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Factors that Affect the Adhesion of Probiotics Bacteria to Resist Rice Starch

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Abstract

Prebiotics such as resistant starch can be included with probiotics to increase their survival during processing. In this study lactic acid bacteria (LAB) isolated from several sources (yoghurt, banana, and human breast milk) were screened for their probiotic properties. Ten species of bacteria overcame the stress to pH 3 and 0.3% bile. The adhesion properties of these LABs to resistant rice starch (RRS) were investigated. All 10 species of bacteria adhered to RRS within 60 min of exposure. Isolates Bn1 and HM2 were highly adhered to RRS with a total of 79% and 77% of the cells adhering, respectively. Moderate adherent

was observed by isolates, YN(70%), CY(48%), FY(55%), HM1(61,5%), HM3(65%), and HM4(50,5%), while isolate YD and Bn2 were poorly adhered to RRS (< 40%adherent). Bacteria adhesion to RRS was positively correlated to time but not to concentration. 37 °C was the ideal temperature for adhesion and Living cells are important for the adhesion.

Keywords: Probiotic, Adhesion, Resistant Starch, Lactic acid bacteria, Prebiotic

1. Introduction

Probiotics are live microorganisms that are created food supplements order to take advantage the health of the consumer by a positive impact on microbial balance in the intestine(Crittenden *et al*., 2001). Lactic acid bacteria, including Lactobacillus spp. And Bifidobacterium spp. become very popular in the dairy industry because of their therapeutic benefits. Some health benefits include improvement in intestinal disorders and lactose intolerance, altered vitamin content of milk, antagonism against various pathogenic organisms and antimutagenic and anti-carcinogenic activities. There is currently much interest in the concept of actively improving the host health by managing the colonic microflora. Traditionally, this has been attempted by using probiotics. An alternative approach is the consumption of food ingredients known as prebiotics (Rycroft *et al*., 2001).

Viability and stability of probiotics are both marketing and technological challenge for industrial producers. Probiotics to be functional, it must be viable and adequate dose levels (Galdeano & Perdigón, 2004). Production of probiotic supplements for food / feed requires that strains to maintain an appropriate level of viable cells during product processing and shelf life. Once culture is determined desirable, technological demands placed on the strains of microorganisms are great and often has new manufacturing process and formulation technologies are needed for the survival and keep healthy and functional properties accordingly. Before being delivered strains of microorganisms in food / feed products, should survive and deal with stress factors and maintain the digestive system and its biological function within the host (Mattila-Sandholm *et al*., 2002).

Prebiotics are carbohydrates of comparatively short chain length (Cummings *et al*., 2001); additionally Carbohydrates that have escaped digestion in the upper gastrointestinal tract are the pillars of the dominant growth of bacteria in the colon (Roberfroid, 2001).

Some starches also can arrive to the colon as fermentable carbohydrate sources for intestinal bacteria without digestion when they are passing through the human small intestine (Cummings & Macfarlane, 1997). Resistant starches synthesized by a number of food plants cannot digested completely because their size and molecular conformation (Vonk *et al.*, 2000).

A group of human intestinal bacteria can ferment soluble starch; such as *Bacteroides*, *Bifidobacterium, Fusobacterium*, and *Butyrivibrio* (Macfarlane & Englyst, 1986). Inclusion of resistant starches in the diet of animal models increases the population of bifidobacteria in the intestinal tract (Silvi *et al.*, 1999). It has been shown that some intestinal bacteria can adhere to starch in vitro (Tancula *et al.*, 1992).

The adhesion of bacteria depends on numerous factors, including morphological and physiological characteristics, the nature of the substrate and the environment (Fernando *et al.*, 2011).

The current work examined the ability of LAB isolated from different sources with potential probiotic properties to adhere to several resistant rice starch granules in an attempt to understand the factors that affect adhesion of these LABs to resistant rice starch.

2. Materials and Methods

2.1. Isolation of Lactic Acid Bacteria from Different Sources

2.1.1 Yoghurt and Fermented Banana

Locally available fermented dairy products, namely: Yoghurt Natural (YN), Children Yoghurt (CY), Yoghurt Drink (YD), Fruit Yoghurt (FY), and Fermented banana, which all claimed to contain lactic acid bacteria (LAB), was used in this study as sources of LAB. Three (3) samples of each product were bought from the several supermarkets were obtained.10 g of sample was added to 90 mL 0.1% peptone water and appropriate dilution was spread plated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid CM0361) plates containing 0.8% calcium carbonate ($CaCO_3$). Plates were incubated anaerobically (in anaerobe jar using Oxoidanaerogen compact) at 37 °C for 48 h. All bacterial strains were preserved in 15% glycerol stock then stored at -20 °C. They were re-cultured in MRS broth (Oxoid CM0359) at 37 °C under anaerobic condition. Each bacterial strain was sub-cultured at least three times before the experiments (1%, v/v) at 24 h (Kheadr, 2006).

2.1.2 Human Breast Milk

Sterile samples were collected from 10 healthy Mothers and then stored on ice until it is delivered to the laboratory .Then they were taken to the procedure for isolation. Isolation the organisms were by using Pour plate technique. Samples were used directly and also diluted to 10^{-1}, 10^{-2} and 10^{-3} using sterile peptone water. 1 ml aliquot of the samples and dilutions were plated into MRS-cystein agar (pH 5.5). The plates were incubated at 37 °C for 3 days under anaerobic conditions (in anaerobe jar using Oxoidanaerogen compact) (Yavuzdurmaz, 2007).

2.2 Probiotic Properties of Isolates

2.2.1 Oxbile Tolerance

The tolerance of LAB strains to oxbile (Fluka Analytical 70168) was tested using sterile flat-bottom 96-well microtiter plate (Falcon, Becton Dickinson and Company, Frankin Lakes,NJ, USA). MRS broth (Oxoid CM0359) with 0.3% w/v oxbile (Fluka Analytical 70168) was prepared, and 150 µL was added to each well inoculated with 30 µL of overnight culture previously diluted 1/1000 in the same broth. Microplate was incubated anaerobically at 37°C for 24 h. Optical densities were read at 600 nm using a biophotometer (Eppendorf Asia Pacific Sdn. Bhd) (Khaedr, 2006).

2.2.2 Acid Challenge

Ten mL of mid-log-phase MRS cultures of each isolates were harvested by centrifugation at 6,000 × g for 15 min at 4°C (AllaegraTM 25R centrifuge, Beckman CoulterTM), added to an equal volume of MRS broth (Oxoid CM0359) (pH 2.0 using 1M HCl) (Fisher Scientific). Then they incubated anaerobically at 37°C for 60 min. by diluting samples in peptone water (0.1% w/v Liofichem 610038) and spread plating appropriate dilutions onto MRS agar viable counts were determined before and after incubation. Plates were incubated at 37°C for 48 h (anaerobically) (Khaedr, 2006).

2.2.3 Oxbile Challenge

Ten mL of mid-log-phase MRS cultures of each isolates were harvested by centrifugation at 6,000 × g for 15 min at 4°C, added to an equal volume of MRS broth (pH 6.5) containing 0.3% (w/v) oxbile. The resuspended cells were incubated anaerobically at 37°C for 90 min. By diluting samples in peptone water (0.1%, w/v) and plating appropriate dilutions onto MRS agar (Oxoid CM0361) viable counts were determined before and after incubation. Then plates were incubated anaerobically at 37°C for 48 h (Khaedr, 2006).

2.3 Determination of Adhesion Level by a Co-sedimentation Assay

Determination of adhesion level by co-sedimentation assay followed the method of Crittenden *et al.* (2001). The cells were washed twice with 10 ml of 0.1 M phosphate buffer (pH 7.0) ,then they are suspended in the same buffer (concentration =10^7 cells ml^{-1}). In a 1-cm-diameter test tube two milliliters of the bacterial suspension were mixed with an equal volume of a suspension of starch granules (10 g $liter^{-1}$) in 0.1 M phosphate buffer (pH 7.0). The suspension was let for 1 h at room temperature to allow the starch to sediment. Two 1.5 mL samples were then taken from 0.5 cm below the liquid surface, and by a spectrophotometer the optical density was measured (at 540 nm). Then find the result as follows:

Percentage of cells adhering to starch = a – b/c

a = OD_{540} of a sample containing starch plus bacteria.

b = OD_{540} of a sample containing starch but no bacteria.

c = OD_{540} of a sample containing bacteria but no starch.

Highly adherent (more than70% of the cells adhered to the starch) as (40% to 70% adhesion) were named as moderate however less than 40% adhesion were named as poor adherent strains.

2.4 The Influence of Time on the Adhesion of Bacteria to Starch Granules

The method described in 2.5 was repeated. Adhesion of the bacteria to starch at 15 min, 30 min, 45 min, and 1 h were determined.

2.5 The Effect of Starch Concentration on the Adhesion of Probiotics

The method described in 2.5 was repeated. The concentration of starch that was added to bacteria was varied at 10 g $liter^{-1}$, 15 g $liter^{-1}$, and 20 g $liter^{-1.}$

2.6 The effect of temperature on the attachment of probiotics to starch granules

The method described in 2.5 was repeated. Adhesion of the bacteria to starch was determined in body temperature (bt), heat-killed cells (hk), room temperature (rt).

2.7 Influence of Growth Phase of Bacteria on Adhesion

The method described in 2.5 was repeated. Adhesion of the bacteria to starch was determined with cells that were in the late lag phase (6 h), the exponential phase (24 h), and the mid-stationary phase (36 h).

3. Results

3.1 Isolation of LAB

39 bacteria were isolated from different sources. From those isolates 18 showed clear zone on modified MRS-$CaCO_3$ agar, catalase test negative and Gram positive and were considered as LAB.

Table 1. Phenotypic characteristics of LAB isolated

NO.	Source	Code	$CaCO_3$[a]	Catalase reaction	Gram reaction	Gas from glucose	Cell morphology
1	Yoghurt Natural	YN1	+	-	+	-	Rod
		YN2	+	-	+	+	Coccid
		YN3	+	-	+	+	Coccid
2	Children Yoghurt	CY	+	-	+	-	Rod
3	Yoghurt Drink	YD1	+	-	+	-	Short rod
		YD2	+	-	+	+	Rod
4	Fruit Yoghurt	FY1	+	-	+	-	Rod
		FY2	+	-	+		Rod
5	Banana	Bn1	+	-	+	-	Short rod
		Bn2	+	-	+	-	Rod
		Bn3	+	-	+	+	Coccid
		Bn4	+	-	+	+	Coccid
		Bn5	+	-	+	+	Rod
6	Human Milk	HM1	+	-	+	+	Short rod
		HM2	+	-	+	+	Short rod
		HM3	+	-	+	+	Rod
		HM4	+	-	+	+	Rod
		HM5	+	-	+	-	Coccid

(+) positive, (-) negative

3.2 Probiotic Properties of Isolates

3.2.1 Oxbile Tolerance

Concentration of bile in the human gastrointestinal tract is different, and believed that the average concentration of bile in the intestine to be 0.3 w/v. Growth was monitored at OD600, and it was observed that all ten isolates showed varied degree of growth when grown on MRS

medium supplemented with concentration (0.3%) of bile salt. From Table 4, it was observed that HM2 showed the highest value of OD600 (1.23) while Bn2 showed the lowest absorbance (0.689). Other isolates were in the range of 0.711 to 1.092.

Table 2. Growth of LAB in 0.3% oxbile[a]

Isolate	OD60
YN	0.961
CY	0.711
YD	1.092
FY	0.933
Bn1	1.004
Bn2	0.689
HM1	0.777
HM2	1.23
HM3	0.867
HM4	0.822

[a] Titer plates were incubated at 37°C for 24 h anaerobically and growth was monitored at OD600 nm

3.2.2 Acid and Oxbile Challenge

Survival of isolates after acid and oxbile stresses is shown in (Table 5). Showed viable counts (log^{10} cfu/mL) of probiotic isolates at the beginning and end of acid and oxbile stress and percentage survival of the isolated strains in the acid and oxbile stress (Table 6). All ten isolates could survive and increased viability in acidic condition by 0.01 to 0.35 Log^{10} cfu/ml.

Table 3. Viable counts (log10 cfu/mL) of LAB isolates at the beginning and end of acid and oxbile stress experiments.

Isolates	Acid stress[a]			Oxbile stress[b]	
	0 min	60 min		0 min	60 min
YN	9.08± 0.06	9.11 ±0.03		9.29 ±0.03	9.34 ±0.01
CY	8.26 ±0.15	8.29 ±0.25		8.81 ±0.21	9.17 ±0.08
YD	9.23 ±0.05	8.98 ±0.16		9.18 ±0.09	9.27 ±0.03
FY	8.55 ±0.08	8.56 ±0.10		8.84 ±0.01	9.11 ±0.06
Bn1	9.12 ±0.00	9.31± 0.06		9.00 ±0.00	9.17 ±0.03
Bn2	7.95 ±0.29	6.00 ±0.00		7.03 ±0.00	7.47 ±0.19
HM1	7.60 ±0.46	7.69 ±0.46		7.40 ±0.32	8.26 ±0.15
HM2	9.70 ±0.02	9.92 ±0.02		8.77 ±0.04	9.37 ±0.05
HM3	8.90 ±0.19	8.98 ±0.09		9.00 ±0.26	9.33 ±0.06
HM4	7.11 ±0.26	7.20 ±0.46		8.34 ±0.28	8.37 ±0.23

(a) cells kept for 60 min in MRS broth (pH 2.0 at 37°C).

b cells let with 0.3% (w/v) oxbile in MRS broth (pH 6.5 at 37°C).

Table 4. Percentage survival of the LAB isolates strains in the acid and oxbile stress

Isolates	Survival (%)	
	Acid stress[a]	Acid stress[a]
YN	3	5
CY	3	36
YD	25	9
FY	1	27
Bn1	9	17
Bn2	35	44
HM1	9	86
HM2	22	60
HM3	8	33
HM4	9	3

3.3 Determination of Adhesion Level by a Co-sedimentation Assay

Ten species of bacteria adhered to rice starch granules with in 60 min of exposure to the granules (Figure 1.).Bn1 and HM2 adhered well to rice starch with a total of 79% and 77% of the cells adhering (highly adherent). YN=70%, CY=48%, FY=55%, HM1=61, 5%, HM3=65%, and HM4=50, 5% species adhered less well (moderate adherent). YD=20% and Bn2=18% species adhered less than 40% (Poorly adherent). It was observed that the type of rice from which the resistant starch was produced have no effect on the percent adhesion of probiotic LAB isolates.

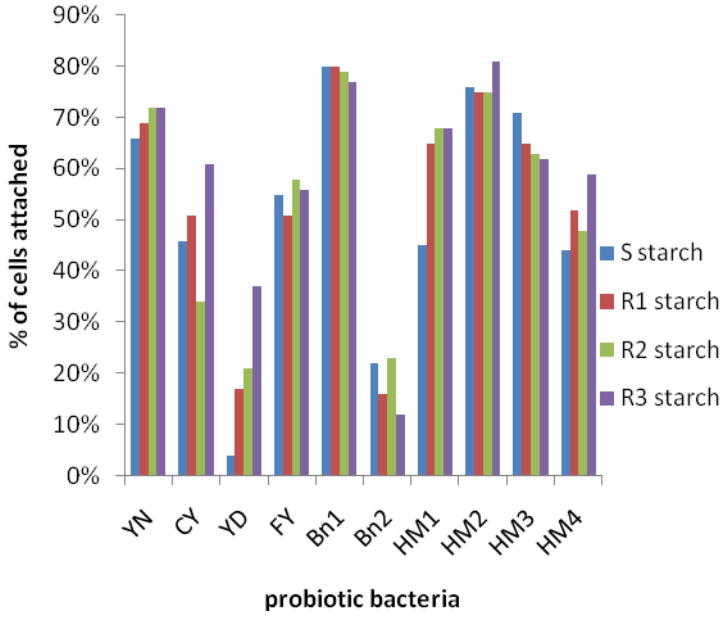

Figure 1. Percentage of cells adhering to resistant rice starch (S=soluble starch, R1=white rice starch, R2=unpolished brown rice starch, R3=grow Cambodian brown rice starch)

3.4 Effect of Time on Adhesion of Probiotics to Resistant Rice Starch

The influence of time on the adhesion of bacteria to starch granules seems clear with all samples Through the next charts (figure. 2) for example, Bn1 had the highest percentage of adhesion at the time of 60 m (80%), while we noted a gradual decline with decrease of time (79% at 45m, 71% at 30 m, and 60% at 15 m).

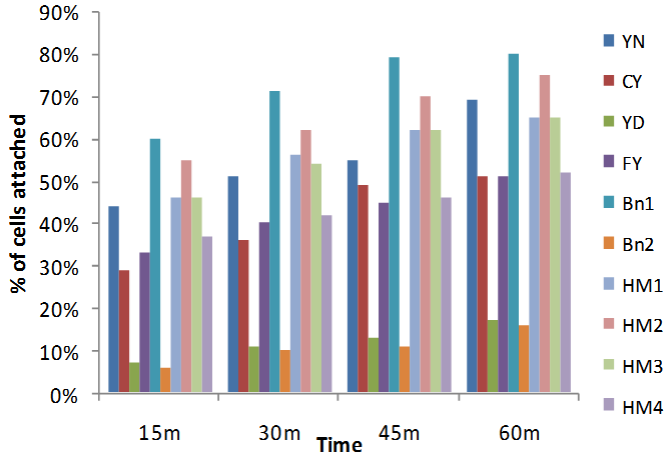

Figure 2. Effect of time on percentage adhesion of LAB to rice resistant starch

3.5 The Effect of the Concentration of Substrate from Rice Starch on the Adhesion of Probiotics

The influence of the concentration of substrate (10 g liter^{-1}, 15 g liter^{-1}, and 20 g liter^{-1}) on adhesion was examined. As you see the increase in the concentration of starch granules did not observe a significant difference in adhesion (Figure 3.)

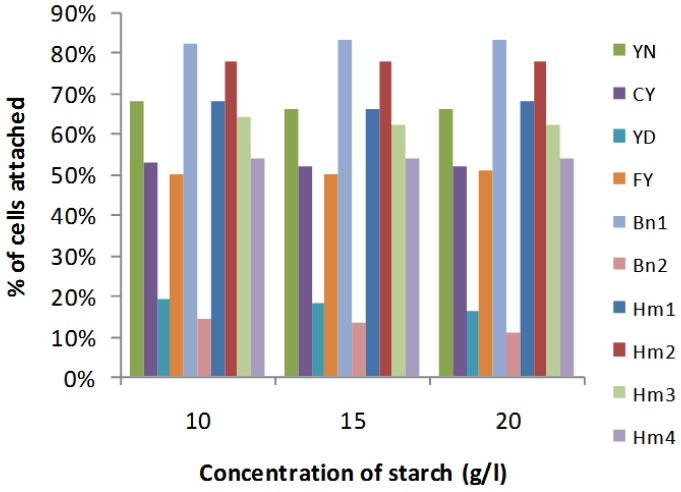

Figure 3. Effect of substrate concentration on adhesion of probiotic bacteria to resistant starch

3.6 The effect of Temperature on Attachment of Probiotics to Rice Starch.

The effect of temperature (body temperature bt, heat-killed cells hk, room temperature rt) on attachment was examined. There are decline in the proportion of cells seen in our trials

adhere to granular starch at 100 ° C compared with 37 ° C and room temperature(figure4.).

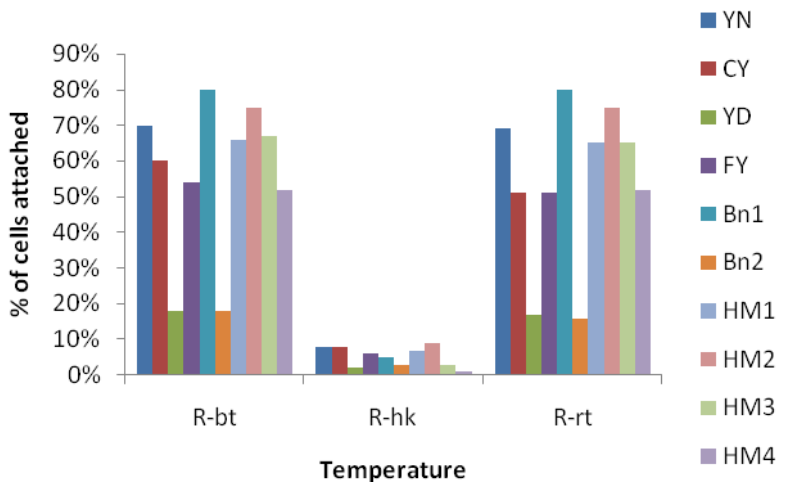

Figure 4. Effect of temperature on adhesion of probiotic to RRS

3.7 The effect of the Bacterial Growth Phase on Adhesion of Probiotic Bacteria to Rice Starch

The effect of the growth phase on adhesion was examined (figure 5.). Adhesion of the bacteria to starch was determined with cells that were in the late lag phase (ll=6 h), the exponential phase (le=24 h), and the mid-stationary phase (ms=36 h).Maximum adhesion occurred in the late exponential phase.

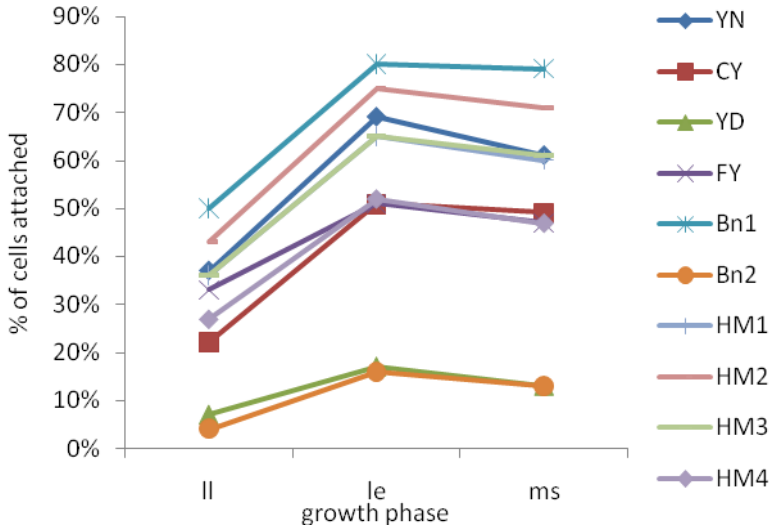

Figure 5. Effect of growth phase on adhesion of cells to RRS

4. Discussion

The use of chemical supplements or antibiotic growth promoters in animals are replaced by Probiotics as health supplements in food and feeds (Kosin & Rakshit, 2006). *Lactobacillus* and *Bifidobacterium* can restore the normal balance of microbial populations in the intestine

(Shah, 2006). One of the technological hurdles that need to be solved is the survival of probiotic during processing. Viability and stability of probiotics has been both a marketing and technological challenge for industrial producers. Probiotics to be functional, it must be sustainable and adequate doses (Galdeano & Perdigón, 2004).

Prebiotics such as resistant starch can be included with probiotics (synbiotic system) as a composite carrier matrix system to increase their survival during processing (Kosin & Rakshit, 2006). Some starches can arrive to the colon as fermentable carbohydrate sources for intestinal bacteria without digestion when they are passing through the human small intestine (Cummings & Macfarlane, 1997). Additionally, resistant starch provides the perfect surface for adherence of the probiotics to the starch granule while processing, storage and transit through the upper regions of the gastrointestinal tract (Crittenden *et al*., 2001).

It was reported that adhesion of different species of probiotics such as *Bifidobacterium* spp to native maize, potato, oat, and barley starch granule showed that starch adhesion was not characteristic of all the bifidobacteria tested (Crittenden *et al*., 2001). Our finding indicated that probiotic bacteria isolated from the various source showed different adhesion level to resistant starch extracted from three type of rice using a co-sedimentation assay. Ten species of bacteria adhered to rice starch granules within 60 min of exposure to the granules. Bn1 and HM2 adhered well to rice starch with a total of 79% and 77% of the cells adhering (highly adherent). YN=70%, CY=48%, FY=55%, HM1=61, 5%, HM3=65%, and HM4=50, 5% species adhered less well (moderate adherent). YD=20% and Bn2=18% species adhered less than 40% (Poorly adherent), adhesion to starch granules was measured by a co sedimentation assay.

The relationship between the time and adhesion is positive correlation (Yavuzdurmaz, 2007). Our findings may also indicate that the influence of time on the adhesion of bacteria to starch granules seems clear with all samples, for example, Bn1 had the highest percentage of adhesion at the time of 60 m (80%), while we noted a gradual decline with decrease of time (79% at 45m, 71% at 30 m, and 60% at 15 m) , that agreed with previous work by Fernando *et al*. (2011) which; studied attachment of Bifidobacteria and Lactobacilli to dietary fiber fractions within 15 to 60 min., and showed positive relationship between time and attachment percentage of Bifidobacteria and Lactobacilli to rice fiber . Imam and Harry (1991) found the number of *Lactobacillus amylovorus* cells bound to cornstarch granules increased with time, reaching a maximum of 60 to 75% in 30 min.

Interestingly, relationship between the surface area obtainable for bound bacteria and concentrations of the substrate is positively. However, the present study did not observe a significant difference in adhesion with an increase in the concentration of starch granules.Our result agreed with Fernando *et al*. (2011), but it Inconsistent with Imam and Harry (1991) who found that; binding of L. amylovorus cells to granules increased proportionally with the concentration of starch present in the incubation mixture.

High temperatures will deactivate the enzymes of cells and effect on cell attachment. This can be the pillars of explains the decline in the proportion of cells seen in our trials adhere to granular starch at 100 ° C compared with 37 ° C and room temperature. High temperatures

kill microorganisms, this observation refers that the micro-organisms that live cells are probably to bond with the granular than dead cells. Optimum growth temperature for these microorganisms and body temperature (37 °C) was perfect temperature to adhere (Fernando *et al.*, 2011; Yavuzdurmaz, 2007).

The influence of the growth phase on adhesion was studied. Adhesion of the bacteria to starch was determined with cells at (6 h, 24h, and 36h). So this notice confirmed that maximum adhesion happen in the late exponential phase when enzyme activity is a maximum (Crittenden *et al.*, 2001; Fernando *et al.*, 2011), it is similar result which reported by Fernando *et al*. (2011), however there is no significantly different (*P.* 0.05) in the percentages of cells adhering to the starch granules for cells harvested in the lag phase, the exponential phase, and the stationary phase (Crittenden *et al*., 2001). The effect of bacterial growth phase on adhesion was carried out with highly adherent strain *E.ludwigii*. Cells in stationary phase were capable to adhere more than 60%. In contrast, cells in exponential growth phase reached only 35% of cells adhesion (Schoebitz *et al*. 2009).

5. Conclusion

The present study focused on adhesion of probiotic to resistant starch and investigating factors affecting on adhesion of probiotics to resistant rice starch. The adhesion level was measured by a co-sedimentation assay. In addition, this study had been shown that probiotic isolates have different level of attachment to several type of resistant rice starch. Bacterial adhesion is complicated, which involve the bacteria, substrate and environment. Adhesion to starch granular may not be influenced by concentration of substrate from rice starch, but the Adhesion level was registered clear difference among different time, temperature, and growth phase.

Bacterial adhesion to starch may also supply benefits in new probiotic technologies that promote submission of viable and metabolically active probiotics to the intestinal tract. It might be possible to trade on adhesion of probiotic bacteria to starch granules in microencapsulation technology and for synbiotic food applications such as bakery products.

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