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Formation of oligosaccharides from whey UF-permeate by enzymatic hydrolysis — analysis of factors

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Two-level fractional factorial experiments were designed to study effects of enzyme (0.05 and 0.1%) and initial lactose concentrations (L_o: 14 and 23%), pH (5.0 and 7.0) and temperature (35 and 45°C) on enzymatic formation of oligosaccharides (OS) from whey UF-permeate in a batch reactor. β-D-galactosidase from *Aspergillus oryzae* (A), *Kluyveromyces lactis* (B) and *K. fragilis* (C) were compared. Hydrolysis with B and C gave comparable yields which were higher than that from A at identical conditions. L_o did not influence reaction time, but concentration of OS significantly increased by increasing L_o for all enzymes. Increasing L_o reduced the yield after hydrolysis with A and B, but improved the yield for C. L_o had negligible effect on degree of hydrolysis (DH) for A and C. However, increasing L_o significantly lowered DH for B. Increasing enzyme concentration significantly reduced reaction time for A, but it had no effect on that for B and C. DH significantly decreased after increasing concentration of A. Consequently, OS and yield were reduced. Applying B and C at higher concentration improved DH, OS and yield. Increasing temperature or pH reduced DH, OS and yield for A, but increased the responses for B and C.

INTRODUCTION

Whey utilisation continues to be a global concern in terms of economic, environmental and health-related aspects. Recently, production of oligosaccharides from lactose present in whey has attracted the attention of food scientists and industry provoked by the potential of oligosaccharides as ingredients in functional foods (Oku, 1994).

The ingestion of oligosaccharides encourages the proliferation of *Bifidobacterium bifidum* in the intestine, which has advantageous physiological effects on the host human (Wijsman *et al.*, 1989). Bifidobacteria inhibits the growth of harmful pathogenic microorganisms such as *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* (Misra and Kuila, 1992; Gibson and Wang, 1994). Moreover, bifidobacteria were reported to possess anti-cancer activity (Yaeshima, 1996). Oligosaccharides, being indigestible, have physiological effects similar to those of the dietary fibre; that is, lowering of blood serum cholesterol, pressure and sugar (Tomomatsu, 1994).

Oligosaccharides are polymeric carbohydrates consisting of two to ten monomer residues joined through glycosidic bonds. They can be obtained from different sources; for instance, fructo-oligosaccharides naturally occurring in a variety of crops (onion, garlic), and galacto-oligosaccharides synthesised during enzymatic hydrolysis of lactose present in whey and whey UF-permeate. Currently, fructo-oligosaccharides are the major oligosaccharides utilised in prebiotic formulations. Galacto-oligosaccharides have, however, been less exploited in spite of the fact that they possess tremendous potential as a prebiotic component.

Formation of galacto-oligosaccharides during enzymatic hydrolysis of lactose in milk, whey and whey UF-permeate is influenced by a number of factors, including nature and initial concentration of substrate, enzyme concentration, pH, temperature, source of enzyme and presence of minerals which may, at a certain level,

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retard the enzyme activity (Prenosil *et al.*, 1987*b*; Zárate and López-Leiva, 1990; Iwasaki *et al.*, 1996; Onishi and Yokozeki, 1996). The process technique, that is free, immobilised or membrane-retained enzyme, may also affect the yield and the degree of lactose hydrolysis (Berger *et al.*, 1995; Gekas and López-Leiva, 1985). The strategies followed to investigate the enzymatic hydrolysis of lactose to oligosaccharides adapt the one-variable-at-a-time paradigm. However, this approach is not efficient since it is unable to segregate and elucidate the effects of individual factors and their interactions on a given response. Moreover, a relatively large number of experiments is required in order to accurately understand the effects of the factors on the response (Joglekar and May, 1987). The drawbacks associated with the one-factor-at-a-time approach can be avoided by using factorial designs which are powerful techniques for planning and analysis of experiments. In a factorial design, the variables are changed simultaneously in a systematic fashion and the results are evaluated using statistical inference; that approach constitutes hypothesis testing.

The objective of this work was to study, using factorial designs, the effects of potential factors on formation of oligosaccharides during enzymatic hydrolysis of lactose in whey UF-permeate. The factors were initial lactose concentration, enzyme concentration, temperature and pH. The enzymes were β-D-galactosidases from *Aspergillus oryzae*, *Kluyveromyces lactis* and *K. fragilis*. The hydrolysis reaction (batch-wise) was monitored in terms of reaction time, oligosaccharides concentration, yield and degree of hydrolysis.

MATERIALS AND METHODS

Preparation of whey UF-permeate

Whey powder (80% lactose) was purchased from Arla Foods, Stockholm, Sweden). The powder was rehydrated to 14% or 23% lactose with distilled water and stirred for 1 h at 40°C.

A batch ultrafiltration (UF) circuit, consisting of a hollow fibre membrane (Romicon MP 10) with an effective area of 0.5 m^2 and cut off 10 000, was used to prepare the permeate. The rehydrated whey was ultrafiltered at 55°C and 1.5 bar. The permeate was thereafter pasteurised (HTST, Alfa-Laval, Lund, Sweden) at 80°C for 15 s, collected in sterilised glass bottles and stored at 4°C for further utilisation.

Enzymes

Three enzymes, indicated A, B and C, were used to hydrolyse lactose in the permeate. The enzymes were:

1. *Aspergillus oryzae* β-D-galactosidase (grade M) from Sigma-Aldrich, Milwaukee, USA;
2. *Kluyveromyces lactis* β-D-galactosidase (Maxilact® L 2000) from Gist-Brocades NV, Delft, The Netherlands;
3. *Kluyveromyces fragilis* β-D-galactosidase (Lactozym® 3000 L) from Novo Nordisk A/S, Bagsvaerd, Denmark.

Experimental design

Four factors were arranged in a two-level fractional (1/2) factorial design (Montgomery, 1984), for each of the enzymes, to investigate their effects on the formation of oligosaccharides. The factors and their corresponding lower and higher levels were: initial lactose concentration (14 and 23%), enzyme concentration (0.05 and 0.1%), pH (5.0 and 7.0) and incubation temperature (35 and 45°C). The experiments were performed in a random manner in order to reduce bias. Four responses, namely, reaction time, concentration of oligosaccharides formed, yield and degree of hydrolysis were evaluated. The experimental designs together with the responses for enzymes A, B and C are shown in Tables 1–3, respectively.

Formation of oligosaccharides

For each experiment, 100 ml of the permeate were dispensed into a 250 ml Erlenmeyer flask and inoculated with the enzyme. The pH was adjusted with NaOH or H_2SO_4. The reaction mixture was incubated in a temperature-controlled water bath for 5.5 h. Samples were withdrawn at 30 min intervals and collected in tubes. The collected samples were immediately immersed in a boiling water bath for 5 min to inactivate the enzymes before sugar analysis.

Analytical methods

Ash contents in the whey permeate (5.1%, 14% and 23% lactose) were determined according to the method described by the AOAC (1984).

Concentrations of lactose, glucose + galactose, and oligosaccharides were determined by HPLC according to the method described by Jeon and Mantha (1985). The mobile phase was made of acetonitrile (75% v/v) and distilled water (25% v/v) filtered though a sterile micro-filter (0.22 μm) and deaerated for 30 min in an ultrasonic equipment before use. The HPLC equipment consisted of a manual injector (20 μl), a pump (Waters, model M-6000 A), a pre-column (Nucleosil 120-7NH_2, 30×4 mm ID) and a column (Nucleosil 120-7NH_2, 250×4 mm ID). The detection was done on a refractive index detector (Varian R1-4, range 16, temperature = 35°C) and the chromatograms were registered on a Crome Jet SP 4400 integrator (AT = 8, C5 = 0.5 cm min^{-1}). The samples were diluted (20 times) with double-distilled water, filtered (0.22 μm), injected at ambient temperature and eluted with the mobile

Table 1. Factors and responses for lactose hydrolysis with *A. oryzae* β-D galactosidase (enzyme A)

Exp.	Factors				Responses			
	Lactose conc. (%)	Enzyme conc. (%)	Temperature (°C)	pH	Reaction time (h)[a]	Oligosaccharides (%)	Yield (%)[b]	Degree of hydrolysis (%)[c]
1	14	0.10	35	7	1.5	2.2	15.7	25.1
2	14	0.05	45	7	2.5	2.1	15.0	26.6
3	14	0.10	45	5	0.5	2.2	15.7	34.1
4	14	0.05	35	5	2.0	2.5	17.9	48.4
5	23	0.10	35	5	1.0	3.9	17.0	35.0
6	23	0.05	45	5	1.5	3.7	16.1	36.1
7	23	0.10	45	7	3.0	3.4	14.8	28.5
8	23	0.05	35	7	3.5	3.6	15.7	41.0

[a]Time at maximum formation of oligosaccharides.
[b]Yield = 100×conc. of oligosaccharides/initial lactose conc.

Table 2. Factors and responses for lactose hydrolysis with Maxilact® L 2000 (enzyme B)

Exp.	Factors				Responses			
	Lactose conc. (%)	Enzyme conc. (%)	Temperature (°C)	pH	Reaction time (h)[a]	Oligosaccharides (%)	Yield (%)[b]	Degree of hydrolysis (%)[c]
1	14	0.05	35	5	4.5	1.9	13.6	42.9
2	14	0.10	45	5	4.0	2.6	18.6	48.6
3	23	0.05	45	5	4.0	3.0	13.0	48.3
4	23	0.10	35	5	3.5	2.8	12.2	54.6
5	14	0.05	45	7	4.0	2.3	16.4	79.7
6	14	0.10	35	7	4.0	2.6	18.6	84.2
7	23	0.05	35	7	4.5	3.1	13.5	63.5
8	23	0.10	45	7	4.5	5.1	22.2	72.1

[a]Time at maximum formation of oligosaccharides.
[b]Yield = 100×conc. of oligosaccharides/initial lactose conc.
[c]Degree of hydrolysis = 100×conc. of (oligosaccharides + glucose + galactose)/initial lactose conc.

Table 3. Factors and responses for lactose hydrolysis with Lactozym® 3000 L (enzyme C)

Exp.	Factors				Responses			
	Lactose conc. (%)	Enzyme conc. (%)	Temperature (°C)	pH	Reaction time (h)[a]	Oligosaccharides (%)	Yield (%)[b]	Degree of hydrolysis (%)[c]
1	14	0.10	35	7	4.0	2.5	17.9	73.4
2	14	0.05	45	7	4.0	2.6	18.6	77.4
3	14	0.10	45	5	4.5	2.8	20.0	49.2
4	14	0.05	35	5	4.0	1.1	7.9	30.8
5	23	0.10	35	5	4.5	2.3	10.0	52.2
6	23	0.05	45	5	3.5	4.0	17.4	75.0
7	23	0.10	45	7	3.5	5.4	23.5	69.5
8	23	0.05	35	7	4.5	4.0	17.4	52.4

[a]Time at maximum formation of oligosaccharides.
[b]Yield = 100×conc. of oligosaccharides/initial lactose conc.
[c]Degree of hydrolysis = 100×conc. of (oligosaccharides + glucose + galactose)/initial lactose conc.

phase at 2.0 ml min $^{-1}$. Three replicates of each sample were analysed.

Statistical analysis

STATGRAPHICS® software was used to generate the experimental designs and calculate the mean effect of each individual factor on the responses. Three-and-higher-order interactions among the factors were neglected. A factor (or interaction) with the least mean square value was considered non-significant and its corresponding mean square was used as an estimate of experimental error for testing the significance of factors at 5% confidence level. The F-value obtained from the F-distribution table, was used to accept or reject the null hypothesis. The significances of the mean effects of the

factors were further confirmed using normal probability plots of standardised effects versus percent commulative probability.

RESULTS AND DISCUSSION

The concentration of a saccharide in the whey permeate hydrolysate (WPH) was calculated from the HPLC chromatograms and reported as a percentage (or, equivalently, as g saccharide per 100 ml WPH). The equipment and conditions at which the HPLC analysis was performed did not allow for detection of the glucose and galactose peaks independently because they had quite similar retention times. Therefore, the concentration of these carbohydrates was reported as the sum of the individual concentrations, indicated as glucose + galactose or, simply, monosaccharides.

Effect of enzyme source

Depletion of lactose, and simultaneous formation of mono and oligosaccharides observed during hydrolysis of lactose in the UF-whey permeate by enzyme A, enzyme B and enzyme C are demonstrated in Figs 1–3, respectively. The hydrolysis conditions associated with the concentration-time curves (Figs 1 to 3) corresponded to the maximum yields of oligosaccharides obtained following the different experimental designs. In general, the lactose concentration decreased with time. In contrast, the concentrations of mono and oligosaccharides increased with time. However, after a certain period, the concentration of oligosaccharides started to drop, most probably as a result of the enhanced inhibitory effect of the monosaccharides. Interestingly, the monosaccharides concentration was higher than that of the oligosaccharides, with a vertical distance between curves, representing the two saccharide groups, increasing with progress in the reaction. This reflected the role of glucose and galactose as competitive inhibitors to the formation of oligosaccharides during enzymatic hydrolysis of lactose. Glucose and galactose, produced during enzymatic hydrolysis of lactose, were reported to restrain the formation of oligosaccharides, when the concentration of the monosaccharides reaches a certain level (Prenosil *et al.*, 1987*a*; Iwasaki *et al.*, 1996).

The maximum yield of oligosaccharides (17.9%) by *A. oryzae* β-D-galactosidase occurred at 14% initial lactose, 0.05% enzyme concentration, 35°C and pH 5.0, and corresponded to 2.0 h reaction time and 48.4% degree of hydrolysis (Table 1). The oligosaccharides concentration was characterised by a sharp increase (from 0 to 2.0%) during the first 30 min of the reaction, followed by a level-off period with non-significant fluctuations (2.2–2.7%) up to 5.0 h, and decreased to 1.9% at 5.5 h (Fig. 1).

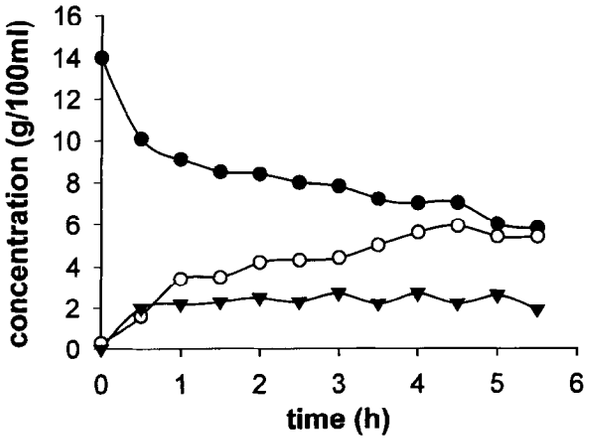

Fig. 1. Formation of oligosaccharides during hydrolysis of lactose in whey UF-permeate with *Aspergillus oryzae* β-D galactosidase. The incubation conditions were 14% initial lactose concentration, 0·05% enzyme concentration, 35°C and pH 5·0. ●, Lactose; ○, glucose + galactose; ▼, oligosaccharides.

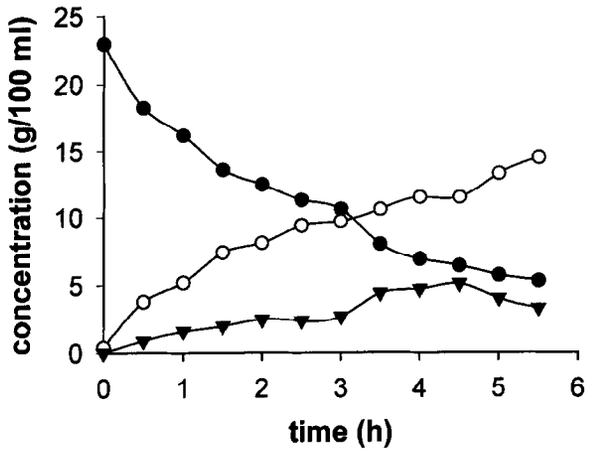

Fig. 2. Formation of oligosaccharides during hydrolysis of lactose in whey UF-permeate with Maxilact®. The incubation conditions were 23% initial lactose concentration, 0·1% enzyme concentration, 45°C and pH 7·0. ●, Lactose; ○, glucose + galactose; ▼, oligosaccharides.

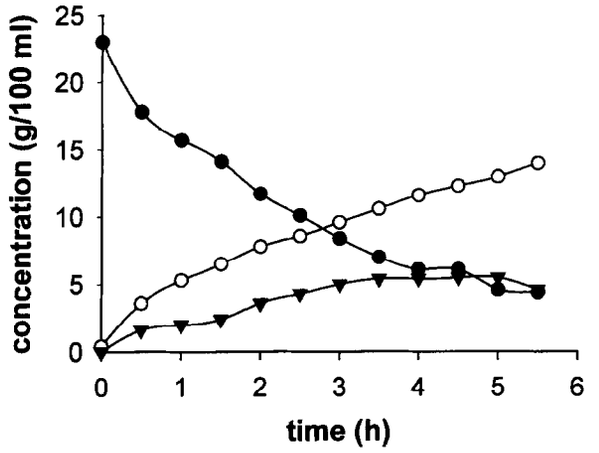

Fig. 3. Formation of oligosaccharides during hydrolysis of lactose in whey UF-permeate with Lactozym®. The incubation conditions were 23% initial lactose concentration, 0·1% enzyme concentration, 45°C and pH 7·0. ●, Lactose; ○, glucose + galactose; ▼, oligosaccharides.

The concentration-time relationships observed during hydrolysis with Maxilact® (Fig. 2) and Lactozym® (Fig. 3) were rather similar. However, the differences in the enzyme origin resulted in different yields, degrees of hydrolysis and reaction times. At similar incubation conditions, Lactozym® gave higher yield and oligosaccharides (23.4%, 5.4%, respectively) than did Maxilact® (22.0%, 5.1%, respectively). Also, the maximum yield was attained after a shorter reaction time when the lactose was hydrolysed with Lactozym® (3.5 h) in comparison with Maxilact® (4.5 h). In general, it was shown that β-D-galactosidases from yeast have a higher capacity for oligosaccharides formation than that of galactosidases from bacteria and fungi (Prenosil *et al.*, 1987*b*; Onishi *et al.*, 1995).

Factors affecting formation of oligosaccharides

The mean effects of each factor with respect to the different responses obtained after hydrolysis with enzyme A, enzyme B and enzyme C are shown in Table 4. A factor with a negative mean effect indicated that changing the factor from its lower to higher level decreased the response, and vice versa. The reaction time (h) is defined as the time at which the maximum formation of oligosaccharides occurred, and after which a decrease or no significant increase in the oligosaccharides concentration was observed. The yield (%) was the ratio of the oligosaccharides to the initial lactose concentration, expressed as a percentage. The degree of hydrolysis is the ratio between concentration of the total sugars formed (oligosaccharides and glucose + galactose) and the initial concentration of lactose, expressed as a percentage.

Initial lactose concentration

The initial lactose concentration did not influence the reaction time when lactose was hydrolysed with enzyme A, enzyme B and enzyme C, as indicated by non-significant mean effects (Table 4). In contrast, the maximum concentration of oligosaccharides formed was significantly increased by increasing the initial lactose concentration from 14% to 23%, with all of the enzymes investigated. The mechanism of lactose hydrolysis by β-D-galactosidases involves several reactions occurring simultaneously. These reactions are the self-transferase reaction of lactose, self-transferase reaction of trisaccharides, hydrolysis of lactose, hydrolysis of trisaccharides, and inhibition of oligosaccharides formation by glucose and galactose (Iwasaki *et al.*, 1996). The transgalactosylic activity of β-D-galactosidases was suggested to be strongly dependent on initial lactose concentration. At low concentration, galactose alone seems to be involved in the transfer reactions. At high concentration, both glucose and galactose are likely to participate in the transgalactosylation (Prenosil *et al.*, 1987*b*). Thus, the proposal made by Prenosil and co-workers might explain the increase in the oligosaccharides yield induced by using a substrate with higher initial lactose concentration, in this experiment.

The statistical analysis (Table 4) revealed that increasing the initial lactose concentration from the lower to higher level resulted in a decrease in the oligosaccharides yield obtained after hydrolysis with enzyme A and enzyme B. In contrast, the yield was increased with increasing the initial lactose concentration when it was hydrolysed with enzyme C. These differences could be attributed to the presence of cations in the whey permeate, which might detain or enhance the activity of some β-D-galactosidases, depending on their sensitivity to specific cations. Mahoney and Adamchuk (1980) found that the activity of lactase from *K. fragilis* in whey was controlled by the ionic environment; Na and Ca ions were inhibitory, while K and Mg ions were activating. Also, Na and Ca inhibited the activity of lactase from *K. lactis* (Mozaffar *et al.*, 1985). Figure 4 illustrates ash content in the whey permeate at different lactose levels. The ash content increased in the whey permeate with increasing initial lactose concentration. Whey permeate was reported (Zall, 1992) to contain (mg $100\,g^{-1}$) high amounts of K (130) and Mg (79), and relatively lower levels of Na (41) and Ca (40). Thus, it was more likely that the substrate with 23% initial lactose concentration contained higher amounts of inhibitory cations than the one with 14% lactose.

Table 4. Mean effects of the factors for enzymes A, B and C

Factor	Reaction time (h)[a]			Oligosaccharides (%)			Yield (%)[b]			Degree of hydrolysis (%)[c]		
	A	B	C	A	B	C	A	B	C	A	B	C
Lactose conc.	0.6	0	−0.1	1.4*	1.2*	1.7*	−0.4	−1.4	1.0	1.6	−4.2*	4.6
Enzyme conc.	−0.9*	−0.3	0.1	−0.1	0.7*	0.3	−0.6	3.7*	2.5	−7.4*	6.3*	2.2
Temperature	−0.1	0	−0.4*	−0.2*	0.6	1.2	−1.1*	3.1	6.7*	−6.1*	0.9	15.6*
pH	1.4*	0.3	−0.1	−0.3*	0.7*	1.1	−1.4*	3.3*	5.3*	−8.1*	26.3*	16.4*

[a]Time at maximum formation of oligosaccharides.
[b]Yield = 100 × conc. of oligosaccharides/initial lactose conc.
[c]Degree of hydrolysis = 100 × conc. of (oligosaccharides + glucose + galactose)/initial lactose conc.
*Significant at 95% confidence level.
Enzyme A: β-D-galactosidase from *Aspergillus oryzae*.
Enzyme B: Maxilact® L 2000 from *Kluyveromyces lactis*.
Enzyme C: Lactozym® 3000 L from *K. fragilis*.

The initial lactose concentration had non-significant effects on the degree of hydrolysis with enzyme A and enzyme C. In contrast, increasing the initial lactose concentration sigificantly reduced the degree of hydrolysis with enzyme B. This could also be attributed to differences in sensitivity of the enzyme to minerals in the whey.

Enzyme concentration

Increasing the enzyme concentration from 0.05% to 0.1% significantly reduced the reaction time for enzyme A. However, the increase in the enzyme concentration had no significant effect on the reaction time for enzyme B and enzyme C (Table 4). Surprisingly, the degree of hydrolysis was significantly reduced as a result of increasing the concentration of enzyme A. Consequently, the amount of oligosaccharides formed and the yield were reduced when the concentration of enzyme A was increased. Generally, in enzyme-catalysed reactions, the reaction rate is directly proportional to the enzyme concentration. However, deviation can occur as a result of the presence of small amounts of some highly toxic impurity or reversible inhibitors in the enzyme preparation. Thus, increasing the enzyme concentration raises the level of the inhibitor in the reaction mixture. The results suggested that it is more economical to use a lower amount (below 0.1%) of enzyme A. However, it might be more beneficial to apply enzyme B and enzyme C at concentrations higher than 0.05%, since that increases the amount of oligosaccharides, yield and degree of hydrolysis (Table 4).

Temperature and pH

Incubation temperature controls, to a great extent, the kinetic parameters of enzyme-catalysed reactions. Increasing the temperature or pH from the lower to higher level reduced the amount of oligosaccharides formed, yield and degree of hydrolysis for enzyme A. In contrast, the responses were increased when the temperature or pH was raised from the lower to higher level for enzyme B and enzyme C, with varying degrees of significance (Table 4). The contradictory effects observed when changing the levels of temperature and pH for enzyme A on the one hand, and for enzyme B and enzyme C on the other hand, could be attributed to differences in the enzyme activities as influenced by their origin, sensitivity to pH and degree of purity (Gekas and López-Leiva, 1985). It is obvious that maximum enzyme activity and consequently higher yields of oligosaccharides were attainable by performing the hydrolysis at temperatures not exceeding 35°C with enzyme A, and at temperatures greater than 35°C with enzyme B or enzyme C. This is in agreement with results obtaied by other workers. For example, the maximum activity of *K. fragilis* lactase was found to be at 47°C and pH range 6.0–7.0 (Lutzen and Norman, 1980), while it was at 40°C for Lactozym®, from *K. fragilis*, (Novo, 1992) and at 35–40°C for Maxilact®, from *K. lactis*, (Gist-Brocades, 1994).

CONCLUSIONS

Hydrolysis with the yeast enzymes (Maxilact® and Lactozym®) produced comparable yields of oligosaccharides which was higher than that obtained using the fungal lactase (from *A. oryzae*). Moreover, the degree of hydrolysis achieved by the yeast enzymes was essentially higher than what occurred using the fungal lactase. The use of a membrane reactor set-up in which the monosaccharides produced during the hydrolysis of lactose can be continuously removed, during the reaction course, can be recommended to eliminate their inhibitory effect, and thus improve the yield and efficiency of the process. Also, removal of minerals from whey prior to hydrolysis may increase the yield since they may adversly influence the enzyme activity. The factors shown to be significant, in this study, for the hydrolysis of lactose, need to be optimised. Moreover, a kinetic study would help understand the mechanism of the hydrolysis, and thus lead to a proper design of the process.

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