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Prebiotic Effect of Cissusquadrangularis and Probiotic Potential against V.parahaemolyticus Isolated from Shrimp Cultivation Pond

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Abstract: *In the present study, the prebiotic potential of Cissusquadrangularisand immobilization ofLAB wasinvestigated against V.parahaemolyticus. The effectiveness of probiotic activity was determined by coaggregation, hydrophobicity and biofilm formation capability of both immobilized and native LAB. It was noted that the Poly saccharide ofCissusquadrangularis acted as prebiotic and stimulate or sustain the viability of LAB under chitosan entrapment. Immobilization of Lactobacillusspshowed effective autoaggregation at the 5 hour by immobilized cell and 24 hour for native LAB.It was noted that entrapment of LAB with natural prebiotic and chitosan polymer have had potent probiotic properties with improved hydrophobicity, coaggregation as well as a strong biofilm production onLactobacillus sp.Immobilized LAB have had a coaggregation 82% and 74% respectively on chitosan and alginate.*

Key word: Prebiotics, Alginate, entrapment, Probiotic, Co aggregation

INTRODUCTION

Vibrio parahaemolyticus causes gastroenteritis as a result of utilization of raw foodor partly cooked food, especially seafood. *V. parahaemolyticus* accounts for the large amount of food-borne illness.Theorder of control in *Vibrio parahaemolyticus* study on function probiotics is much needed in poultry field. Lactobacilli is a part of the commensal microbial flora of the humans, mammals and main representatives in the probiotic bacteria, might be useful candidates in prevent and treatment of infections cause by MDR bacteria due to the ability of modulate the immune responses of the host and to protect the host from pathogens by competitive exclusion (Brachkova et al., 2010; Mohammadi et al., 2011). Survival of Probioticsare affected by many factors including pH, post-acidification during products fermentation, hydrogen peroxide production and storage temperatures (Krasaekoopt et al., 2014). Providing probiotic living cells with a physical barrier against adverse conditions is an approach currently receiving considerable interest (Favaro-Trindade et al., 2011). During oral intake of commercial probiotic products, the probiotic bacteria must survive the harsh physicochemical conditions in the gastrointestinal tract, such as acidic pH of gastric juice and bile secretions of intestinal fluid and reach the colon, where it can exert its effects. Encapsulation occurs naturally when bacterial cells grow and produce exo-polysaccharides. The LABs are entrapped within their own secretions that act as a protective structure, reducing the permeability and therefore capable to survive on adverse environmental factors. Many lactic acid bacteria synthesiseexo-polysaccharides, but they produce insufficient exo-polysaccharides to be able to encapsulate themselves fully (Shah and Ravula, 2000). Probiotic encapsulation is an exciting and emerging in biopharma and developed rapidly.

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Encapsulation is the process also known as immobilisation which refers to the trapping of bacteria with a polymer material within or throughout a matrix. Encapsulation tends to stabilize, sustain and increase viability of cells during production, storage and handling. The alginate is linear polymer of the heterogeneous structure are composed by the two monosaccharide units: acid α-l-guluronic (G) and acid β-d-mannuronic (M) linked by β (1–4) glycosidic bonds. Carrageenan is another linear polymer consisting of D-galactose units alternatively linked by α(1–3) and β(1–4) bonds (Gaaloul et al., 2009).Prebiotics are non-digestible short- chain carbohydrates that acts as substrates for known prebiotics include galactooligosaccharides, fructo oligosaccharides and inulin (Al-Sheraji et al.,2013) and can be found naturally in various foods and fruits can be supplemented as prebiotic for survival of LAB. In this study a comparative analysis between starch and natural polysaccharide as prebiotic was evaluated. Chitosan biocatalysts are stable in phosphate-buffered solutions, and cross-linked low-molecular-weight counter ions results in globules in which the cells are entrapped.

MATERIALS AND METHOD

Sample Collection

The curd sample was collected from the local market.

Isolation of Lactic acid Bacteria

The MRS agar was prepared and poured into the Petridis and they were allowed to solidify. The sample was taken by using the loop and streaked into the plate. The plates were incubated at 37^{o}c for 24 hrs. Curd sample was diluted upto 10^{7} and plated on MRS agar to find out the aerobic count.

Sub Culture

The LB broth was prepared, the culture was taken from the plate using the loop and they were added into the broth. The broth was incubated for 24 h under static and shaking and OD values were recorded.

Isolation of Polysaccharides

20 g of dried sample *Cissusquadrangularis*dis solve 20 ml distilled water andheated at 50° C for 30 min. The aqueous phase filtered and mixed with 20 ml ethanol and kept under refrigeration overnight. The precipitate was collected by centrifugation and sugar was estimated.

Estimation of Reducing Sugar by DNS Method

Standard Glucose 0.2 to 1 mg/ml was prepared in a volume of 1 ml and 2ml of DNS reagent is added into the each test tubes. 0.2 ml of sample extract was makeup to one ml and mixed with 2ml DNS reagent. Distilled water used as blank. The test tubes are subjected to heating process in the heating mantle at 75^{0}C for 10 minutes. After heating, 1ml of sodium potassium tartrate solution is added each tubes and the OD value is taken.

Immobilization

Freshly grown LAB was centrifuged and the cell pellet was diluted in PBS buffer to obtain an OD 1 at 600 nm. About one ml of LAB cell suspension was mixed with polymers for entrapment. The entrapment of cells were grouped as follows

Group 1:0.3% alginates in cacl2

Group 2:05 % chitosan in 0.5 m NaOH

Group 3: alginate+ starch

Group 4:Alginate+ OPS extract

Group 5:Chitosan+Starch

Group 6: Chitosan+ OPS extract

Group 7: control LAB alone

All the entrapped cells maintained in phosphate buffer for 72 h and cells were recovered by manual lysis and sub cultured on MRS broth followed by filtration. The non-immobilized cells and immobilized LAB probiotic potential was studied by Biofilm and co aggregation test.

Biofilm Assay(Borges et al. 2012)

The wells of a sterile 12-well polystyrene microtiter plate were filled with 2 ml of MRS broth, absorbance (A600 nm) of bacterial suspensions in MRS was adjusted to 0.25 ± 0.05 in order to standardize the number of bacteria (107–108 CFU/ml) and 200 µl of overnight was added to each well. The plates were incubated aerobically for 48 h at 30°C. To quantify the biofilm formation, the wells were gently washed three times with 2 ml of sterile distilled water. The attached bacteria were fixed with 2 ml of methanol for 15 min, and then, micro plates were emptied and dried at room temperature. Subsequently, 2 ml of a 2% (v/v) crystal violet solution was added to each well and held at ambient temperature for 5 min. Excess stain was then removed by placing the plate under gently running tap water. Stain was released from adherent cells with 2 ml of 33% (v/v) glacial acetic acid. The optical density (OD) of each well was measured at 595 nm using a plate reader.

Aggregation and Coaggregation

The test organism was isolated from pond of shrimp using CLED agar. Clean sterile test tubes were taken and labeled as T1toT8 respectively. 5ml of Lactobacillus suspension was added into the T1. The bacterial suspension of V.parahaemolyticus was added into tube T2. LABandV.parahaemolyticus were taken in T3 and T8. All the cultures were previously adjusted to OD 1 at 600 nm to get a uniform cell suspension. The tubes were allowed to kept undisturbed for 24 h and were observed for aggregation and the OD values of aqueous are taken for 5 and 12 h

Autoaggregation (%) = $((A_0 - A_t)/A_0) \times 100$,

where A_0 indicates the absorbance at time 0 h and A_t indicates the absorbance every hour, up to 5 h.

Coaggregation (%) = $[(Ax + Ay)/2 - A(x + y)]/[(Ax + Ay)/2]$,

where x and y indicate the absorbance of strains in the control tubes and $(x + y)$ indicates the absorbance of the mixtures.

Surface Hydrophobicity

The test was determined using the microbial adhesion to solvents (MATS) method of Bellon-Fontaine et al (1996) Briefly, overnight cultures of Lactobacillus strains were harvested by centrifugation at 5,000 × g for 10 min, washed twice, and re suspendedin buffer to get 1 ($A0$) at OD600. Five milliliters of bacterial suspension was mixed by 2 min of vortexing with 2 ml petroleum ether (the solvent) in a 10-ml glass tube. The tubes were incubated statically for 15 min at room temperature to allow phase separation of the mixture. The aqueous phase was collected, and its OD600 was measured ($A1$). The cell surface hydrophobicity (CSH) is presented as the percentage of microbial cells retained in the solvent and calculated as

$[1 - (A_1/A_0)] \times 100$.

RESULTS AND DISCUSSION

From the curd sample pure bacterial colonies were isolated. Bacteria in plate 1b were observed by compound microscope followed by gram and capsule stain. It is clear that the bacteria was gram positive, non-capsulated, coccobacilli (Image a), occurring singly or in chains. Based on the biochemical results isolated bacteria was found to belactobacillusacidophilus and the total viable aerobic count was 7.2 × 106 CFU. Oligopolysccharide(OPS) was extracted from Cissusquadrangularis fiber and its sugar was estimated as 28 mg/g by reduction of DNS reagent. Cells were immobilized successfully with alginate and chitosan with and without extracted of Cissusquadrangularis. Growth rate of isolated LAB was found to be moderate at static and significant at 150 rpm with an OD 0.2 and 1.5 at 600 nm. Further immobilized LAB growth rate was recorded and chitosan + extracted polysaccharide LAB showed enhanced growth and maximum growth of 2.7 was observed under 150 rpm with in 20 h (Fig 1)Further this study clears that the addition of starch to alginates and chitosan showed a significant negative changes in growth of LAB.

The percentage of auto aggregation among immobilized LAB was given in table 1. Lactobacilli tested in this study showed around 68 % auto aggregation after 5 h of incubation and 70 % at 12 h incubation. Similarly the auto aggregation of V.parahaemolyticusranges between 15 and 16 % respectively for 5 and 12 h incubation. In the present study, lactobacilli showed moderate auto aggregation close to or above those found for lactobacilli isolated from other studies. The efficiency of auto aggregation followed by immobilization was evaluated and found to be increasedfrom 70 to 80% along with natural prebiotic such as LAB+Chitosan+extractedoligo polysaccharide (OPSe). The percentage of aggregation was maximum at chitosan entrapment with OPSe followed by alginate and ineffective among starch as prebiotic. The auto-aggregation is an important bacterial feature in several ecological niches, especially in human and animal

mucosa, where probiotics display their activities. The potential of auto aggregation is a crucial factor for the maintenance of significant probiotic activity among oral, gastrointestinal and urogenital tracts (Nikolic et al., 2010). Most of the Lactobacilli isolate auto aggregates ranging from low to moderate.The co aggregation ability of LAB against V.parahaemolyticuswas58%. The ability of immobilized cells and effect of prebiotic such as starch and extracted polysaccharide among co aggregation was given in figure 2. The data's give in table 2 shows that the best co aggregation 82% was found among chitosan immobilized cells enriched with OPSefollowed by 74% in alginate+OPSeentrapment under static condition. Further it was noted that addition of starch does not favour the probiotic potential of LAB.

In this study, hydrophobicity was evaluated by the microbial adhesion to xylene (non polar solvent) and, after 12 h of incubation results obtained and highly hydrophobic (75 %) of LAB alone. Where as 82% of hydrophobicity was recorded at OPSeenrichedchitosan polymer trapping and 80% by alginate+OPSe. These values of hydrophobicity are much higher than those found for other lactobacilli isolated from curd not reported by elsewhere. Immobilization with prebiotic (OPSe) enhance the hydrophobicity of LAB. Testing a strain of L. fermentum and three strains of L.plantarum, obtained hydrophobicity values ranging from zero to 1.4% reported by many studies (Bouchard et al., 2015). The result obtained in hydrophobicity, aggregation, and co-aggregation tests correspond with previous works like García-Cayuela et al. (2014) a connection between hydrophobicity of cell surface and bacterial attachment, colonization, and biofilm formation. In the present study, the selected LAB showed coaggregation abilities with the indicator strains tested but the percentages differed, depending on entrapment condition of LAB. Isolated entraped LAB showed greater biofilm formation and was compared with a standard biofilm forming Klebsiellapneumoniae. Isolates showed bio film formation but its distribution is different. The Biofilm formation was 25, 40, 72 and 76% respectively for GP 3 to GP6 at 24 h. among the different groups less than 10% biofilm activity was recorded in starch and non immobilized cells. maximum biofilm showed 95% biofilm formation which showed it as a potential probiotic.Thus, biofilm formation by probiotic bacteria is considered a beneficial property because it could promote colonization and longer permanence in the mucosa of the host, avoiding colonization by pathogenic bacteria. It has also been demonstrated that the EPS produced by some biofilm forming strains is able to inhibit the formation of biofilms by certain pathogenic bacteria (Ramos et al., 2013)

CONCLUSION

These results demonstrate that there is relationship between immobilization and co aggregation ability of *LAB* with the tested indicator strain.It has been suggested that inhibitor producing LAB, which coaggregate with pathogens, may constitute an important host defiance mechanism against infection. Biofilm formation by secretion of exopolysaccharide (EPS) was enhanced by immobilization. Subsequently, encapsulation of LAB with chitosan and polysaccharide from *Cissusquadrangularis* enhance the cell viability and probiotic potential is a future impact among poultry biomedicines.

Table 1:Auto-aggregation during 5 and 12 hours incubation

S.No	Organism	Auto aggregation percentage	
		5h	12h
T1	*V.parahaemolyticus*	15	16
T2	*Lactobacillus acidophilus*	68	70
T3	*LAB+Chitosan+OPS*	78	80
T4	*LAB+Alginate+OPS*	66	72
T5	*LAB+ Chitosan+Starch*	46	54
T6	*LAB+Alginate+ Starch*	44	58
T7	*LAB+Chitosan*	67	72

T8	*LAB+Alginate*	65	70

Table 2: Immobilization effect on Co aggregation of *V.parahaemolyticus*

Treatment	% OF CO AGGRIGATION(5 h)
V.parahaemolyticus +LAB	58
Alginate immobilized	61.00
Chitosan	72.62
Alginate+starch	29.72
Alginate+OPS extract	74
Chitosan+starch	36
Chitosan+OPS extract	82

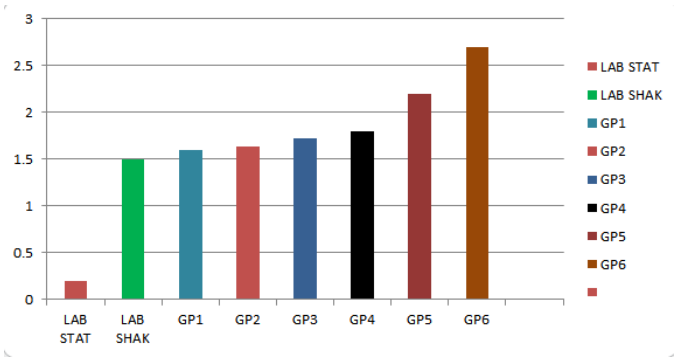

Figure 1: Growth rate on immobilized and non immobilized cells

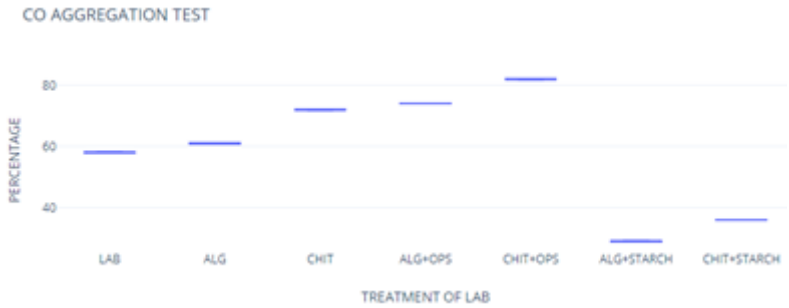

Figure 2: Effect of immobilization on co aggregation with *V.parahaemolyticus*

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