Production of *Lactobacillus salivarius*, a new probiotic strain isolated from human breast milk, in semi-industrial scale and studies on its functional characterization

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Probiotic are living microorganisms when applied to humans or animals, beneficially affect the health of the host by influencing activity of microflora of the gastrointestinal tract or indigenous microbial balance. For industrial production of probiotics belong to *lactobacilli*, it is necessary to obtain high biomass in a short time and low cost. Seven media screened for effective production of high biomass were evaluated using shake flask and incubated at 37°C, pH 7.0. The best medium supports high biomass and low lactic acid was further used to optimized using different concentrations of medium components. Furthermore, the optimized medium was used for batch cultivation of *L. salivarius* in bioreactor under controlled and uncontrolled pH conditions. This medium was composed of (g L^{-1}): glucose, 20.0, yeast extract, 20 and meat peptone, 35. Data showed that *L. salivarius* grew well in this medium with specific growth rate of about 0.179 h^{-1} in shake flask, and 0.249 h^{-1} in controlled pH bioreactor. The maxiamum biomass of 5.71 g L^{-1} and 7.57 g L^{-1} , were obtained in shake flaks and controlled pH bioreactor, respectively. To evaluate the potential use of this *lactobacilli* strain as probiotics, studies on the effect of gastric juice, pH and bile salts were conducted. Cell tolerancey to acidity and bile salt are important factor that affect the probiotics to remain and exert their potential functionalities in a host. *L. salivarius* showed higher resistant to SGJ with cell viability of 22.9%, 38.8%, 63% and 65% at pH 1,2,3 and 4,respectively. *L. salivarius* also has good functionality because of its tolerant to wide range of bile salt concentrations ranged from 0.5% to 4%. Moreover, *L. salivarius* was susceptible to antibiotics like erythromycin, rifampicin, ampicillin, and resistance to streptomycin and gentamycin. In conclusion, *L. salivarius*, new isolated from mother milk, has a big potential use as starter culture for probiotic application based on its high stability and could has potential use especially as probiotic supplement for infant milk formulation.

Keywords probiotics; *Lactobacillus salivarius*; growth kinetic; high cell density cultivation; breast milk

1. Introduction

Probiotics are viable non-pathogenic microorganisms that colonize the intestine, modify the intestinal microflora and their metabolic activities positively affect the health of the host [1]. Nowadays, many research activities have focused on the benefits of administering live microbial feed supplement to restore the normal intestinal microbial balance in human gut. To date, the increasing interests in some of these breast milk *lactobacilli* such as *L. gasseri, L. salivarius,* and *L. rhamnosus*. According to Heikkila and Saris [2], those commensal bacteria, isolated from human milk, has been identified for their potential use as bacteriotherapeutic agents in preventing neonatal and maternal breast infections which is caused by pathogenic bacteria such as *Staphylococcus aureus*. *L. salivarius* is a gram-positive, oxidase and catalase-negative, rods-shaped non-spore forming bacillus with the size of approximately 0.9 μm × 1.5-3.0 μm [3].

This bacterium belongs to homofermentative type, those produces lactic acid as the only acid produced from carbohydrates metabolism. *L. salivarius* can only be found naturally in the human oral cavities, intestines, vagina. Thus, this potential probiotic was successfully isolated from human origin such as human feces and human milk [3]. *L. salivarius* is a moderate heat tolerant microorganism and lose viability after storage under non-refrigerated temperature. However, *L. salivarius* isolated from human gastrointestinal tract was tolerant to high salt conditions [4]. Moreover, *L. salivarius* also showed tolerance when exposed to bile salts [5]. According to the research, done by Juarez et al. [6], they found that *L. salivarius* can survive in acidic conditions mimic to the stomach because this bacteria can growth at low pH. The probiotic should be resistance to *in vivo* conditions. This because of that after administration of probiotics, the number of viable bacteria should be not highly reduced by the defense mechanism of the host and also should be resistant to the specific conditions of gastrointestinal tracts.

3. Materials and method

3.1 Bacterial strains and culture conditions

Lactobacillus salivarius WICC-BO8, originally isolated from human breast milk and obtained from Wellness Industry Culture Collection, Chemical Engineering Pilot Plant, Universiti Teknologi Malaysia (Johor, Malaysia) was used throughout this study. Cells obtained from the master cell bank were used to make working cell bank through preservation of cells in glycerol culture and stored at -80°C until further use.

3.2 Growth and production media

Based on literature review for the media applied for *L. salivarius* cultivations, seven different types of broth media were used in this study. These media were composed as follows (g L^{-1}): Medium (1), soy peptone, 25; glucose, 25; yeast extract, 25 [7]; Medium (2), glucose, 45; yeast extract, 20; NaCl, 0.01;sodium acetate, 0.5;tri-ammonium citrate, 0.2;KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.2; $MnSO_4.7H_2O$, 0.05 [8]; Medium (3), Glucose, 22; Yeast extract, 5; Peptone, 10; $MgSO_4.7H_2O$, 0.1; $MnSO_4.4H_2O$, 0.038; Tween 80,1.0; tri-ammonium citrate, 2.0;sodium acetate, 8.29, KH_2PO_4, 2.0 [9]; Medium (4), Glucose, 11.0; Yeast extract, 6; Peptone, 30; $MgSO_4.7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.05; tri-ammonium citrate, 2.0; Na_2HPO_4, 2; Tween 80, 1.0 [10] ; Medium (5), glucose, 19.8; yeast extract, 5.0; sodium acetate, 18.57; KH_2PO_4, 1.007; $MgSO_4.7H_2O$, 0.197; $MnSO_4.4H_2O$, 0.045; citric acid, 0.826; Tween 80, 1; Na_2HPO_4, 2.002 [11]; Medium (6), glucose, 19.8; sodium acetate, 18.57; KH_2PO_4, 1.007; $MgSO_4.7H_2O$, 0.197; $MnSO_4.4H_2O$, 0.045; citric acid, 0.826; Na_2HPO_4, 2.002; Vitamins: L-alanine, 1.1; L-arginine, 0.5; L-asparagine, 0.7; L-cysteine, 2.5, L-isoleucine, 0.8; L-lysine,0.6; L-methionine,0.7; L-phenylalanine,0.6; L-proline,0.9; L-threonine,0.8; L-tryptophan,0.5; L-tyrosine,0.6; L-valine, 0.9; riboflavin, 0.27 [12], Medium (7), glucose, 33.24; yeast extract, 43.1;K_2HPO_4, 2.0; MgSO4.$7H_2O$, 0.02;Tween 80, 1 [13].

3.3 Inoculum preparation and cultivations conditions in shake flask

For the initial bacterial cell propagation, 1 cryogenic vial from stock culture, stored at -80°C, (contain 1 ml) was taken and used to inoculate 250 ml Erlenmeyer flask of 50 ml working volume. For inoculum preparation, MRS broth was used for vegetative growth. After inoculation, flasks were incubated on rotary shaker (Innova 4080, New Brunswick, NJ, USA) at 200 rpm and 37ºC for 24 h. The obtained cells were used to inoculate either shake flask or bioreactor to obtain cell concentration of 0.1 OD_{600} in culture.

3.4 Bioreactor cultivations

Cultivations with growing cells were carried out in a 16-L stirred-tank bioreactor with working volume of 8-L (BioEngineering, Wald, Switzerland). The bioreactor is equipped with pH probe, oxygen probe, foam sensor, and stirrer of two–four bladed Rushton turbines. For controlled pH cultivations, the pH was maintained at 7.0 by addition of 4 M NaOH and 2 M HCl solution. For uncontrolled pH experiments, the pH was also adjusted to 6.5 before inoculation and monitored only during the cultivation time. During the experiments, temperature, aeration rate and the agitation speed were controlled at 37ºC, 1 vv^{-1} min^{-1} and 400 rpm, respectively. The dissolved oxygen was adjusted to 100% saturation before inoculation and kept uncontrolled during cultivation process. The total time of fermentation was approximately 24 hours. Samples were taken at approximately 1 hour intervals and analyzed immediately for optical density and lactic acid concentration. Antifoam reagent (Silicone antifoam, Sigma, USA) was added to suppress the foaming when necessary. All medium components were prepared as in shake flask except C-source. Glucose was prepared and autoclaved separately in 1000 ml and added aseptically to the bioreactor before inoculation.

3.5 Survival of *L. salivarius* in simulated gastric juices and bile solution

The Simulated Gastric Juice (SGJ) was prepared by suspending of 3.5 g D-glucose, 2.05 g NaCl, 0.6 g KH_2PO_4, 0.11 g $CaCl_2$, 0.37 g KCl, 0.05 g oxgall bile (Difco, Lab., Detroit, MI, USA) and 13.3 g pepsin in 1000 ml distilled water according to the method of Kim *et al.* [14]. The artificial gastric juice was adjusted to different pH values (1, 2, 3, and 4) using 1M HCl. One percent cell suspension was used to inoculate MRS medium without bile salts as control. The prepared salt solution was sterilized by autoclaving at 121°C for 10 min. Both SGJ and bile solution were prepared fresh daily Samples were withdrawn and enumeration of viable bacteria was counted on MRS agar (pH 7.2) after 48 hours incubation at 37°C. 0.1 ml aliquot of bacterial suspensions was inoculated into 10 ml sterile artificial gastric juice of different pH ranged between 1 and 4. Samples were withdrawn periodically at 0, 30, 60, 90 and 120 min. The enumeration of viable bacteria was conducted on MRS agar (pH 7.2) after 48 hours incubation at 37°C. Following incubation, colony forming units were counted and recorded. Control samples without acidification were also prepared. Serial dilutions were made by using distilled water. All experiment for cell viability was conducted in triplicates.

3.6 Cell viability of *L. salivarius* in bile salt

To evaluate the survival of *L. saliva*rius in bile salts oxgal (Difco, Lab., Detroit, MI, USA), 0.5, 1.0, 2.0, 3.0 and 4.0 % concentrations of bile salts in MRS broth were prepared. MRS without bile salts was used as the control. All the solutions were sterilized at 121°C at 15 minutes. After sterilization, bile solutions and MRS broth (control) were stored at room temperature until needed. One percent inoculations were injected into fresh culture medium with MRS medium without bile salts as a control. Store solutions of each bile concentration (10 ml) were transferred into sterile universal bottle containing 0.1 ml *L. salivarius* inoculums (1%). The mixtures were plated with MRS. After plating for initials counts, mixtures were incubated for 48 hours. *L. salivarius* were then enumerated again to test for survival rates after 2 hours incubation, by intermittent plating after 0, 30, 60, 90 and 120 min. Following incubations, colony forming units were counted and recorded. Three replicates were conducted with each concentration.

3.7 Determination of antibiotic susceptibility

Cell-free supernatant was collected by centrifugation at 6000 rpm for 15 min. The antibiotics used for antibiotic susceptibility assay were tetracycline, gentamycin, erythromycin, streptomycin, ampicillin, and rifampicin. Each of the antibiotic powders was carefully weighed, dissolved, diluted in appropriate diluents filtered sterilized through a 0.2 µm cellulose nitrate membrane filter (Millipore Corp., Billerica, MA, U.S.A). Serial dilutions of antibiotics ranging from 0 to 20 mg/l were prepared. 20 µl of culture (0.5 McFarland Standard) was added into the test tube containing 20 ml of melted MRS agar, mixed gently, and the agar was poured into Petri dishes. After solidification, sterilized paper disks (8mm; Whatman, Florham Park, NJ, U.S.A) were placed aseptically on the agar surface, and the serial 2-fold solutions of the antibiotic solution (20µl) were immediately applied to each disk. Agar plates with antibiotics disks were incubated for 24-48 hours at 37°C. The inhibition zone was then measured. The results were expressed as S (sensitive), I (Intermediate) and R (resistant) of each antimicrobial.

3.8 Optical density determination

The optical density was measured by using spectrophotometer (Model DR/2500, Hach Company, Loveland, CO, USA) at 600 nm after proper dilution**.** For all samples the cultivated broth were diluted to give values less than (1 OD_{600}) for better accuracy**.** The OD of culture was converted to dry cell mass through a linear correlation standard curve. 1 OD_{600} was almost equivalent to 0.3 g L^{-1}.

3.9 Glucose and lactic acid determination

Both glucose and lactic acid were determined by High Performance High Chromatography (HPLC) obtained from Waters. For lactic acid, a 250 mm × 4.6 mm ID Spherisob Octyl Column (Waters, Milford, MA, USA) and a UV detector (210 nm) were used. The adsorbed substances were eluted with 0.2 M H_3PO_4 at flow rate of 0.8 ml/min at room temperature. For glucose, a 300 × 4 mm ID µ Bondapak/Carbohydrate column (Waters, Milford, MA, USA) with IR detector were used. The mobile phase use was acetonitrile:water (80:20) at a flow rate 1.0 ml min^{-1} at room temperature.

4. Result and Discussion

4.1 Effect of different medium composition on cell mass and lactic lactic production

The highest cell mass of about 2.27 g L^{-1} at 12 hours and 2.28 g L^{-1} for 24 hours was obtained in medium number 1, Soy Peptone Yeast (SPY) which contains, glucose, yeast extract and peptone. Therefore, this medium was selected in our primary screening based on its greatest potential as an industrial growth medium for probiotics and used further to study the growth cell growth kinetics in shake flask and bioreactor.

4.2 Kinetics of cell growth and lactic acid production by *L. salivarius* before medium optimization

L. salivarius was cultivated in SPY broth in shake flasks to determine the kinetics parameters for growth and to determine the time span of each growth phase. Cell growth, lactic acid production and change in culture pH were followed hourly for 24 hours at temperature 37°C (Figure 1). As shown, cells grew solwly during the first 2 hours followed by a rapid exponential growth phase for the next 6 hours. The stationary growth phase was observed after 12 hours growth. The cell density of *L. salivarius* increased from 0.09 to 2.64 g L^{-1} during 24 hours of growth. On the other hand, the pH decreased from 6.96 to around 5.02 with the growth profile, which was acidic to the cells growth and remained constant throughout the stationary growth. These results show that cells entered stationary phase after approximately 12 hours, as cell mass was ranged between 2.58-2.64 g L^{-1} from 12 to 24 hours. It is also worthy to note that, production of lactic acid increased from 0.26 g L^{-1} after 3 hour of cultivation to 5.4 g L^{-1} during the following 6

hour. The growth profile of *L. salivarius* followed a typical Monod type kinetic. During the exponential growth phase, the specific growth rate [μ] of *L. salivarius* was 0.084 h^{-1}

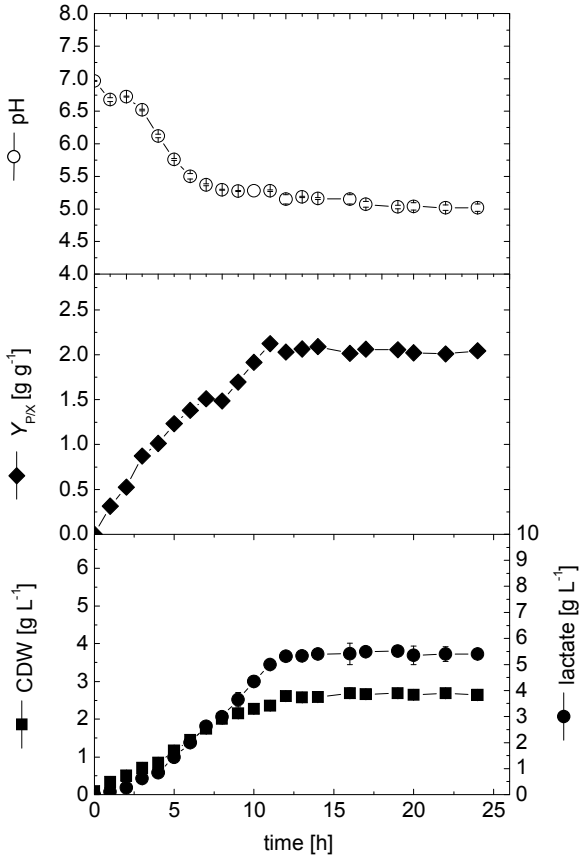

Fig. 1 Growth kinetics of *L. salivarius* in SPY medium before medium optimization in shake flask culture.

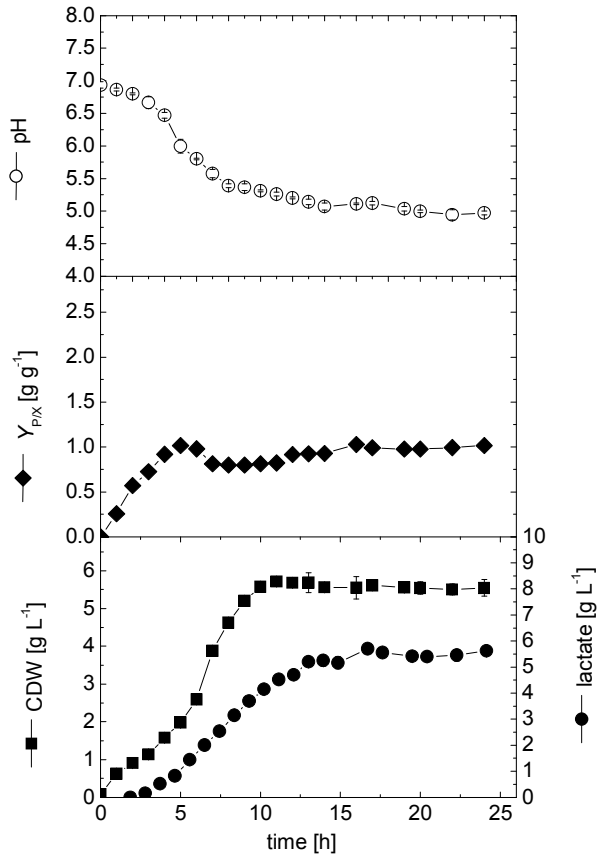

Fig. 2 Kinetics of cell growth, lactic acid production and change in pH for optimized SPY medium in shake flask culture

4.3 Kinetics of cell growth and lactic acid production by *L. salivarius* using optimized medium

Based on the data from previous experiments, the concentration of three main key nutrients were determined and therefore the new formulation was utilized in the following experiments for better understanding of production process of *L. salivarius*. The optimal medium supporting the highest cell mass was composed of (g L^{-1}): glucose, 20; yease extract, 20.0 and peptone, 35. Therefore, the kinetics of cell growth in this optimized medium were studied in more details. Figure 2 demonstrates the growth profile, change in pH and lactic acid production as a function of time when cells cultivated in shake flasks at temperature 37°C for 24 hours in optimized medium.
The data showed decline in biomass concentration from about 11 hours. As shown in figure 2, after 2-3 hours, the cell mass production increased and entered exponential phase after 5 hours and reached maximal cell dry weight of about 5.8 g L^{-1} after only 10 hours cultivation. Concomitantly, the pH of medium decreased gradually and reached its minimal value of about 4.97 after 24 hours. This decrease was associated with cell growth and lactic acid production. The specific growth rates [μ] obtained from this experiment was 0.18 h^{-1}.

4.4 Effects of pH-uncontrolled fermentation on kinetics of cell growth and lactic acid production in 16- L bioreactor

Cell growth, lactic acid production and change of pH during cultivations under uncontrolled batch fermentation in 16.L bioreactor are represented in Figure 3. *L salivarius* began to multiply almost immediately after inoculation, but in a slower rate during the first 2 hours at lag phase followed by a rapid exponential growth phase in the next 3 hours. The stationary growth phase was observed after 9 hours of growth. Experiments were performed completely exposed to 100% oxygen and under aerobic conditions. The dissolved oxygen fell rapidly during the first 12 hours. In aerobic conditions, the specific growth rate [μ] of *L. salivarius* growth was 0.23 h^{-1}. Under batch fermentations in uncontrolled pH environment, the microorganism continued growing but with at a lower growth rate.

The maximal cell growth of *L. salivarius* was about 5.38 g L^{-1} after 9 hours. On other hand, during the growth phase the pH of culture decreased significantly. As lactic acid produced, the pH of the medium decreased and resulted in the decrease in *L. salivarius* growth. Thus, pH needs to be controlled to optimize the fermentation. In the early phase, the *L. salivarius* growth was directly associated with lactic acid production. In the later after achieved stationary phase, the concentration of *L. salivarius* was approximately constant in biomasss production. In consequence, the lactic acid production was increased in parallel to the increase in the biomass. The maximal lactate production of 7 g L^{-1} was obtained after 12 h. In the case of batch fermentation under uncontrolled pH, the culture reached pH 4.75 at the end of the exponential growth phase and kept more or less constant for the rest of cultivation time. The drop of pH in uncontrolled cultivations was mainly due to the formation of lactic acid concomitantly with cell growth.

4.5 Effects of pH-controlled fermentation on kinetics of cell growth and lactic acid production in 16-L bioreactor

In this study, the growth kinetics of cell for high cell mass production in large scale 16-L bioreactor with controlled pH strategy was conducted. Under controlled pH, the growth profile is showed in Figure 4. From this profile, the culture took approximately 2 hours for begin the log phase followed by a rapid exponential growth phase in the next 5 hours. The stationary phase was observed after 10 hours of growth profile. Optimized medium under controlled pH conditions of fermentation medium resulted in a maximum specific growth rate of 0.2492 min^{-1} and a yield coefficient of 0.55 g/g. The results showed that the growth of this *L. salivarius* was greatly influenced by pH. Previous experiment resulted a low biomass count and increasing organic acid production was observed when the pH was left uncontrolled during cultivations. The growth performance was dramatically improved when the culture pH was held constant at 7.0. A maximum biomass of 7.57 g L^{-1} and maximum specific growth rate of 0.25 h^{-1} were achieved when the pH was maintained at 7.0. The concentration of lactic acid was lower and biomass increasing when the medium fermentation controlled to pH 7.0. It also observed that after 11 hours of fermentation the biomass production was constant approximately in a range between 7.1 to 7.5 g L^{-1}. The dissolved oxygen was rapidly decreased from 100% to 15.3% during 10 hours cultivation, and at this dissolved oxygen 15.3% gives the maximum lactic acid of 4.32 g L^{-1}.

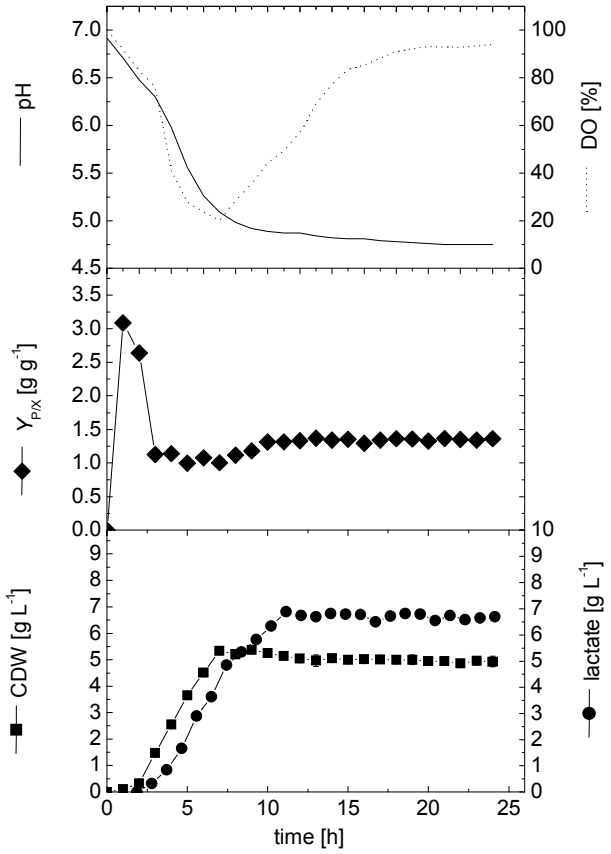

Fig. 3 Kinetics of cell growth, lactic acid production and change in pH for optimization medium in bioreactor (un-controlled pH) for 24 hours incubation

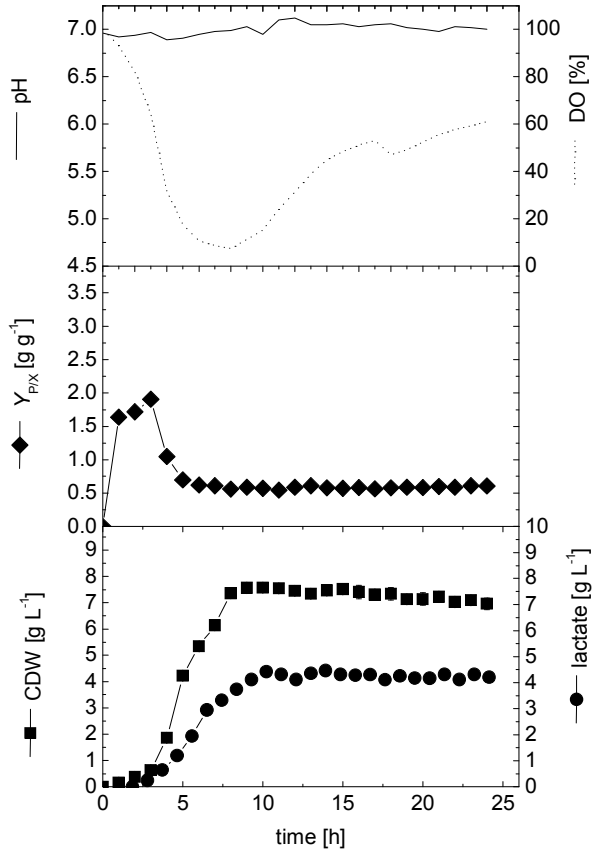

Fig. 4 Kinetics of cell growth, lactic acid production and change in pH for optimization medium in bioreactor (Controlled pH) for 24 hours incubation

4.6 Viability of L. salivarius in Simulated Gastrointestinal tract conditions

Probiotics should have the ability to withstand in acidic environments in order to be effective. Before reaching the gastrointestinal tract, probiotic bacteria must first survive transit through the stomach and have their health promoting effects as metabolically viable active cells when they arrive in the colon [15]. In our study, *L. salivarius* exhibited higher tolerance to simulated gastric juice (SGJ) and pH as well and can withstand lower pH in certain limit. When cells incubated at pH 4, 3, 2 and 1, cell viability was 65%, 63%, 38.8% and 22.9%, respectively (Table 1). In general, *L. salivarius* showed high resistance to SGJ under all pH tested. In general, *L salivarius* may survive passage through the digestive system that has specific condition such as the low pH of the stomach. Most of probiotic grow more slowly at low pH, with low viability loss. However, *L salivarius* remained stable at low pH for 2 hour with percent of survival. In similar studies, Martin *et al.* [3] reported survival of *L salivarious* CECT showed a survival rate of approximately 55% after exposition to conditions simulating these found in the gastrointestinal tract at pH 2.5. The strain viability in probiotic commercial preparation is usually varied from 41% (*Lactobacillus rhamnosus* GG and *Lactobacillus johnsonii* La1) to more than 70% (*Lactobacillus caseimunitass* [3].

Table 1. Viability in synthetic gastric juice (pH 1, pH 2, pH 3 and pH 4) of the *L. salivarius* after 0 min, 30 min, 60 min and 2 h incubation at 37°C

SGJ + varied of pH incubation at	Gastric emptying fraction Log Cfu ml $^{-1}$ (% survival)				survival after 2h Log Cfu ml $^{-1}$
	0 min	30 min	60 min	90 min	120 min
SGJ + pH 1	8.70 (100%)	2.49(28.6%)	2.31(26.59%)	2.12 (24.3%)	2.0 (22.9%)
SGJ + pH 2	8.73 (100%)	3.79(43.4%)	3.64 (42%)	3.69(42.2%)	3.39(38.8%)
SGJ + pH 3	10.7(100%)	9.95(92.3%)	9.90 (91.8%)	7.76 (72%)	6.79 (63%)
SGJ + pH 4	10.17(100%)	9.47(93.1%)	8.77 (86.2%)	7.80 (77) %	6.61 (65%)

4.7 Viability of *L. salivarius* in bile salts conditions

Bile tolerance is one of the essential properties required for lactic acid bacteria to survive in the small intestine and to be functionally effective in intestine [16,17]. According to Suskovic *et al.*[18] and Goldin and Gorbach [19], probiotic bacteria should be able to grow in 0.15%-0.30% oxgall. *L. salivarius* tested in this study was either resistant or tolerant to 2 hour of incubation in MRS broth supplemented with 0.5% to 4% oxgall. This result demonstrates that *L. salivarius* is more tolerant to bile salts at all tested concentration (Table 2). As shown, the number of viable cells was reduced from 12.44 to 12.21 (1.84% inhibitions), 12.33 to 12.29 (0.32% inhibitions), 10.51 to 10.16 (3.33%), 10.72 to 10.23 (4.57%), when incubated in 0.5%, 1%, 2%, 3% and 4% bile salt solution, respectively. These results demonstrate that this strain is more tolerant to bile salt and were in agreement with previous studies which reported by Vasala *et al.*[5] and Martin *et al.* [3].

Table 2. Survival of *L. salivarius* in MRS at varied of bile salt concentration at 37 °C.

MRS + varied of bile salt concentration incubation at 37°C	Log Cfu ml $^{-1}$					Inhibition [%] after 2h	Survival [%] after 2 h
	0 min	30 min	60 min	90 min	120 min		
MRS+ 0.5%	12.44	12.40	12.38	12.21	12.21	1.84%	98.16%
MRS+ 1%	12.33	12.32	12.23	12.26	12.29	0.32%	99.68%
MRS+ 2%	12.32	12.30	12.34	12.36	12.06	2.11%	97.89%
MRS+ 3%	10.51	10.34	10.28	10.26	10.16	3.33%	96.67%
MRS+ 4%	10.72	10.46	10.33	10.24	10.23	4.57%	95.43%

4.8 Antibiotic resistance

Many researchers propounded that changes may be effected in the normal human intestinal flora by the administration of therapeutic antibiotics and the intestinal microbial balance can be disturbed [20]. In our study, *L. salivarius* antibiotic susceptibility was examined and evaluated according to the Kirk-Bauer scale [21]. As shown in Table 3. *L. salivarius* was resistant to streptomycin and gentamycin and susceptible to ampicillin, tetracycline, rifampicin and erythromycin. Although, this potential probiotic bacteria, *L. salivarius* resistance to gentamycin and streptomycin, the above result will raise the confidence to use these strains as starter culture and added product for human supplement based of safety issues and stability. Salminen *et al.* [22], stated that this strain is good probiotic bacteria with excellent safety record for human consumption. This is also in agreements with those results obtained by Boyle *et al.* [23] and Ishibashi and Yamakazi [24]. In fact, probiotic should be susceptible to antibiotic to prevent or create the chances of transmitting the antibiotic resistance genes to another pathogenic bacteria in intestine [25].

Table 3. Antibiotic resistance of *L. salivarius*

Antibiotics	Concentration (μg/mL)	*L. salivarius*
Erythromycin	5	+
	10	+
	15	+
	20	+
Rifampicin	5	+
	10	+
	15	+
	20	+
Tetracyline	5	+
	10	+
	15	+
	20	+
Ampicillin	5	+
	10	+
	15	+
	20	+
Streptomycin	5	-
	10	-
	15	-
	20	-
Gentamycin	5	-
	10	-
	15	-
	20	-

References

[1] Weizman Z, Facn, MD, Alsheikh A, MD. Safety and tolerance of a probiotic formula in early infancy comparing two probiotic agents: a pilot study**.** *Journal of the American College of Nutrition.* 2006;25:415-419.

[2] Heikkhala MP and Saris PEJ. Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. *Journal of Applied Microbiology*. 2003;95:471-478.

[3] Martin R, Jimenez E, Olivares M, Marin ML, Fernanderz L, Xaus J, Rodriguez JM . *Lactobacillus salivarius* CECT 5713, a potential probiotic strain isolated from iInfant feces and breast milk of a mother-child pair. *International journal of Food Microbiolog.* 2006;122:35-43.

[4] Charteris WP, Kelly PM, Collins JK. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract*. Journal of Applied Microbiology*.1997;84:759-768.

[5] Vasala A, Panula J, Neubaurer P. Efficient lactic acid production from high salt containing dairy by-products by *Lactobacillus Salivarius Ssp. Salicinius* with pretreatment by proteolytic microorganism. *Journal of Biotechnology*. 2005;117:421-431.

[6] Juarez T, MS, Bru E, Wiese B, De Ruiz Holgado A A, Nader-Macias ME. Influence of Ph, temperature, and culture media on the growth and bacteriocin production by vaginal *Lactobacillus Salivarius* CRL 1328. *Journal of Applied Microbiology*.2001;93:714-724.

[7] Heenan CN, Adams MC, Hosken RW, Fleet GH. Growth medium for culturing probiotic bacteria for applications in vegetarian food products, *Lebensem-Wiss, U- Technoogyl.* 2002;35:171-176.

[8] Li Z, Ding S, Li Z, Tan T . L-Lactic acid production *by lactobacillus casei* fermentation with corn steep liquor-supplemented acid- hydrolysate of soybean meal. *Biotechnology Journal*. 2006;1:1453-1458.

[9] Schiraldi C, Adduci V, Valli V, Maresca C, Giuliano M, Lamberti M, Carteni M, Rosa M D. High cell density cultivations of probiotics and lactic acid production, B*iotechnology and Bioengineering*. 2002; 82:213-222.

[10] Avonts L, Uytven E V, Vuyst L.D. Cell growth and bacteriocin production of probiotic l*actobacillus* strains in different media, *International Dairy Journal.* 2004;14:947-955.

[11] Petry S, Furlan S, Crepeau M J, Cerning J, Desmazeaud M. Factors affecting exocellular polysaccharide production by *Lactobacillus Delbruekiii Subsp. Bulgaricus* grown in a chemically defined medium. *Appplied and Environmental Microbiology. 2000* 66:3427-3431.

[12] Grobben GJ, Chin-Joe IV, Kitzen A, Boels IC. Boer F, Sikkema J, Smith M R, de Bont J A M . Enhancement of exopolysaccharide production by *Lactobacillus Delbruekii Subsp. Bulgaricus* NCFB 2772 with a simplified defined medium, *Journal of Applied and Environmental Microbiology*, 1998;64:1333-1337.

[13] Lim CH, Rahim R A, Ho Y W and Arbakariya B A . Optimization of growth medium for efficient cultivation of *Lactobacillus Salivarius* I 24 using response surface method. *Malaysia Journal of Microbiology*. 2007;3:41-47.

[14] Kim P, Young Jung M, Hyo Chang Y, Kim S, Jae-Kim S, Ha Park Y. Probiotic properties of *Lactobacillus* and *Bifidobacterium* strains isolated from porcine gastrointestinal tract. *Applied Microbiology and Biotechnology*. 2007;74:1103-1111.
[15] Sung-Mee L, and Soon I M D. Screening and characterization of probiotics lactic acid bacteria isolated from korean fermented foods. *Journal of Microbiology and Biotechnology*. 2009*;*19:178-186
[16] Park H S, Lee S H, Uhm T B. Selection of microorganism for probiotics and their characterization. *Kor.J.Food Nutr*. 1998; 27:433-440.
[17] Ibrahim S A, Benzkorovainy A. Survival of b*ifidobacteria* in the presence of bile salts. *Journal of the science of Food and Agriculturec***.** 1993;62:351-354.
[18] Suskovic J, Kos B, Matosic S, Besendorfer V. The Effect of Bile salts on Survival and Morphology of a Potential Probiotics Strain *Lactobacillus acidophilus* M92. *World Journal of Microbiology and Biotechnology*. 2000;16:673-678.
[19] Goldin B R & Gorbach S L. Probiotics for humans. In Probiotics ± the Scienti®c Basis, ed. Fuller, R., pp. 355±376.London: Chapman & Hall. ISBN 0-412-40850-3; 1992.
[20] Kurmann J A. Starters for fermented milks. Section 5: Starters with selected intestinal bacteria. IDF Bulletin. 1988;227:41-55.
[21] Bauer AW, Kirby W M M, Sherris JC,Turck M . Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical and Pat*hology. 1996;45:493-496.
[22] Salminen S, Von Wright A, Morelli L, Marteau P, Brassart D, De Vos WM, Fonden R, Saxelin M, Collins K, Mogenson G, Birkeland S, and Mattila-Sandholm T. .Demonstration of safety probiotics-a review. *Internaional Journal of food. Micr*obiology.1998;44:93-106.
[23] Boyle R J, Robins-browne R M, and Tang M L K . Probiotics use in clinical practice: What are the risks? *The American Journal Clinical Nutrition*. 2006;83:1256-64
[24] Ishibashi N, and Yamakazaki S. Probiotics and safety. *The American Journal of Clinical Nutrition.* 2001;73: 465S-470S.
[25] D'Aimmo MR, Modesto M, Biavati B. Antibiotic resistance of lactic acid bacteria and *bifidobacterium* spp. isolated from dairy and pharmaceutical products. *International Journal of Food Microbiology*.2007;115:35-42.

