CHAPTER 4

RESULTS AND DISCUSSIONS

4.0 Results and Discussion

4.1 Choice of Isolation Media

The isolates were obtained through an enrichment process using MRS media and different sugars. All lactic acid bacteria are nutritionally fastidious requiring rich organic media. De Man and co-workers (1960) described a non-selective medium, which in general will support good growth of lactobacilli in general. This MRS medium contains peptone, meat extract, yeast extract and various stimulatory constituents that enhance the growth of lactic acid bacteria. The role of yeast extract in the medium is as a supplier of vitamins to the cells. Some lactobacilli have been noted to have a high requirement of riboflavin. (Rogosa *et al.*, 1961) Constituents such as acetate, citrate, polysorbitan monooleate (Tween 80), Mg^{2+} and Mn^{2+} have been described to have stimulatory effects on the growth of lactic acid bacteria. It was observed that these nutrients appear to shorten the lag phase when lactobacilli are cultured in peptone water containing these nutrients (Evans & Nivens, 1951).

Growth and metabolism of lactic acid bacteria utilise a number of enzymes that directly metabolise oxygen. Here, Mn^{2+} plays an important role in scavenging $O_2^{\cdot}$. This fact made Mn^{2+} an absolute requirement of lactic acid bacteria in the culture media in addition to the Mn^{2+} dependence of the RNA polymerases. If H_2O_2 producing systems are more active than the H_2O_2 destroying ones under aerobic conditions, the bacteria are inhibited by their own products of oxygen metabolism. (Teuber, 1993). A low pH setting of between 6.5 to 4.5 for the MRS medium would mediate favourable growth for lactobacillus as they are

aciduric or acidophilic in nature.(Hammes *et al.*, 1992). The type of carbohydrate or sugars plays a crucial role in the isolation of certain species of lactobacilli. Carbohydrate metabolism leads to the production of lactic acid or of lactic acid, CO_2, and acetate and/or ethanol. The homofermentative species utilise hexose by the glycolytic or Embden-Meyerhof pathway and the heterofermentative species by the 6-phosphogluconate pathway. The facultative heterofermentative species possess both systems, which enable the cells to utilise both hexoses and pentoses. (Kandler, 1983).

The *Lactobacillus* can be divided into three groups, namely the obligate homofermenters, facultative heterofermenters and obligate heterofermenters. *Lactococcus* species are microaerophilic and only produce L-(+) lactic acid from their fermentation of glucose (Teuber, 1992). The rationale of using different types of sugars or carbohydrates for isolation is that different types of lactic acid bacteria, especially the lactobacilli have, different carbohydrate fermentation patterns within the genus (table 4.1), this will help in isolating different species of the lactic acid bacteria.

4.2 Isolation and Screening of Indigenous Lactic Acid Bacteria from Food Sources

The isolates obtained from the enrichment process were screened for characteristics of lactic acid bacteria. The isolates were put through the catalase test and Gram's stain. With these two methods, the isolates were screened for the general characteristics of lactic acid bacteria. The four food sources screened for lactic acid bacteria were tempoyak (fermented durian paste), tapai (fermented tapioca), chili boh (Chili paste) and fresh goat's

Table 4. 1: Some of the species of lactobacilli with capabilities of fermenting certain carbohydrates. (Adapted and modified from Hammes, 1992)

Species	Fermentative Category	Lactic acid isomers	Type of Carbohydrate fermented			
			Glucose	Lactose	Mannitol	Sucrose
L.*delbrueckii* subsp. *delbrueckii*	Obligate fermentative	D	+	-	-	+
L.*delbrueckii* subsp. *bulgaricus*	Obligate fermentative	D	+	+	-	-
L.amylophilus	Obligate fermentative	L	+	-	-	-
L.casei subsp. *casei*	Facultative heterofermentative	L	+	+	+	+
L.plantarum	Facultative heterofermentative	DL	+	+	+	+
L.fructivorans	Obligate heterofermentative	nd	+	-	-	v
L.brevis	Obligate heterofermentative	nd	+	+	-	v

Indicators: **nd** ; not determined
v variable
+ ; able to ferment
- ; not able to ferment

milk. From these four sources, 126 isolates were obtained and were screened for characteristics of lactic acid bacteria. Out of the 126 isolates, tempoyak contributed 53 isolates, while tapai, contributed 36 isolates, chili boh and fresh goat's milk contributed 24 and 13 isolates respectively.

4.2.1. Catalase Test

Catalase test was performed as first line screening for lactic acid bacteria. This procedure uses 3% H_2O_2 dropped on lactic acid bacteria streaked on glass microscope slide. Lactic acid bacteria would not produce bubbles (indication of O_2 release). Lactic acid bacteria are all aero-tolerant anaerobes that grow readily on the surface of solid media exposed to air. However, they are unable to synthesise ATP by respiratory means, a reflection of their failure to synthesise cytochromes and other heme containing enzymes. One consequence of the inability to synthesise heme proteins is that the lactic acid bacteria are catalase negative, and hence cannot mediate the decomposition of H_2O_2 according to the reaction :

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

(Stainer *et al.*, 1987).

Out of the 126 isolates, 55 were found to be catalase negative.

4.2.2 Gram Staining and Microscopic Observation of the Isolates

Lactic acid bacteria according to Bergey's manual of determinative bacteriology are gram positive. 55 of the catalase negative isolates were stained. All were found to be gram positive. All of the gram-positive isolates were found to be rod shaped (plate 4.1), except the isolate from fresh goat's milk which was found to be coccus shaped (plate 4.2).

4.2.3 Gel Plug Test

Only the isolates, which tested negative for catalase and gram-positive were put through the gel plug test. This test was done to screen for the producers of CO_2. Homofermenters and facultative heterofermenters do not produce CO_2 from fermentation of glucose as do obligate heterofermenters. A positive result for obligate heterofermenters would be when the gel plug is forced up the tube by the CO_2 evolved from the fermentation of glucose. This test would thus differentiate between the homofermenters (and facultative heterofermeters) and obligate heterofermenters. From the 55 isolates that were identified earlier as lactic acid bacteria, only 16 were found to be homofermenters or facultative heterofermenters while the rest (39 isolates) were found to be obligate heterofermenters.

Teuber (1993) described the physiological categories in which the *Lactobacillus* species can be categorised into: (1) obligate homofermentative, in which more than 85% lactic acid is produced from glucose via the Embden-Meyerhof pathway (glycolysis), pentoses and gluconates are not fermented. (2) Facultative heterofermentative, in which

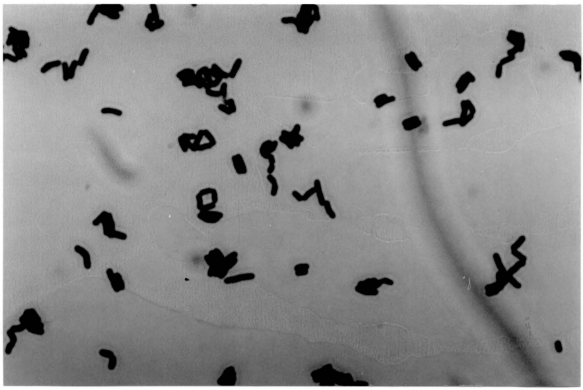

Plate 4.1: A Gram positive rod shaped isolate (1000X magnification)

Plate 4.2: A Gram positive ovoid shaped isolate at (1000X magnification)

Plate 4.3: Shows a negative (left), positive (centre) and the control (uninoculated) (right) results of the gel plug test.

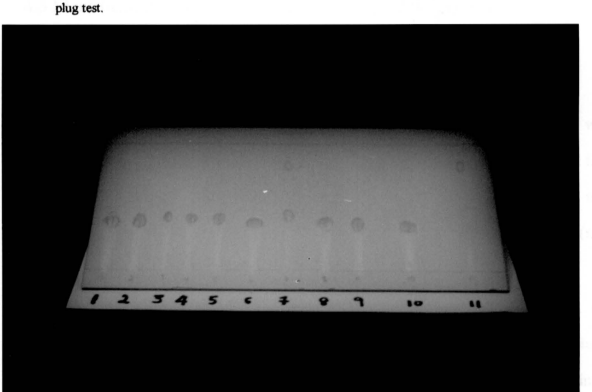

Plate 4.4: Shows the different chromatogram of seven isolates tested for D and L lactic acid isomers. (1) L-lactic acid standard (2) TapLac (3) TemSor (4) FM (5) TemMan (6) L-lactic acid standard (7) CB (8) TapSuc (9) TemGlu (10) L-lactic acid standard (11) D-lactic acid standard

85% lactic acid is produced from the Embden-Meyerhof pathway. Pentoses and gluconates are fermented via the pentose phosphate pathway. (3) Obligate heterofermentative, in which equimolar amounts of CO_2, lactic acid, and acetic acid and / or ethanol are produced. Obligate heterofermenters ferment hexoses via the 6-P-Gluconate pathway (Pentose Phosphate Pathway). For example, glucose in this pathway is phosphorylated to glucose-6-phosphate. In turn, glucose-6-phosphate is oxidised to gluconate-6-phosphate. Gluconate-6-phosphate, in turn is decarboxylated, yielding CO_2 and pentose-5-phosphate. The pentose-5-phospate is split into a C-2 and C-3 moiety. Thus equimolar amounts of CO_2, lactate, and acetate or ethanol are formed from glucose (Kandler, 1983)

4.3 Identification of Lactic Acid Isomer(s) by Thin Layer Chromatography

Out of the 16 homofermenters (or facultative heterofermenters), only seven were selected to test for the lactic acid isomer produced. These seven isolates were obtained from different food sources and isolated using different sugars. The codes for the isolates were given on the basis from which sources and the sugars used in the enrichment process (table 4.2)

The run on thin layer chromatography plates yielded blue spots. Each spot represents one type of lactic acid isomer. A single spot originating from a single point of origin shows that only a single isomer existed, while two spots from a single point of origin, show that both the D and L isomers were produced by the isolate. The spots formed on the thin layer chromatogram corresponded to the R_f of the D and L lactic acid standards run on the same thin layer chromatograph. From the seven homofermentative isolates

tested, 6 were found to be L-lactic acid producers while the isolate from chili boh was found to produce D and L lactic acid isomers (table 4.3). None of the isolates produces the single type D lactic acid isomer.

The Rf of lactic acid was calculated as follows:

a) Solvent Front = 9.7cm

b) Distance travel from point of origin for D - Lactic Acid standard = 8.4cm

c) Distance travel from point of origin for L- Lactic Acid standard = 4.8cm

R_f for D-lactic Acid = Distance travel from point of origin for D - Lactic Acid standard / Solvent Front

R_f for D-lactic Acid = 8.4cm/9.7cm = 0.866

R_f for L-lactic Acid = Distance travel from point of origin for L - Lactic Acid standard / Solvent Front

R_f for L- Lactic Acid = 4.8cm/9.7cm = 0.495

From the observation of the lactic acid isomers produced from the seven isolates, six of the isolates could be consider for lactic acid producers with the intention of making poly(lactic acid) (PLA) as they only produced only one type of lactic acid isomer. Six of the isolates, which produce single lactic acid isomer are TapLac, TapSuc, TemSor, TemGlu, TemMan and FM. These six isolates only produce the L form of the isomer. This trait of producing a single form of lactic acid isomer is desirable as it will help in the downstream process of making poly(lactic acid). Polymerisation of a single isomer is attractive compared to polymerisation of a mixture of both isomers. This is because, the control blending of poly (D-lactic acid) and poly(L-lactic acid) will help to determine the rate of degradation of the bio-polymer (Kulkarni, 1971). One isolate, CB produces both

types of the lactic acid isomer and deemed unsuitable for the lactic acid production for making PLA.

4.4 Identification of the Bacterial Isolates

The API strips were interpreted by observing the colour change of the APICHL 50 medium in the cupules. Each of the bacterial isolates was subsequently identified (table 4.4) using the API software provided by the manufacturer. The ability to ferment different sugars in the kit has been the basis for identifying different species of the lactic acid bacteria group. Each species of lactic acid bacteria generally is able ferment certain set sugars, this ability to ferment certain types of sugars is inherently connected to their metabolic pathway and this is used to set a basis for identification of lactic acid bacteria species. From the results, interpreted from the APICHL50 kit, six of the isolates were identified from the *Lactobacillus* genera. TapLac, TapSuc, TemSor, TemGlu and TemMan were identified as *Lactobacillus casei* and CB was identified as *Lactobacillus plantarum*. FM was subsequently identified as *Lactococcus lactis* on the basis of its ability to ferment a certain set of sugars and the shape of its cell.

4.5 Screening and Selection for Potential Industrial Lactic Acid Bacteria

The purpose of these tests was a rapid survey of the survival of the lactic acid bacteria isolates under several environmental conditions. Growth and metabolic activity were indicated by acid production denoted by colour change of the pH indicator.

Table 4. 2: Shows the name of the homofermentative isolates, isolation sources and substrate used in the enrichment process.

Isolate Code	Food Source from which isolated	Sugars used in MRS medium
TapLac	Tapai	Lactose
TapSuc	Tapai	Sucrose
TemSor	Tempoyak	Sorbitol
TemGlu	Tempoyak	Glucose
TemMan	Tempoyak	Mannitol
CB	Chili Boh	Glucose
FM	Fresh Goat's Milk	none

Table 4.3 : The types of lactic acid isomer produced by the isolates

Isolate	FM	TapLac	TapSuc	TemSor	TemGlu	TemMan	CB
Type(s) of Lactic Acid isomer produced	L	L	L	L	L	L	DL

Table 4. 4: The identified bacterial isolates and each of their probabilities.(API 50CHL, Biomerioux)

Isolates	FM	TapLac	TapSuc	TemSor	TemGlu	TemMan	CB
Identification	Lactococcus lactis	Lactobacillus casei	Lactobacillus casei	Lactobacillus casei	Lactobacillus casei	Lactobacillus casei	Lactobacill plantarum
Probabilities (%)	95.9	99.3	99.6	95.9	95.9	94.5	99.9

4.5.1 The Growth of Isolates and Acidification of Bacterial Cultures at Various Temperatures

(Table 4.5)

Five of the seven isolates were able to change the colour of the pH indicator from purple to yellow. This change indicated that, these five isolates could produce sufficient acid at 15°C. TapLac, TaSuc, TemSor, TemMan and CB were able to change the pH indicator while TemGlu and FM did not. Although there were changes observed in the pH value of these isolates, apparently they did not produce enough acid to positively change the pH indicator from purple to yellow. This might be due to the decrease of enzyme catalysed reaction at low temperature (Wouters et al; 2000).

All of the isolates produce sufficient acid to enable positive colour changes by the pH indicator from purple to yellow at temperature of 30°C, 37°C and 45°C. This seems to indicate the suitability for acid production of the seven isolates at these temperatures. At 50°C, only FM produces positive colour change while the other six isolates did not. Changes of pH did occur for the six isolates, but was not sufficient to create a positive change. This could be due to the heat stress on the enzyme system of the isolates at high temperature.

Table 4.5: Acidification and final pH of bacterial cultures at different temperatures after 48 hours.

	Isolates						
Temperature	FM	TapLac	TapSuc	TemSor	TemGlu	TemMan	CB
15°C	-	+	+	+	-	+	+
	(5.85)	(4.65)	(4.61)	(4.63)	(4.86)	(4.64)	(4.58)
	(0.03)	(0.02)	(0.03)	(0.02)	(0.01)	(0.03)	(0.04)
30°C	+	+	+	+	+	+	+
	(4.41)	(3.63)	(3.63)	(3.84)	(3.87)	(3.91)	(3.89)
	(0.02)	(0.05)	(0.04)	(0.05)	(0.04)	(0.04)	(0.02)
37°C	+	+	+	+	+	+	+
	(4.24)	(3.67)	(3.63)	(3.84)	(3.83)	(3.78)	(3.82)
	(0.03)	(0.02)	(0.02)	(0.04)	(0.03)	(0.06)	(0.04)
45°C	+	+	+	+	+	+	+
	(4.24)	(3.78)	(3.82)	(3.79)	(3.79)	(3.83)	(4.29)
	(0.04)	(0.03)	(0.03)	(0.02)	(0.03)	(0.05)	(0.03)
50°C	+	-	-	-	-	-	-
	(4.52)	(4.94)	(4.99)	(4.97)	(4.97)	(5.22)	(5.54)
	(0.05)	(0.03)	(0.02)	(0.03)	(0.04)	(0.03)	(0.04)

Standard deviation is in the lower bracket
pH indicator used was Bromocreosol Purple
Indicators: + (postive change); indicate colour changes from purple to yellow
- (negative change); indicate no colour changes from purple to yellow

Table 4.5: Acidification and final pH of bacterial cultures at different temperatures after 48 hours.

	Isolates						
Temperature	FM	TapLac	TapSuc	TemSor	TemGlu	TemMan	CB
15°C	-	+	+	+	-	+	+
	(5.85)	(4.65)	(4.61)	(4.63)	(4.86)	(4.64)	(4.58)
	(0.03)	(0.02)	(0.03)	(0.02)	(0.01)	(0.03)	(0.04)
30°C	+	+	+	+	+	+	+
	(4.41)	(3.63)	(3.63)	(3.84)	(3.87)	(3.91)	(3.89)
	(0.02)	(0.05)	(0.04)	(0.05)	(0.04)	(0.04)	(0.02)
37°C	+	+	+	+	+	+	+
	(4.24)	(3.67)	(3.63)	(3.84)	(3.83)	(3.78)	(3.82)
	(0.03)	(0.02)	(0.02)	(0.04)	(0.03)	(0.06)	(0.04)
45°C	+	+	+	+	+	+	+
	(4.24)	(3.78)	(3.82)	(3.79)	(3.79)	(3.83)	(4.29)
	(0.04)	(0.03)	(0.03)	(0.02)	(0.03)	(0.05)	(0.03)
50°C	+	-	-	-	-	-	-
	(4.52)	(4.94)	(4.99)	(4.97)	(4.97)	(5.22)	(5.54)
	(0.05)	(0.03)	(0.02)	(0.03)	(0.04)	(0.03)	(0.04)

Standard deviation is in the lower bracket
pH indicator used was Bromocreosol Purple
Indicators: + (postive change); indicate colour changes from purple to yellow
- (negative change); indicate no colour changes from purple to yellow

4.5.2 The Growth of Isolates and Acidification of Bacterial Cultures at Various Lactic Acid Concentrations

(Table 4.6)

All of the bacterial isolates were observed to show positive change in 5% (w/v) lactic acid concentration. At 7.5%(w/v) lactic acid concentration, only TapLac and TapSuc manage to show positive change. At 10% and 15% (w/v) of lactic acid concentration, none of the isolates managed to display a positive change. It was also observed that even though none of the isolate display positive change at 10% and 15% (w/v), pH changes were observed but not enough to cause a positive change. The inability to cause the positive change could be due to the product inhibition exerted by lactic acid on the isolates.

4.5.3 The Growth of Isolates and Acidification of Bacterial Cultures at Various Salt Concentration

(Table 4.7)

It was observed that all of the isolates were able to growth at lower NaCl concentrations until 5% (w/v). This was seen by the positive changes displayed by all the isolates. The isolates were able to produce sufficient acid to enable a change from purple to yellow to occur, indicating that the culture broth has become acidic. At 7.5% (w/v) NaCl concentration, only FM manage to display a positive change while the other isolates did not. By looking at the pH values of each isolate, only FM showed substantial change in pH. This ability by FM, to produce acid at 7.5%(w/v) NaCl concentration might be due to its ability to regulate osmotic pressure between inside and outside the cell (Kaskhet, 1987).

Table 4.6: Acidification and final pH of bacterial cultures grown at different lactic acid concentrations after 48 hours.

	Isolates						
Lactic Acid Concentration (%w/v)	FM	TapLac	TapSuc	TemSor	TemGlu	TemMan	CB
2.5	+ (4.67) (0.02)	+ (4.42) (0.04)	+ (4.04) (0.03)	+ (4.06) (0.04)	+ (4.05) (0.05)	+ (4.15) (0.04)	+ (4.10) (0.03)
5	- (5.22) (0.05)	+ (4.42) (0.03)	+ (4.38) (0.02)	+ (4.51) (0.03)	+ (4.43) (0.02)	+ (4.64) (0.02)	+ (4.37) (0.03)
7.5	- (5.53) (0.02)	+ (4.64) (0.04)	+ (4.56) (0.03)	- (5.03) (0.02)	- (4.91) (0.05)	- (5.25) (0.03)	- (4.62) (0.02)
10	- (5.78) (0.05)	- (4.75) (0.02)	- (4.77) (0.01)	- (5.24) (0.04)	- (5.11) (0.02)	- (5.38) (0.04)	- (4.97) (0.03)
15	- (5.94) (0.02)	- (5.42) (0.05)	- (5.33) (0.04)	- (5.51) (0.04)	- (5.90) (0.03)	- (5.77) (0.05)	- (5.56) (0.04)

Standard deviation is in the lower bracket
pH indicator used was Bromocreosol Purple
Indicators: + (postive change); indicate colour changes from purple to yellow
- (negative change); indicate no colour changes from purple to yellow

Table 4.7: Acidification and final pH of bacterial isolates each isolate at different salt concentrations after 48 hours

	Isolates						
NaCl Concentration (%w/v)	FM	TapLac	TapSuc	TemSor	TemGlu	TemMan	CB
1.5	+ (4.11) (0.04)	+ (3.51) (0.02)	+ (3.52) (0.02)	+ (3.50) (0.02)	+ (3.51) (0.01)	+ (3.64) (0.02)	+ (3.54) (0.04)
2.5	+ (4.02) (0.03)	+ (3.54) (0.04)	+ (3.56) (0.03)	+ (3.49) (0.05)	+ (3.53) (0.03)	+ (3.61) (0.02)	+ (3.48) (0.05)
5	+ (4.09) (0.02)	+ (4.42) (0.03)	+ (4.42) (0.03)	+ (4.60) (0.05)	+ (4.61) (0.04)	+ (4.59) (0.02)	+ (4.65) (0.03)
7.5	+ (4.22) (0.04)	- (6.62) (0.02)	- (6.57) (0.03)	- (6.69) (0.02)	- (6.63) (0.01)	- (6.57) (0.05)	- (6.58) (0.04)
10	- (6.70) (0.03)	- (6.65) (0.05)	- (6.74) (0.03)	- (6.67) (0.04)	- (6.65) (0.02)	- (6.67) (0.02)	- (6.74) (0.03)

Standard deviation is in the lower bracket
pH indicator used was Bromocreosol Purple
Indicators: + (postive change); indicate colour changes from purple to yellow
- (negative change); indicate no colour changes from purple to yellow

However, at 10% (w/v) NaCl concentration, none of the isolates produces positive changes or manage to produce significant changes in the value of their pH.

4.5.4 The Turbidity of Isolates and Acidification of Bacterial Cultures at Various Initial pH Condition

(Table 4.8)

Three pH value were chosen representing three different condition which were acidic (pH 4.5), neutral (pH 7) and basic (pH 9) conditions. From observation made at pH 4.5, all the isolates displayed a change in the turbidity except FM. This was supported that FM showed the least change in the pH compared to the other isolates. The failure of FM to display change here could be attributed to the free acid (H^+) in the culture broth. FM, identified as *Lactococcus lactis*, has been described to have a lower tolerance to free acid compared to the *Lactobacillus* species (Kashket, 1987). At pH 7, all the isolates showed positive change. This was supported by significant changes in their pH. At pH 9, only TapSuc did not display a change in turbidity. Consequently this was noticeable in the pH value. While the other bacterial isolates manage to produce or display a noticeable change or pH, FM did not display the same results. This failure to produce a significant change could be due to the inability of TapSuc internal system to tolerate very high pH (Rhee & Pack; 1980).

Table 4.8: Acidification and final pH of bacterial cultures at different initial pH conditions after 48 hours.

	Isolates						
pH	FM	TapLac	TapSuc	TemSor	TemGlu	TemMan	CB
4.5	-	+	+	+	+	+	+
	(4.35)	(3.54)	(3.53)	(3.54)	(3.59)	(3.80)	(3.54)
	(0.01)	(0.02)	(0.02)	(0.01)	(0.02)	(0.05)	(0.04)
7	+	+	+	+	+	+	+
	(4.21)	(3.99)	(3.93)	(3.98)	(3.97)	(3.99)	(3.96)
	(0.03)	(0.02)	(0.04)	(0.03)	(0.03)	(0.05)	(0.03)
9	+	+	-	-	+	+	+
	(4.31)	(4.36)	(7.81)	(4.50)	(4.24)	(4.56)	(4.20)
	(0.01)	(0.03)	(0.04)	(0.02)	(0.02)	(0.05)	(0.03)

Standard deviation is in the lower bracket
Indicators: +; indicate changes in turbidity
-; indicate no changes in turbidity

4.6 Biomass, pH, Glucose Consumption and Lactic Acid Production Profiles of a few Selected Bacterial Isolates at Various Culture Conditions.

Three isolates were selected for characterisation at various growth conditions. The three isolates chosen were FM, TapLac and TapSuc. The values of pH, cell dry weight, lactic acid production and glucose consumption were noted after 48 hours incubation time. The isolates chosen for these tests were based on their abilities to tolerate high lactic acid concentration, grow at high temperature and to produce one type of lactic acid isomer. They were cultured in basal MRS broth with 2% (w/v) glucose and incubated for 48 hours in a shaking water bath set at 37°C.

4.6.1 Effect of Temperature on Culture Profiles.

Temperature plays an important role in the production of lactic acid as it could influence productivity of an isolate. Furthermore, temperature could influence the growth rate and evolution of products. Its importance warrants an investigation of an optimum temperature for potential isolates before they could be used in the industry. Choosing an isolate that could tolerate high temperature is advantageous as the risk of bacterial contamination is lowered. (Tsai *et al.*, 1993)

4.6.1.1 Biomass and Final pH Values of the Cultures at Various Cultivation Temperatures

(Figure 4.1 and Figure 4.2)

Observing from figure 4.1, shows that all three isolates exhibited maximum growth profiles at temperature of 37°C. Two isolates, Taplac and TapSuc exhibited good growth at temperatures of between 30°C and 45°C. This was reflected in the amount of biomass generated between the two temperatures. At temperature of 15°C and 50°C, the biomass accumulation were significantly lower compared to the others. Similar results shown by other workers have found that the maximum growth rate for *Lactobacillus casei* and *Lactococcus lactis* are at 37°C. (Ishizaki *et al.*, 1990a; Krischke *et al.*, 1991; Hujanen & Linko, 1996). The isolate FM, which was identified as *Lactococcus lactis* (Table 4.4) was observed to exhibit a lower biomass profile comparing with the other isolates. This could be attributed to the size and weight of individual cells. Teuber and co-workers (1992) described the *Lactococcus* genus as spherical or ovoid cells, 0.5 to 1.0μm in diameter, in pairs or in chains. Comparatively, the *Lactobacillus* genus is about 8 to 10μm in size and rod shape and in chains. Increase in growth rate would subsequently show significant differences in weight between the two genus.

There was significant acid production as indicated by lowering of pH when the isolates were grown in 30°C, 37°C and 45°C. Less acid was produce when the isolates were cultivated at 15°C and 50°C (figure 4.2) This could be due at low temperature (15°C), enzyme reaction that catalysed the formation decreases due to the unfavourable temperature (Wouters *et al.*, 2000). High temperature might not be conducive for these isolates, as seen

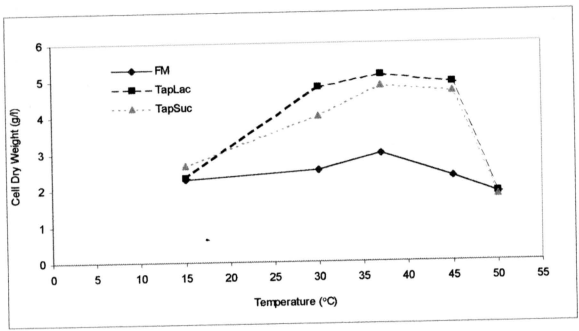

Figure 4.1: Biomass profile at various cultivation temperatures after 48 hours

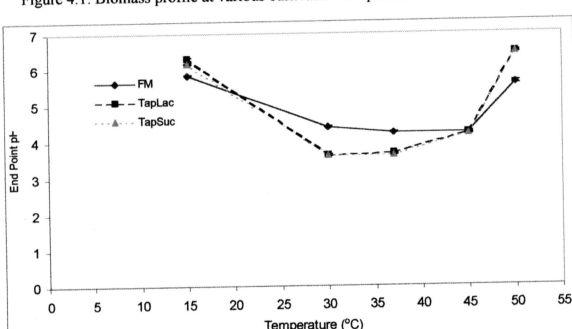

Figure 4.2: Shows the end point pH profiles at various cultivation temperatures after 48hours

Since the value of the standard deviation is to small to be observed on the figures, the values and standard deviation are included as tables in the addendum

in their biomass and end point pH profiles displayed less biomass and higher pH values compared with at 30°C, 37°C and 45°C. This could be due to heat stress excerted on their enzyme and metabolic systems. A difference in the end point pH profiles between the coccus shaped isolate (FM) and rod shaped isolates (TapLac and TapSuc) were observed between 15°C and 30°C. FM displayed a lesser inclination if compared with TapLac and TapSuc. This difference in inclination could be cause by the pH tolerance threshold of the isolates. Generally, Lactobacillus species could tolerate acidic pH, down to 3.5 while Lactococcus and Streptococcus species could only tolerate acidic pH down to 4.5. (Kaskhet, 1987)

4.6.1.2 Glucose Consumption and Lactic Acid Production at Various Cultivation Temperatures

(Figure 4.3 and Figure 4.4)

All of the isolates displayed the ability to grow, metabolise glucose and produce lactic acid at low temperature (15°C). However, the glucose consumption value for FM was higher compared to the other two isolates. This could be due the adaptability of FM at 15°C. Wouters and workers (2000) reported that a set of genes in a lactococci species was induced to increase glycolytic activity at low temperature. For FM, glucose consumption saw only a slight increase between 15°C and 30°C, while TapLac and TapSuc, a significant difference was observed. This trend was also seen in the lactic acid profile. At 37°C, maximum glucose consumption and lactic acid concentration were observed (figure 4.3 & 4.4). By comparing the three isolates at 37°C, TapLac consumed the largest amount of glucose and produced the highest concentration of lactic acid. At higher temperatures (45°C

to 50°C), saw a sharp decline in glucose consumption and lactic acid production by all the isolates. Other researchers have revealed that the optimum temperature for lactic acid production by *Lactobacillus casei* is at 37°C (Bruno-Barcena *et al.*, 1999).

Figure 4.5 shows the ratio between lactic acid production and glucose consumption in the cultures at various cultivation temperatures. The ideal ratio between lactic acid formation and glucose consumption is 2, since 1 mol of glucose could yield 2 mol of lactic acid.

1mol glucose ⟶ 2mol lactic acid

Ideal ratio: 2/1 = 2

The best ratio value displayed by FM was at 37°C, while for TapLac and TapSuc, their best ratio values were at 45°C. However, TapLac displayed better ratio values between 37°C and 45°C. The value of this ratio only displayed the conversion of lactic acid from glucose and does not reflect on the amount of glucose consumed and lactic acid produced. Although TapLac and TapSuc, displayed the best ratio values at 45°C, the amount of lactic acid produced at this temperature was much lower than at 37°C. At 50°C, the ratio values for all the isolates were at the lowest. This could be attributed to the temperature stress on the activities of enzymes involved in the Embden-Meyerhof pathway. The overview of all the isolates indicated that all of them fell into the category of mesophilic bacteria. Teuber (1993) describes mesophilic bacteria as having a temperature range between 28°C and 45°C.

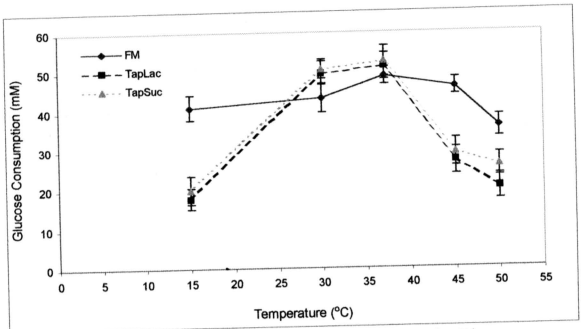

Figure 4.3: The glucose consumption (mM) at various cultivation temperatures after 48 hours

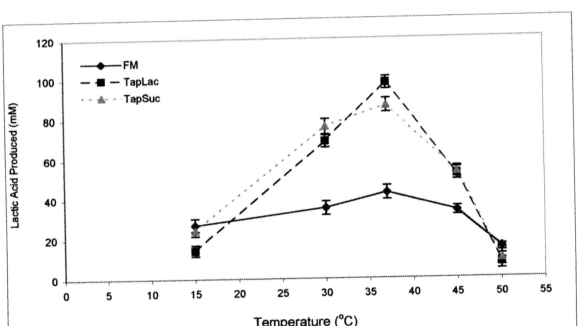

Figure 4.4: The lactic acid produced (mM) at various cultivation temperatures after 48 hours

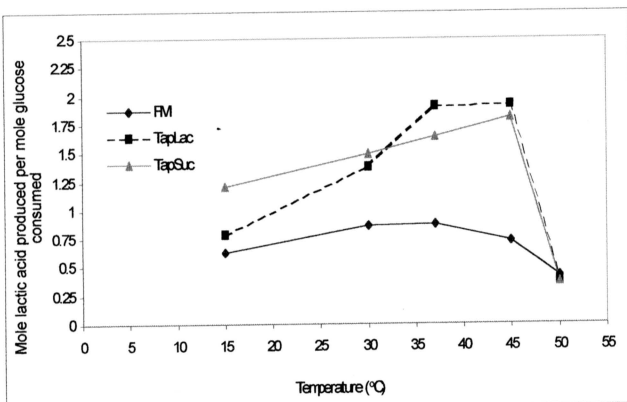

Figure 4.5: The ratio between lactic acid produced (mM) and glucose consumed (mM) at various cultivation temperatures.

4.6.2 Effect of Various Initial pH Conditions on Profiles of Lactic Acid Bacteria Cultures.

pH plays a crucial role in lactic acid fermentation. Unsuitable pH will result in low growth yield, which ultimately would influence the overall productivity of lactic acid fermentation.

4.6.2.1 Biomass and Final pH Profiles of Several Isolates at Various Initial pH Conditions.

(Figure 4.6 and 4.7)

Low pH environment is very conducive for the growth of lactic acid bacteria. Researchers prescribed pH between 5.5 to 7 as a good range for cultivation of lactic acid bacteria (Hammes *et al.*, 1992). Although an acidic environment is conducive for growth and cultivation of lactic acid bacteria, a low pH of a high concentration of H^+ could inhibit growth (Amrane & Prigent, 1999). At low pH, most of the energy derived from the metabolic pathway are being diverted from growth related purposes towards proton (H^+) extrusion system to expel H^+ from the cytoplasm to outside the cell. This could be seen in figure 4.6.

The biomass profiles of the isolates cultivated at pH 4.5 were less if compared to the same isolates when cultivated at pH 7. Also noted were the difference of biomass production between the cocci shaped isolate (FM) and the rod shaped isolates (TapLac and

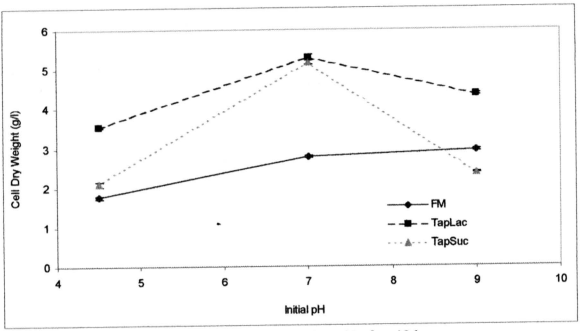

Figure 4.6: Biomass profile at various initial starting pH after 48 hours

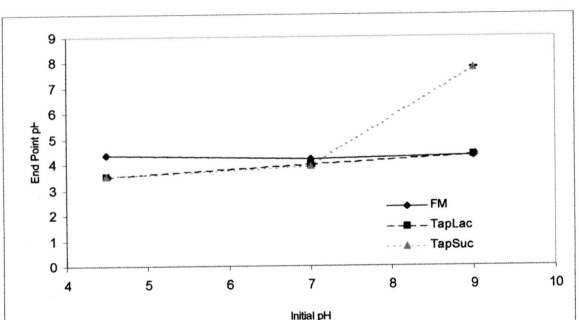

Figure 4.7: End Point pH profile at various initial starting pH after 48 hours

Since the value of the standard deviation is to small to be observed on the figures, the values and standard deviation are included as tables in the addendum

TapSuc) at pH 4.5. One contributing factor to this result could be due to the level of tolerance of the isolates to high concentration of H^+. Lactococci and streptococci have a low tolerance to high concentration of H+ than lactobacilli. This could be related to the end point pH of the isolates. When cultivated at pH 4.5, end point pH for FM displayed little change if compared with the initial pH, while TapLac and TapSuc manage to produce significant changes. When cultivated in a suitable environment, lactic acid bacteria would be able to display maximum biomass gain. This could be seen when the isolates were cultivated at pH 7. All of the isolates displayed a substantial amount of biomass production when compared at pH 4.5.

The end point pH of all the isolates displayed a slight difference when compared at pH 4.5. Here, pH threshold again might be the contributing factor. Lactic acid bacteria maintain a cytoplasm that is more alkaline than the medium, but whose pH decreases as the medium is acidified during growth and fermentation. Lactococci and streptococci generally acidified the cytoplasm from approximately pH 7.6 to 5.7 with the external pH 4.5 before growth and then fermentation ceases (Bender *et al.*, 1985). The internal enzyme machinery of these anaerobic fermenters thus tolerates a fairly wide range in internal proton concentration. Lactobacilli tolerate a significantly more acidic cytoplasmic pH of 4.4 while the external pH 3.5. However when cytoplasmic pH decreases below a threshold pH, which depends on the organisms, cellular functions are inhibited. When cultivated at pH 9, the isolates displayed different characteristics. Both TapLac and FM manage to grow while TapSuc displayed little biomass gains. This could be due to the failure of its metabolic system to function at pH 9.

4.6.2.2 Glucose Consumption and Lactic Acid Production at Various Initial pH Conditions.

(Figure 4.8 and Figure 4.9)

The value of pH plays an important aspect in lactic acid production. Low pH inhibits the growth, metabolism of substrates and product formation in lactic acid bacteria. At low pH, lactic acid exists mostly in a dissociate form. This dissociate form of lactic acid in the cell disrupts the membrane potential of the cell. Lactic acid formed brings about the acidification of the cytoplasm below the permissible. Lactic acid, which act as protonphores, or solvents, which perturb membrane phospholipids at high concentrations, increase the inward leak of H^+ so that H^+ efflux is not rapid enough to alkalinise the cytoplasm. The membrane pH gradient is thus dissipated. (Kaskhet, 1987)

This condition was seen when isolates were cultivated at pH 4.5. All the isolates displayed low metabolism of glucose and lactic acid production if compared at pH 7. This shows that a low pH inhibit the metabolism and end product forming of the cell. Most bacterial cells operate within a range of optimal environment. When cultivated outside the optimal environment, the growth, metabolism and production of primary products could be disrupted. The similar situation was observed when the isolates were cultivated at pH 9. It was observed that the consumption of glucose and lactic acid production was smaller than at pH 7. High pH could inhibit the function of enzymes in the cell such as lactate dehydrogenase, which has an optimum pH of 6.5. Also reported that some

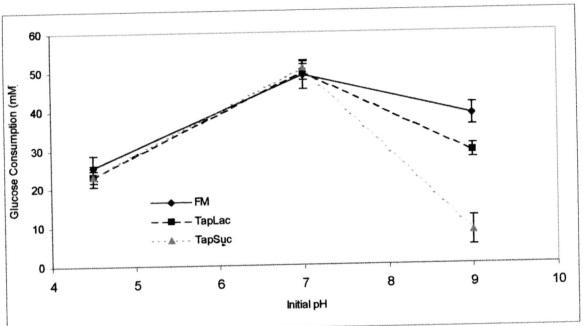

Figure 4.8: The glucose consumption (mM) at various initial starting pH values after 48 hours

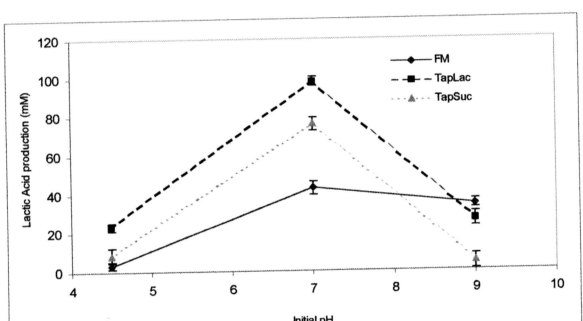

Figure 4.9: The lactic acid production (mM) at various initial starting pH values after 48 hours

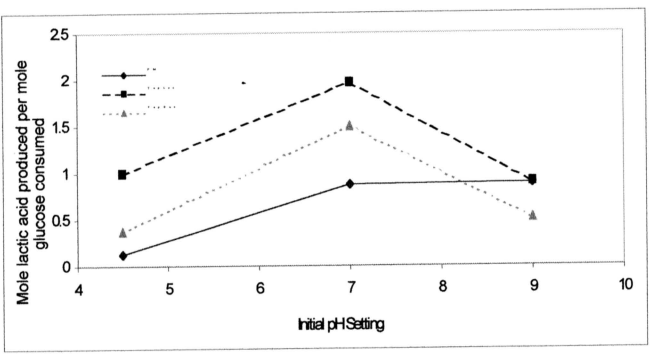

Figure 4. 10: The ratio between lactic acid production and glucose consumption at various initial pH setting after 48 hours

homofermentative lactic acid bacteria could shift into heterofermentative mode, producing acetate, ethanol and formate at high alkaline environment (Rhee & Pack, 1980).

At pH 9, different profiles of each isolate could be seen for glucose consumption and lactic acid production. Two of the isolates, TapLac and FM were able to tolerate high pH. This tolerance to high pH enable these isolates to grow at initial pH 9 and change the initial alkaline to an acidic environment by producing acids. TapSuc, did not seem to be able to tolerate the high alkaline environment. This is reflected in its low glucose consumption and lactic acid production.

4.6.3 Effect of Various NaCl Concentration on Culture Profiles.

The rational of the test was to ascertain the level of osmotolerance of the isolates. During the production of lactic acid, alkali is pumped into the production media to control the pH. This ultimately would raise the level of pH as free acid is changed into its salt form. This would increase the osmotic pressure exerted on the culture. This osmotic pressure would increase overtime as more salt is formed (Imhoff, 1986).

4.6.3.1 Biomass and Final pH Profiles at Various NaCl Concentrations

(Figure 4.11 and Figure 4.12)

Bacterial cells cultivated in a high salt concentration would experience a loss of turgor pressure. The loss of turgor pressure would affect the physiology, the enzyme activity, water activity and metabolism of the cells (Liu *et al.*, 1998). Some of the bacterial

cells overcome this effect of salt stress by intracellular accumulation of low molecular weight compatible solutes, such as amino acid derivatives, trehalose, and polyols (Brown, 1990). The accumulation of solutes or osmolytes, prevents the turgor pressure from decreasing in the high salt environment.

When the isolates were cultivated in low NaCl concentrations (> 5% (w/v) NaCl concentration), the biomass production decreases as the NaCl concentration increases (figure 4.11). However, the end point pH values for the isolates remain low, indicating that acids being produced. At 5% (w/v) NaCl concentration and higher, the biomass gain for all the isolates remains minimal. This is consistent with the reports on several strains of lactococci (Uguen *et al.*, 1999) and lactobacilli (Hutkins, 1987; Glaaskar, 1998) that their growth rate and growth yield decreased with increasing osmolarity of the medium. Their pH profiles increase with increased NaCl concentration. At 7.5% (w/v) NaCl concentration, it was observed that the biomass profile for FM was comparable as at 5% (w/v) but the pH value for the isolate was considerably lower if compared to TapLac and TapSuc. This difference could be due to the presence of osmoprotectants in FM cells. It was reported that lactococci grown in high NaCl concentration, had increase presence of glycine betaine, an osmolyte in cells (Uguen *et al.*, 1999). At this point, acids produced by TapLac and TapSuc were not enough to change the initial pH significantly.

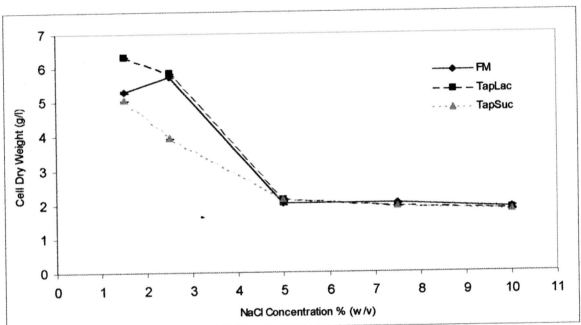

Figure 4.11: The biomass (g/l) profile at various NaCl concentrations after 48 hours

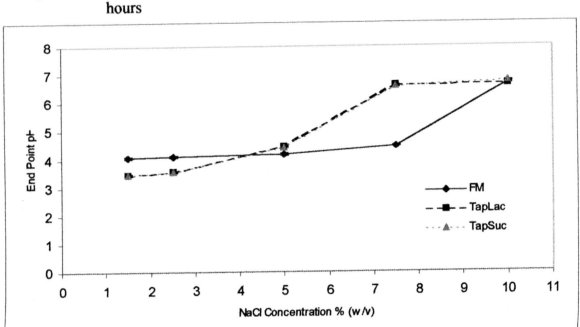

Figure 4.12: The end point pH profile at various NaCl concentrations after 48 hours

Since the value of the standard deviation is to small to be observed on the figures, the values and standard deviation are included as tables in the addendum

4.6.3.2 Glucose Consumption and Lactic Acid Production Profiles at Various NaCl Concentration

(Figure 4.13 and Figure 4.14)

When cultivated in increasing salt concentration, the glucose consumption profiles saw a decline for all the isolates, Researchers have reported that at high salt concentrations or in a reduced water activity environment, bacterial cells stop growing but still able to metabolise substrates. Adaptation employed by the cells was to induce an uptake of osmolytes such as glycine betaine into the cytoplasm (Liu *et al.*, 1998). This is to compensate the osmotic balance between inside and outside the cells. However, at very high salt concentration, growth and metabolism of cells have been reported to be completely inhibited (Zayed & Zahran, 1991).

At lower NaCl concentration, FM consumed less glucose compared to TapLac and TapSuc, but as the isolates were cultured at 5% (w/v) NaCl concentration onwards, the glucose consumption profile for FM became much higher than other isolates. As for lactic acid profiles, at lower NaCl concentration, the profiles for TapLac and TapSuc were higher than FM. However, at 5% (w/v) to 10% (w/v) NaCl concentration, the lactic acid profile for FM was higher than the other two isolates. These two events seem to indicate that some metabolic systems in FM still could function even at high salt concentration. This phenomenon could be attributed to the rigorous uptake of osmoprotectants into the cells (Kaskhet, 1987).

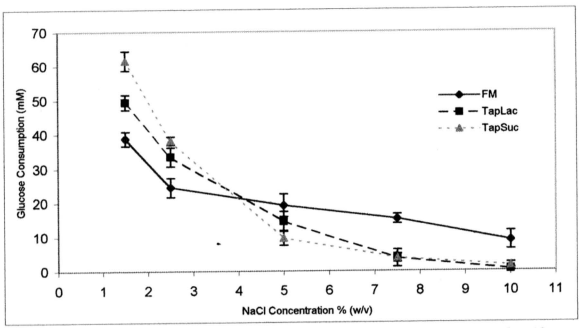

Figure 4. 13: The glucose consumption (mM) at various NaCl concentrations after 48 hours

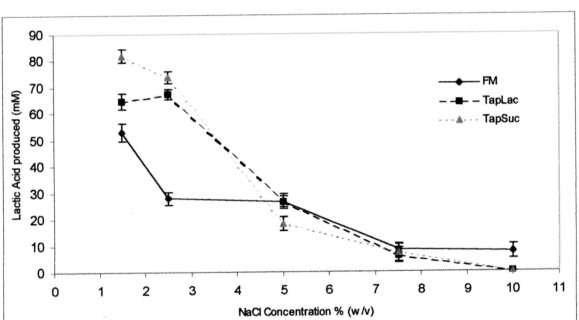

Figure 4.14: The lactic acid produced (mM) at various NaCl concentrations after 48 hours

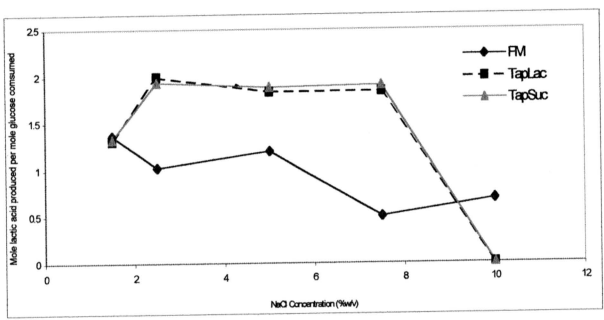

Figure 4.15: The ratio between lactic acid concentration and glucose consumption at various NaCl concentrations

The ratio between lactic acid produced and glucose consumed for TapLac and TapSuc showed good ratios between 2.5% 7.5% (w/v) NaCl concentration (figure 4.15). However, these ratios do not reflect on the amount of lactic acid produce or amout of glucose consumed. The ratio for FM, shoed a downward trend as the isolate was cultured in an increasing NaCl concentrations. As for, TapLac and TapSuc, between NaCl concentration of 5% and 7.5% (w/v), the uncoupling of growth and lactic acid production was observed. Lactic acid is a product of primary metabolism, which means, it is produced during the growth of the organism. The isolates have stopped their growth but continue to produce lactic acid. The amount of lactic acid produced decreases with increasing NaCl concentration.

4.7 Time Course Study of Several of Selected Isolates

The isolates FM, TapLac and TapSuc were cultured in 1 litre Erlenmeyer flask containig 400ml MRS broth with 2% (w/v) glucose. Initial pH of the broth was adjusted to 7. The flasks were incubated at 37°C and rotated at 100rpm

4.7.1 Biomass and End Point pH Profiles During the Time-Course Study

(Figure 4.16 and Figure 4.17)

The observation made from the biomass profiles displayed different profiles for different genus. FM, identified as *Lactococcus lactis*, displayed a short log phase for the first 6 hours and reached its stationary phase during the second 6 hours. This stationary phase continued throughout the period of cultivation (fig 4.16). This was also reflected in

its pH profile (figure 4.17). The pH profile displayed a rapid decrease in pH value during the first 6 hours with gradual decreases over subsequent period from the 6th to the 48th hour. For Taplac and TapSuc, which were identified as *Lactobacillus casei*, they displayed a longer log phase extending from zero hour to the 24th hour. Both of the isolates reached stationary phase after the 24th hour period with TapLac displaying a higher biomass profile than TapSuc (figure 4.16). The pH profiles for both of the isolates, decreased rapidly during the a 12 hours period with gradual decreases until both of them reached their stationary phase period at the 24th hour. It was also observed that, TapSuc display a lower pH profile than TapLac. FM produce less biomass compared to TapLac and TapSuc. TapLac and TapSuc produced double the amount of biomass as FM at stationary phase. This might be due to the ability of lactobacilli to tolerate a lower pH compared to lactococci as had been indicated in earlier experiments (Tables 4.6 and 4.8). As mentioned previously, streptococci could only tolerate pH approximately around 4.5, while lactobacilli are able to tolerate low pH of 3.5 (Kashket, 1987). This intolerance to low pH, could influence the specific growth rate (μ). The specific growth rate for the isolates was calculated using the natural log biomass.

The specific growth rate (μ) of the isolates was calculated as follows (Stainer *et al.*, 1987):

$dx/dt = (\ln X_t - \ln X_o)/t_1 - t_o$

where as $\ln X_t$ is ln biomass at time t_1,

$\ln X_o$ is ln biomass at time t_o,

t (hours) is time between t_1 and t_o hr

$\mu = dx/dt \,.1/x$

where x is the concentration of biomass between time t_1 and t_o (g/l)

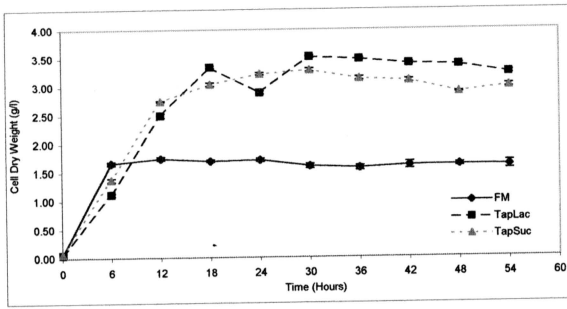

Figure 4.16: The biomass profiles (g/l) during the time course study

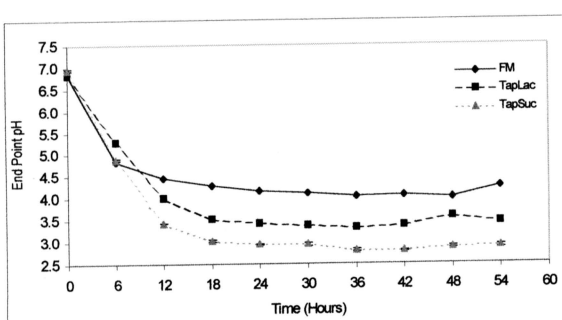

Figure 4.17: Shows the pH profiles during the time course study

Since the value of the standard deviation is to small to be observed on the figures, the values and standard deviation are included as tables in the addendum

Table 4.9: Specific growth rate (μ) and doubling time (T_d)

Isolate	μ (hr^{-1})	T_d (hr)
FM	0.36	1.93
TapLac	0.48	1.45
TapSuc	0.38	1.84

Doubling time (t_d) was calculated as follows:

$T_d = \ln 2/\mu = 0.6931/\mu$.

From table 4.9, the isolate TapLac was observed to have the highest specific growth rate (μ) and the shortest doubling time (t_d), this was followed by TapSuc and FM. Here, the role of pH could be seen playing a crucial role in growth. FM, which could not tolerate very low pH, displayed a lesser value in specific growth rate compared to the other isolates. Consequently, pH also plays an important part in cell growth. Excessive low pH could inhibit cell growth.

4.7.2 Glucose Consumption and Lactic Acid Production Profiles During the Time-Course Study

(Figure 4.18 and Figure 4.19)

Glucose consumption (figure 4.18) and lactic acid production (figure 4.19) profiles of the isolates were similar in the early stages of the cultures. The glucose consumption and lactic acid production for FM, displayed higher profile than TapLac and TapSuc during the first 6 hours. During the second 6 hours, TapLac and TapSuc have overtaken FM in the value of glucose consumption and lactic acid production. This phenomenon is related to the tolerance towards free acid concentration. It was noticed that FM displayed a higher pH profile than the other isolates. This higher profile was due to the low tolerance of *Lactococcus* to high concentration of free acids. This in turn will inhibit growth and lactic acid production. TapLac and TapSuc, in the other hand are from the *Lactobacillus* genus, which could tolerate low pH, displayed higher glucose consumption and lactic acid production. This was reflected in the amount of lactic acid produced by the isolates. It has

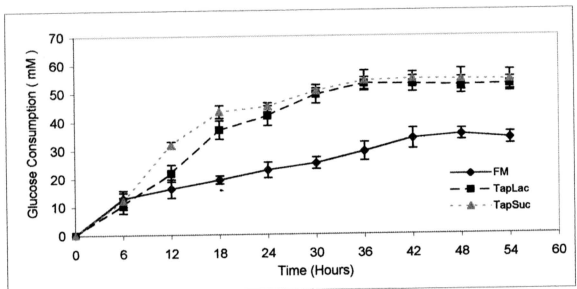

Figure 4.18: The glucose consumption (mM) profiles during the time course study

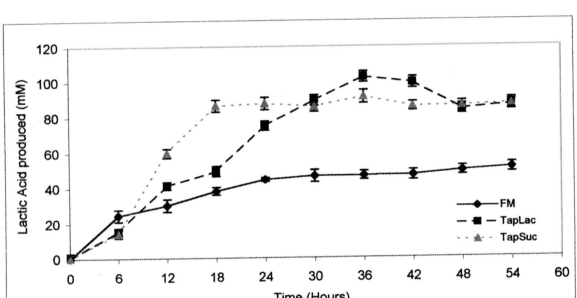

Figure 4.19: The lactic acid production (mM) profiles during the time course study

been suggested that, the capacity of lactobacilli to tolerate lower pH values than other lactic acid bacteria, such *Streptococcus* and *Leuconostoc*, originate from their more basic cytoplasmic pH values, allowing higher concentrations of non-dissociated acids to be tolerated (McDonald *et al.*,1996). This tolerance to low pH, enables TapLac and TapSuc to produce lactic acid amounted to double of that of FM.

During rapid growth phase, the amount of lactic acid produced was closely in proportion to the ideal ratio (figure 4.20), as 1 mol of glucose would ultimately yield 2 mol of lactic acid. At 24th hour, glucose consumption and lactic acid production ratio starts to diverge more from the ideal ratio. Before the 24th hour period, the ratio values were consistently close to the ideal ratio value, which is 2. The decreasing ratio values indicate that glucose that was consumed was not converted entirely into lactic acid. Glucose consumption continued to rise indicting glucose was constantly being utilised. The mechanism, which allows this to happen, is the co-metabolism of glucose and citrate in the cultures (Magni *et al.*, 1995). This mechanism of co-metabolism between glucose and citrate was induced by the presence of lactic acid. The co-metabolism of glucose and citrate enable the cells to generate ATP for cell maintenance, active transport of substrate and proton extrusion by the ATP -link proton pump.

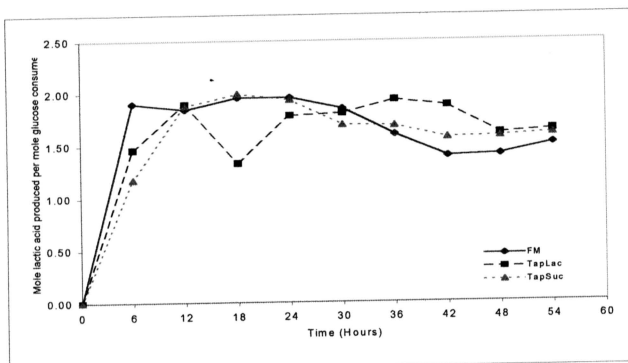

Figure 4.20: The ratio between lactic acid produced and glucose consumption during the time course study