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Lactulose production from milk concentration permeate using calcium carbonate-based catalysts

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ABSTRACT

Milk concentration permeate (MCP), a low-value by-product of ultrafiltration plants and calcium carbonate-based catalysts were used for lactulose production. The results obtained show the effectiveness of oyster shell powder and limestone for lactose isomerisation as a replacement for egg shell powder. With the reaction conditions of 12 mg/ml catalyst loading, reflux time of 120 min at 96 °C, a maximum yield of 18–21% lactulose was achievable at a cost of <50% of original lactose degradation (measured by HPLC). De-proteination of MCP by acidification prior to isomerisation helped lactulose formation in the earlier stages, but did not significantly increase the yield. The resulting lactulose MCP (40 °B) incorporated at the rate of 3–4% was effective in enhancing the growth rate and acid production of *Lactobacillus acidophilus* (LA-5) in probiotic products.

1. Introduction

A large volume of milk concentration permeate (MCP) is produced in Australia by dairy processing plants as a consequence of the increased utilisation of ultrafiltration (UF) for preconcentration of milk. Milk permeate is a low-value by-product which contains predominantly 4.5–4.8% lactose (4-O-β-D-galactopyranosyl-D-glucose) and 0.44–0.47% mineral salts (Suarez, Lobo, Alvarez, Riera, & Alvarez, 2006). It is not suitable for human consumption and having a high Biological Oxygen Demand (BOD) needs appropriate treatment before disposal as a waste. The existing practices are to use MCP for feeding animals, milk standardization, blending with other dairy liquids and recovering lactose for use in food formulation. It can also be used as a cheap lactose source to isomerise lactose into non-absorbable lactose-derivatives e.g. lactulose, lactitol and lactobionic acid (Harju, 2001; Mahran, Haggag, Mahfouz, Zaghloul, & Abd El-Salam, 1995; Montilla, del Castillo, Sanz, & Olano, 2005; Olano, Corzo, Paez, & Martinez-Castro, 1987; Villamiel, Corzo, Foda, Montes, & Olano, 2002).

Lactulose (4-O-β-D-galactopyranosyl-D-fructose) is a ketose disaccharide used in both pharmaceutical and food industries. As a laxative, it is applied for the prevention and treatment of chronic constipation, portal systemic encephalopathy and other intestinal or hepatic disorders (Holsinger, 1999). As a prebiotic food additive, lactulose is utilised in infant formulas and health foods. Its chemical and physiological properties are close to other prebiotics wherein the linkage of galactose and fructose is neither split by human enzymes nor absorbed in the small intestine. Unchanged lactulose reaches the colon where it stimulates the growth of *Lactobacillus* spp. and *Bifidobacteria* spp., generating a large number of short chain fatty acids (Schumann, 2002).

In alkaline conditions, lactulose is formed via the Lobry de Bruyn–Alberda van Ekenstein molecular rearrangement in which the glucose moiety of lactose is isomerised to fructose (Andrews, 1989; Andrews & Prasad, 1987; Martinez-Castro, Olano, & Corzo, 1986). Although there have been a number of studies for lactulose isomerisation using different catalytic systems, little is known about the suitability of calcium carbonate-based catalysts. In a recent study by Montilla et al. (2005), the feasibility of using egg shell powder (ESP) for isomerisation of MCP has been addressed with a lactulose yield of 1.18 g/100 ml of MCP.

In the present study we investigated the suitability of other calcium carbonate-based catalysts i.e. oyster shell powder (OSP) and limestone as a replacement to ESP for lactose isomerisation in MCP and de-proteinated MCP. A preparation of 4% lactose solution was used as control. The OSP was chosen due to the fact that oyster shells are a waste product from mariculture, causing unpleasant smells in shell-harvesting areas. Apart from the use of oyster shells as a fertilizer and as a mineral source in chicken feed and fish pellets, there is a need for the food industry to find alternative ways for using the shells that are beneficial to the environment. The OSP has comparable chemical composition to ESP and can be easily removed after the isomerisation using centrifugation without a

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need for chromatographic purification. Therefore, this study is aimed to turn a large surplus of MCP and oyster shells as by-products into a high value-added product through enrichment of lactulose. Moreover, we conducted experiments to determine the suitability of resulting lactulose syrup as growth promoters and to assess the feasibility of development of yoghurt supplemented with lactulose syrup.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals and reagents

Analytical-reagent grade limestone (pure calcium carbonate; $CaCO_3$, BDH Chemicals, Australia Pty. Ltd., Kilsyth, VIC) and lactic acid (Purac Bioquimica, Barcelona, Spain) were used for lactulose isomerisation. High purity chemicals were employed for HPLC analysis, including HPLC-grade acetonitrile (Baker Analyzed®, J.T. Baker, USA), Carrez I and Carrez II reagents (Univar®, Ajax Chemicals Ltd., Melbourne, Australia) and lactose and lactulose standards (>95% pure, Sigma, St. Louis, MO, USA). Distilled water was filtered through a Milli-Q-ultrapure water purification system (Millipore, Australia) before using for HPLC analysis.

2.1.2. Egg shell powder (ESP)

Egg shell powder was prepared from chicken eggs after removing the shell membrane and washing off the residual albumen under tap water, following the procedure of Montilla et al. (2005). The raw egg shells were then dried overnight in an oven at 102 °C, ground after cooling the following day in a micro hammer mill (Glen Mills Inc., USA) at 600 rpm and sieved through a 120 mesh screen (Greening and Sons Ltd., Hayes, Middx, England). The ESP thus obtained was a cream-coloured powder with an average particle size of 117 μm.

2.1.3. Oyster shell powder (OSP)

Oyster shells obtained from sea food restaurants in Melbourne, Victoria, Australia were cleaned, washed and dried overnight at 102 °C. The dried shells were broken into smaller pieces using a mortar, followed by grinding and sieving as described in Section 2.1.2, resulting in an off-white coloured fine powder.

2.1.4. Milk concentration permeate (MCP)

Milk concentration permeate was collected from the UF plant of a local dairy processor in Victoria Australia and kept in an ice bath during transportation, followed by freezing. To obtain MCP, pasteurised milk was passed through a 30,000 MWCO polyethersulfone membrane (Synder™ MK-4333, Synder Filtration, New Zealand) at 45 °C and 120 kPa. The MCP used in this study contained 4.5% (w/v) solid-non-fat (SNF) including ca. 0.5% protein and 4% lactose. It is not uncommon to have some proteins contaminating the MCP especially at the start of the process, which was the case with MCP used in this project. Prior to processing, the frozen MCP was thawed and filtered through Whatman No. 1 filter paper. In this manner, a turbid green-yellowish liquid was produced ready for isomerisation.

2.1.5. Lactose solution

Lactose (α-form, 95% pure) was dissolved in distilled water to 4% (w/v), the pH of the solution was adjusted to 6.7 using 0.1 M NaOH, filtered and used as reference (control) solution.

2.1.6. Starters, yoghurt milk and growth media

Low-heat skim milk powder (Bonlac Foods Limited, 636, St. Kilda Road, Melbourne, Victoria, Australia) was reconstituted with distilled water at different concentration levels (12%, 13%, 14% and 15%) for yoghurt making. The freeze-dried cultures of *Lactobacillus acidophilus* (LA-5), *Lactobacillus casei* (LC-01) and *Bifidobacterium bifidum* (BB-12) used for direct vat inoculation to produce set-type yoghurts were obtained from Chr. Hansen Pty. Ltd. (49, Barry Street, Bayswater, Victoria, Australia). Growth media were obtained from Oxoid Ltd. (Basingstoke, Hampshire, UK). All other supplements were obtained from Sigma Chemical Co. (St. Louis, MO, USA), Merck (Darmstadt, Germany) and BDH Laboratory Supplies (Poole, England).

2.2. Treatments

2.2.1. Isomerisation

An aliquot of 100 ml MCP was transferred to a 250-ml round-bottom flask. The selected catalyst was added to the vessel (12 mg/ml) and mixed well with magnetic stirrers at room temperature. The flask was then placed in a glycerol bath, stirred and refluxed under the constant temperature of 96 °C for 120–150 min. The zero time of process was taken when the mixture reached 96 °C. Aliquots of 10 ml were taken every 30 min and rapidly placed in an ice bath to stop the reaction. At the completion of heating, the catalyst was removed from the aliquots by centrifugation at 3600*g* and 20 °C for 10 min. The collected supernatants were stored at 4 °C and used for the determination of pH and sugar content within 24 h.

2.2.2. Effect of the type of catalyst on lactose isomerisation

Limestone, OSP and ESP were compared for their catalytic power in MCP and the control lactose solution. Each trial was performed in triplicate. Blank trials (without catalyst addition) were also conducted in both solutions, following the procedure described above.

2.2.3. Effect of de-proteination on lactose isomerisation

Before isomerisation, the protein contaminants in MCP were removed by acidification and mild heat treatment. The pH of MCP was adjusted to 4.5 with 1% lactic acid. To prevent the hydrolysis of lactose, the acidified MCP was heated for 2–3 min at <60 °C, followed by filtration through Whatman No. 1 filter paper, resulting in a green-yellowish clear liquid. The pH was then re-adjusted to 6.7 using 0.1 M NaOH. The de-proteinated MCP was then isomerised, following the procedure described in Section 2.2.1.

2.3. Determination of the prebiotic effect of lactulose-containing MCP

2.3.1. Fermentation in broth

Lactulose is normally sold in syrup form because of difficulties in its crystallization and drying. Therefore, the isomerised MCP was further concentrated in a rotary evaporator (Rotavapor-R, Buchi, Switzerland) at ⩽70 °C to obtain lactulose syrups with 40 °B soluble solids. For comparison, concentrated de-proteinated MCP (40 °B) and 40% lactulose solution were used as control and reference substrates, respectively.

Initial testing for the ability of selected probioitc strains to grow on lactulose was performed in modified MRS broth. Due to difficulty in accurately weighing small amount of freeze-dried cultures when small volume of broth was used, a mother culture was prepared. To this end, 0.01 g of each freeze-dried culture (LC-01, BB-12 and LA-5) was propagated into 100 ml of de Man, Rogosa Sharpe (MRS) broth (Oxoid), incubated aerobically overnight at 37 °C and stored at 4 °C until required for use. Prior to each trial, the mother cultures (10 ml) were sub-cultured at least three times into 90 ml of MRS broth at 37 °C for 12–16 h.

Carbohydrate-free MRS broth was prepared as a basal growth medium which contained per litre: 10 g peptone from casein, 4 g

yeast extract, 8 g Lab-Lemco powder, 2 g triammonium citrate, 1 ml Tween 80, 2 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 4H_2O$ and 5 g sodium acetate. The medium was sterilised at 121 °C for 15 min and divided into sterilised test tubes (8.5 ml in each) the basal MRS was then supplemented with 2.5 ml of various carbohydrate sources (40 °B) to give a final concentration of 1%. All carbohydrate solutions were sterilized by passing through 0.45 m filter (Millex®-HA, Millipore Corporation, Bedford, MA, USA) prior to addition to sterile media which was then inoculated with 1 ml of probiotic culture. All trials were performed in triplicate. The media tubes for LA-5 were incubated aerobically overnight at 37 °C while those for LC-01 and BB-12 were transferred into anaerobic jars containing AnaeroGenTM 2.5 l sachets (Oxoid, Basingstocke, UK) and incubated overnight at 37 °C.

2.3.2. Fermentation in reconstituted skim milk

The suitability of the lactulose syrup as a prebiotic supplement was determined by supplementing reconstituted skim milks (RSM) at 12%, 13%, 14% and 15% solids content with different levels (4%, 3%, 2% and 1%) of lactulose syrup to a final solids content of 16%. The control (non-supplemented RSM, 16% w/v) and lactulose supplemented RSMs were heat treated at 90 °C for 10 min with constant stirring and cooled to 42 °C. All samples were inoculated aseptically with selected freeze-dried probiotics at a concentration of 0.005% (w/v) and were aseptically transferred into 100 ml plastic containers, tightly covered and incubated at 37 °C overnight. All trials were duplicated. Duplicate determinations of bacterial counts, pH and titratable acidity (as measure of the effectiveness of lactulose syrup in modulating growth rate and metabolism of each probiotic) were conducted on duplicate samples taken from each batch after overnight incubation.

2.4. Analytical determinations

2.4.1. Measurement of lactose and lactulose

Lactose and lactulose contents were determined in all samples before and after isomerisation using high performance liquid chromatography (HPLC) method according to Zokaee, Kaghazchi, Zare, and Soleimani (2002). A 5-ml aliquot of the isomerised sample was pipetted into a 25-ml volumetric flask and added with 500 μl each of Carrez I and II reagents to remove protein and fat. The solution was then made up to 25 ml with a mixture of acetonitrile and Milli-Q water (50:50 v/v) and left to rest at least 30 min, followed by filtration through Whatman No. 1 filter paper. The filtrate was kept for HPLC analysis. Sugar standards were dissolved in Milli-Q water before adding the acetonitrile (50:50 v/v). Prepared samples were filtered through 0.45 μm PTFE Philic PP filters (Bonnet Equipment Pty. Ltd., NSW, Australia), before introducing into the HPLC system by a Waters Model 6000A pump (Waters Associates Inc., Milford, MA, USA) via an injection valve fitted with a 20-μl injection loop (Rheodyne®, Cotati, CA, USA). The column chosen was a 3.9 × 300 mm stainless steel Carbohydrate Analysis column (Waters Co., Milford, MA USA), eluted with degassed and filtered 80:20 (v/v) acetonitrile-Milli-Q water mix at a flow rate of 0.8 ml/min. The column was kept at ambient temperature and the detection was carried out in a Waters Model 401 Refractive Index Detector (RID).

Once the separation of sugars was achieved, peaks were identified by comparing their retention times with standard sugars. The amount of individual sugars reported in g/100 ml was estimated from peak heights, instead of peak areas because the HPLC operating conditions applied did not provide a baseline separation of lactose and lactulose. Calibration curves were prepared using each sugar standard in a range of concentration between 2.5 and 10 mg/ml.

2.4.2. Measurement of pH

Measurements of pH were carried out using a bench-top pH-meter (model 520A, Orion Research Inc., Boston, USA), at 20 °C. The pH-meter was calibrated before use with buffer solutions of pH 7.0 and 4.0.

2.4.3. Measurement of titratable acidity (TA)

The milk sample was titrated with 0.1 N NaOH until the pinkish end point of phenolphthalein was noticed (AOAC, 1990). Titratable acidity was reported as the percentage equivalent of lactic acid.

2.4.4. Microbiological tests

One millilitre of samples was diluted in 9 ml peptone water (0.1% w/v) and 10-fold serial dilutions (10^{-7}–10^{-9}) were prepared. Viable counts of BB-12 and LC-01 were enumerated using pour-plate technique on MRS agar (Oxoid Ltd., Hampshire, UK) under anaerobic condition (Gas-pack, Anaerogen, Oxoid, Basingstocke, UK) at 37 °C for 72 h, while counts of LA-5 were determined on MRS agar after aerobic incubation at 37 °C for 48–72 h.

2.5. Statistical analysis

Statistical analysis was carried out using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA, 2004). One-way analysis of variance (ANOVA) was applied to investigate differences between means of more than two groups. When ANOVA indicated differences, the Duncan's multiple range test (DMRT) was used to distinguish groups of means at the 95% and 99% confidence level.

3. Results and discussion

Under our experimental conditions, heating a mixture of MCP and calcium carbonate-based catalysts resulted in a loss of lactose in favour of lactulose formation. Heating under reflux allowed the soluble solids of MCP mixture to remain relatively unchanged at ca. 4–5 °B as measured by Erma Abbe Refractometer (Model ER-98, Tokyo). Any extension of heating duration beyond maximum isomerisation resulted in the formation of reaction by-products e.g. galactose derived from the rapid decomposition of lactulose. In addition, a drop in solution pH and darkening of solution colour were evident, confirming the formation of acidic and brown compounds as by-products of lactose isomerisation.

Fig. 1 shows typical chromatograms of sugar analysis by the HPLC-RID method. The chromatogram of MPC before isomerisation (Fig. 1a) shows solvent and lactose peaks with the retention times of 4.2 and 17.6 min, respectively, while after isomerisation two new peaks were detected with retention times of ca. 10 and 16 min (Fig. 1b). The first peak was tentatively assigned as galactose and the second peak was that of lactulose. Although the present HPLC-RID method achieved the separation of the three saccharides, chromatographic peaks are not always as sharp as is desirable. Peaks corresponding to lactose and lactulose were not completely resolved and therefore reported concentrations of sugars were taken from peak heights as these provide a suitable means of estimating the amounts. The concentration of by-product saccharides was not reported in the current study because of the poor sensitivity associated with refractometry which did not allow the determination of epilactose. The peak assigned to galactose may also include glucose due to their close retention times.

3.1. Effect of the type of catalyst on lactose isomerisation

The conversion of lactose into lactulose in MCP and in the control lactose solution by the catalytic activities of limestone, OSP and ESP is shown in Fig. 2. In the absence of catalyst (blank) the

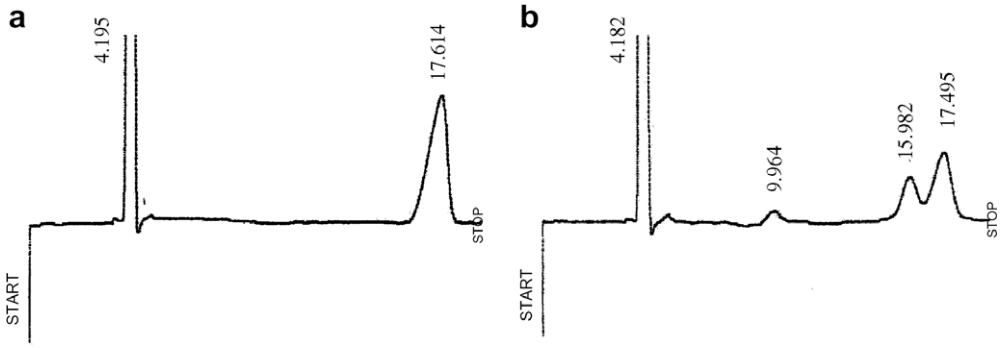

Fig. 1. HPLC-RID chromatograms of MCP treated with 12 mg/ml ESP (a) before and (b) after isomerisation at 96 °C for 120 min. The peaks correspond to: solvent (4.20 and 4.18), lactose (17.61 and 17.50), galactose and glucose (9.96) and lactulose (15.98).

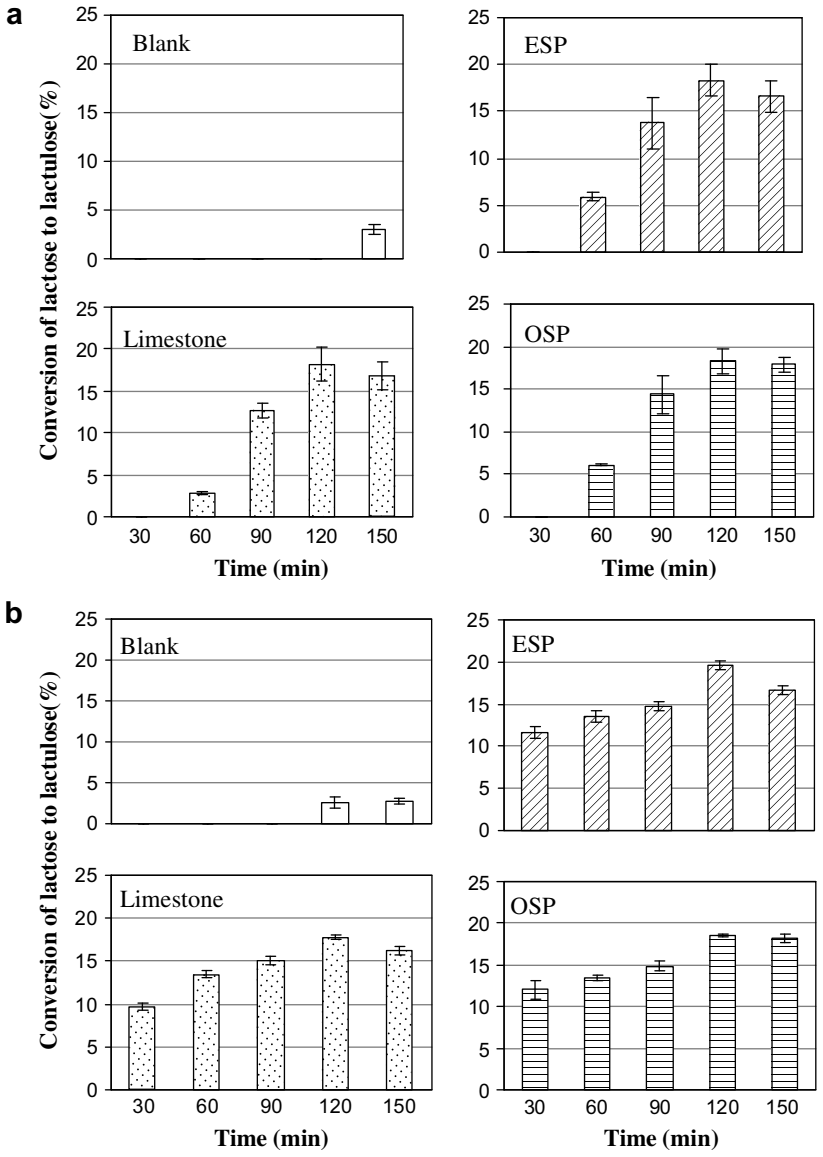

Fig. 2. Conversion of lactose to lactulose from (a) MCP and (b) lactose solution using different catalysts. Data represent the mean ± standard deviation of three different experiments.

isomerisation rate was slow and only ca. 3% conversion (0.12–0.13 g lactulose/100 ml) was detected in MCP after 150 min, and in control lactose solution after 120 min. In the presence of catalyst a significantly higher level of conversion was achieved in both solutions and at earlier stages of heating, i.e. the maximum conversion detected was 13.5% in control but only 6.2% in MCP after

60 min. However, as the treatment time progressed, the conversion in both solutions reached 12–15% at 90 min, rising to 18–20% after 120 min but dropped back to 16–18% after 150 min of heating.

The catalyst type had only small effect on the conversion level. It was found that limestone gave 3% less conversion than OSP and ESP after 60 min, and 2% less in the control lactose solution after 30 min. However, all catalysts showed similar conversion levels between 90 and 150 min in both solutions. These findings support the effectiveness of OSP or limestone for lactose isomerisation as a replacement to ESP. Interestingly, it was found that calcium carbonated-based catalysts could be reused at least twice without significant loss of their catalytic performances (data not shown) which is another advantage compared with soluble catalysts such as NaOH, $Ca(OH)_2$ and KOH.

The lower level of lactulose formation in MCP compared to control lactose solution could be attributed to the buffering effect of proteins and/or other miscellaneous constituents present in MCP. Upon the addition of catalysts to control lactose solution the pH rose from the adjusted initial value of 6.7 to ca. 9 before dropping below 8 with reaction time (data not shown). Under these conditions, lactulose was formed within 30 min of heating (ca. 9.6% conversion). On the other hand, the pH of the MCP did not change much upon catalyst addition and took 60 min to rise >8, with the maximum pH reaching <8.5 after 90 min, probably due to protein denaturation and loss of their buffering capacity. Our findings agree with previous reports by Olano et al. (1987) and Claeys, van Loey, and Hendrickx (2002) who found that lactulose did not form in the earlier stages of heating when the pH was close to that of milk, but formed faster when the pH of reaction mixture was near or above 8.0. Furthermore, residual proteins in MCP could complex lactulose into lactosyl-amino compounds, thus lowering lactulose yield (O'Brien, 1997). Therefore, it was deemed necessary to de-proteinate the MCP prior to isomerisation.

Fig. 3 shows the degradation of lactose during isomerisation of MCP and control lactose solution using different calcium carbonate-based catalysts. In the absence of catalyst, no lactulose was formed but lactose content diminished by 4.8–10% in MCP and only 0.7–3.3% in control lactose solution. The higher loss of lactose could be ascribed to partial condensation of lactose with residual proteins (Maillard reaction). According to Berg and van Boekel

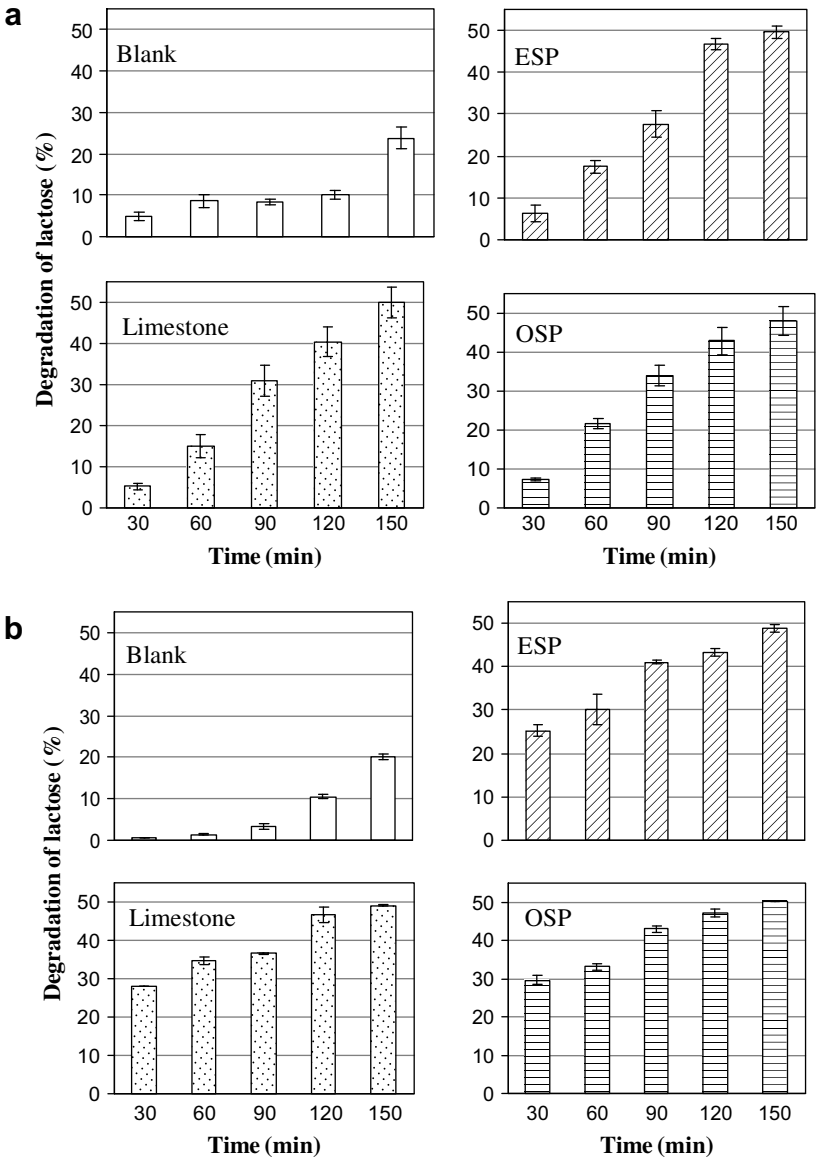

Fig. 3. Degradation of lactose of (a) MCP and (b) lactose solution using different catalysts. Data represent the mean ± standard deviation of three different experiments.

(1994), 80% of lactose in heated milk undergoes Lobry de Bruyn–Alberda van Ekenstein transformation, but 20% enters the Maillard reaction. In the presence of catalyst a considerable loss of lactose in both solutions was observed in favour of lactulose formation. After 150 min, the degradation of lactose in both solutions was equivalent to ca. 50% of its initial mass.

The OSP gave comparable yield of lactulose to ESP and limestone. Undoubtedly, the use of ESP and OSP is economical due to their origin being waste materials, and is beneficial to the environment by alleviating shell disposal problems. However, oyster shells may contain higher levels of heavy metals i.e. lead, mercury and cadmium than egg shells, but on the other hand egg shells may be contaminated with pathogens e.g. Salmonella therefore, both catalysts need to be treated appropriately before using in food applications. Moreover, both catalysts may have variable chemical composition compared to pure limestone. Oyster shells come from the sea therefore its mineral content cannot be controlled, while the constituents of egg shell depend closely on the formulation of the feed.

3.2. Effect of de-proteination on lactose isomerisation

To overcome the retarding effect of protein contaminants of MCP on lactose isomerisation, the MCP was treated by acidification under mild heat treatment and filtration to remove proteins prior to isomerisation. Fig. 4 shows changes in lactose and lactulose contents and pH of de-proteinated MCP during the isomerisation at 96 °C for 150 min. The results revealed that lactulose was formed rapidly within the first 30 min of isomerisation (0.38 ± 0.04 g/100 ml) which doubled after 60 min (0.69 ± 0.15 g/100 ml) and reached a maximum (0.90 ± 0.12 g/100 ml), equivalent to 21.9% of initial lactose content, within 120 min (Fig. 4a). Lactose degradation followed a similar trend dropping from the initial amount of 4.11 ± 0.31 g/100 ml of MCP at time 0, to 2.09 ± 0.21 g/100 ml after 150 min (49.5% degradation). The same trend was observed in the isomerisation of control lactose solution (Fig. 3).

An interesting observation was that upon the addition of catalysts the pH of de-proteinated MCP remained similar to the pH of original MCP. This indicates that besides the buffering action from the residual proteins in MCP, other miscellaneous constituents e.g. citrates and phosphates may also help maintain the pH at the start of process. However, within 30 min of heating the pH rose significantly from 6.78 ± 0.03 to 8.65 ± 0.10 (Fig. 4b) before dropping back to 8.36 ± 0.15 after 60 min and stabilising between 7.89 and 7.76 from 90 to 150 min. The observed pH drop at the final stages of isomerisation could be due to the formation of organic acids i.e. isosaccharinic acid (Moreno, Villamiel, & Olano, 2003) and formic acid (Berg & van Boekel, 1994), derived from degradation of lactulose with prolonged heating.

3.3. Prebiotic effect of lactulose-containing MCP

The addition of calcium carbonate-based catalysts to MCP provided necessary conditions for lactulose formation. This process may be applied to increase lactulose level in other milk-based lactose rich streams e.g. whey and whey permeate and to improve the functional characteristics of food products. Lactulose plays a beneficial role in human gut environment as a prebiotic that promotes the growth of probiotics. Similarly, in probiotic dairy products lactulose is used up by added probiotics as a preferred source of carbon and energy. To evaluate the prebiotic power of the lactulose syrup developed in this project and to determine its possible application in dairy products, we studied the growth of three different probiotic strains in modified MRS media and RSM containing lactulose syrup.

Comparative viable counts of probiotics in modified MRS broths supplemented with de-proteinated MCP (control), lactulose syrup or reference lactulose as carbon sources at 1% concentration are summarised in Table 1. Counts of LA-5 and LC-01 were maximal in media with reference lactulose, followed by those with lactulose syrup and de-proteinated MCP, respectively. The capacity of lactulose syrup to enhance the growth of both strains was lower than that of reference lactulose which could reflect the presence of isomerisation by-products. Previously Montilla et al. (2005) reported ca. 9% of lactose degradation into by-products (0.34 g/100 ml galactose, 0.09 g/100 ml epilactose and 0.02 g/100 ml or-

Table 1
Growth of LA-5, LC-01 and BB-12 in the modified MRS broth supplemented with various carbon sources

Probiotic strain	Carbons source	Bacterial count (log CFU/ml)
LA-5	De-proteinated MCP	8.60 ± 0.35^{b}
	Lactulose syrup	8.71 ± 0.27^{ab}
	Reference lactulose	8.92 ± 0.31^{a}
LC-01	De-proteinated MCP	9.63 ± 0.28^{b}
	Lactulose syrup	9.77 ± 0.42^{ab}
	Reference lactulose	10.21 ± 0.38^{a}
BB-12	De-proteinated MCP	8.88 ± 0.45
	Lactulose syrup	8.74 ± 0.51
	Reference lactulose	8.99 ± 0.18

Results shown are mean ± standard deviation for three experiments each with three replications.
Different letters in the same column of each probiotic differ significantly at $P < 0.05$ by DMRT.

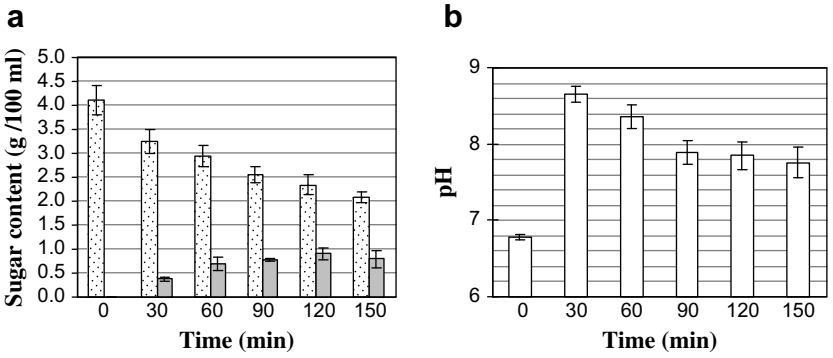

Fig. 4. Changes of lactose (▨), lactulose (■) and pH (□) during the isomerisation of de-proteinated MCP added with 12 mg/ml ESP at 96 °C for 150 min. Data represent the mean ± standard deviation of three different experiments.

Table 2
Counts of LA-5, LC-01 and BB-12 and acid production after overnight incubation in control and supplemented yoghurts with 40 °B isomerised MCP syrup

Probiotic strain	Concentration (%)	Bacterial count (log CFU/g)	pH	TA (% lactic acid)
LA-5	0	7.94 ± 0.17[b]	4.45 ± 0.05[ab]	0.78 ± 0.01
	1	7.91 ± 0.12[b]	4.51 ± 0.19[a]	0.79 ± 0.06
	2	7.78 ± 0.25[b]	4.49 ± 0.15[a]	0.79 ± 0.11
	3	8.52 ± 0.06[a]	4.27 ± 0.04[bc]	0.80 ± 0.06
	4	8.50 ± 0.19[a]	4.18 ± 0.01[c]	0.85 ± 0.01
LC-01	0	8.48 ± 0.18	5.72 ± 0.01	0.49 ± 0.04
	1	8.28 ± 0.06	5.62 ± 0.01	0.46 ± 0.08
	2	8.16 ± 0.28	5.57 ± 0.10	0.47 ± 0.01
	3	8.38 ± 0.72	5.71 ± 0.06	0.46 ± 0.04
	4	8.10 ± 0.28	5.68 ± 0.05	0.48 ± 0.02
BB-12	0	8.65 ± 0.12[a]	5.31 ± 0.01	0.44 ± 0.02
	1	8.65 ± 0.06[a]	5.20 ± 0.11	0.42 ± 0.02
	2	8.31 ± 0.02[b]	5.31 ± 0.06	0.48 ± 0.01
	3	8.11 ± 0.03[b]	5.34 ± 0.15	0.44 ± 0.01
	4	8.09 ± 0.23[b]	5.48 ± 0.06	0.42 ± 0.05

Results shown are mean ± standard deviation for two experiments each with two replications.
Different letters in the same column of each probiotic differ significantly at $P < 0.05$ by DMRT.

ganic acids) under isomerisation conditions close to those of our study. Therefore, a purification step using chromatography appears to be necessary to obtain high purity lactulose syrup. When lactulose syrup and reference lactulose were added to broths, there were no significant changes in count of BB-12 (8.7 and 9.0 log CFU/ml) compared to the control MCP (8.9 log CFU/ml). These results indicate that lactulose may work for LA-5 and LC-01 but not with BB-12. Our findings are in accordance with those of Kontula, Suihko, Von Wright, and Mattila-Sandholm (1998) and Kneifel, Rajal, and Kulbe (2000) whereby *L. acidophilus* strains, including LA-5 grew well on lactulose-containing broth, and those members of *L. casei*, including LC-01 were shown to utilise lactulose pronouncedly. No report is found in literature on the utilisation of lactulose by BB-12, however, addition of lactulose was found to promote the growth of some bifidobacteria cultured in broth e.g. *Bifidobacterium longum* (BB-46), but not *Bifidobacterium lactis* (Crittenden et al., 2001; Kneifel et al., 2000).

Table 2 shows the viable counts and acidifying activity of LA-5, LC-01 and BB-12 grown in RSM containing various levels of lactulose syrup after overnight incubation at 37 °C. Similar to observations in broth, the prebiotic effect of lactulose syrup was strain-specific. Among the probiotics tested, the growth and activity of LA-5 was progressively enhanced as the level of lactulose increased whereas the corresponding values for LC-01 and BB-12 were not affected. The highest counts of LA-5 (ca. 8.5 log CFU/g) were achieved with 3% and 4% lactulose compared to ca. 7.8–7.9 log CFU/g with 1% and 2% lactulose and ca. 8 log CFU/g with control. At the rates of 3% and 4% lactulose addition, a significant drop in yoghurt pH (4.2–4.3) and a slightly higher level of acid production by LA-5 (0.80–0.85%) was observed compared to control (pH 4.5, 0.78%). Similar results were noted by Ozar, Akin, and Ozar (2005) who found that lactulose powder when added to yoghurt at 0.25% and 2.5% promoted the counts of LA-5 to a great extent. The above study also showed a significant increase in the counts of *Bifidobacterium bifidum* (BB-02) with lactulose addition. In contrast, our results showed that increasing levels of lactulose syrup beyond 2% caused the BB-12 populations drop to 8.1–8.3 log CFU/g from 8.7 log CFU/g of the control. This variation could possibly be due to the differences in bifidobacteria strains chosen for the two studies. Bruno, Lankaputhra, and Shah (2002) reported that the probiotic action on lactulose was strain dependent, and that lactulose was effective in stimulating the growth of *Bifidobacterium longum*, *Bifidobacterium animalis* and *Bifidobacterium pseudolongum* cultured in RSM but not *Bifidobacterium infantis*. According to Smart, Pillidge, and Garman (1993) bacterial strains having high -galactosidase or phospho–galactosidase activity had more potential to utilise lactulose. In the case of LC-01, the growth (ca. 8.1–8.4 log CFU/g) and acidification (pH 5.6–5.7, 0.46–0.48%) in the presence of 1–4% lactulose syrup were similar to those obtained in the control ($P > 0.05$). Lactulose did not promote the growth of LC-01 in RSM, as it did in broths, possibly due to the effect of aerobic incubation. According to Saarela, Hallamaa, Mattila-Sandoholm, and Matto (2003), lactulose supplemented in skim milk was well utilised if *Lactobacillus* strains were grown anaerobically.

4. Conclusion

Milk concentration permeate, a low-value waste stream can serve as a cheaply and readily available lactose source for lactulose production. Proteins if present in MCP can affect the isomerisation rate only in the earlier stages without adverse effect on yield of lactulose at longer heating periods. Lactulose yield did not exceed 22% of the initial lactose during heat treatment with 12 mg/ml catalysts at 96 °C and 120 min. Other calcium carbonate-based agents i.e. OSP and limestone were shown to be equally effective as catalysts for lactulose production as ESP. They are insoluble reagents which could be easily removed from the reaction mixture for subsequent re-use. The isomerisation process from MCP with OSP or ESP assistance is ecologically sound by offering an alternative way to use those waste materials. Preliminary studies in modified MRS broths indicated that lactulose syrup (40 °B) could stimulate the growth of two commercial probiotic strains i.e. LA-5 and LC-01, but not BB-12 similar to reference lactulose. However, the addition of concentrated lactulose syrup to RSM in sufficiently high concentration (3–4%) appeared to have a positive effect on the growth and acid production by LA-5.

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