against all 5 microbial indicators. The antimicrobial activities of these strains are broad inhibitory spectrum of both gram-negative and gram positive (Du ward *et.al*., 2002). We showed that all 6 strains were have a interesting probiotic properties such as bile resistance, BSH activity, Exopolysaccharide production, tolerance to antibiotics, antimicrobial activity, production of lactic acid, ability of auto aggregation and Hydrophobicity. Further studies are needed to identify the species and to evaluate their immuno modulatory capabilities

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IJREB
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