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Utilization of UF-Permeate for Production of β-galactosidase by Lactic Acid Bacteria

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Abstract

Four lactobacilli strains (*Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacilus casei* and *Lactobacillus reuteri*) were grown in MRS broth and three lactococci strains (*Streptococcus thermophilus*, *Lactococcus lactis* subsp. *Lactis* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis*) were grown in M17 broth. *L. reuteri* and *S. thermophilus* were chosen on the basis of the best mean β-galactosidase activity of 10.44 and 10.01 U/ml respectively, for further studies on permeate-based medium. The maximum production of β-galactosidase by *L. reuteri* was achieved at lactose concentration of 6%, initial pH 5.0–7.5, ammonium phosphate as nitrogen source at a concentration of 0.66 g N/L and incubation temperature at 30°C/24 hrs to give 6.31 U/ml. While in case of *S. thermophilus*, maximum β-galactosidase production was achieved at 10% lactose concentration of permeate medium, supplemented with phosphate buffer ratio of 0.5:0.5 (KH_2PO_4:K_2HPO_4, g/L), at initial pH 6.0–6.5, ammonium phosphate (0.66g N/L) as nitrogen source and incubation temperature 35°C for 24 hrs to give 7.85 U/ml.

Key words: *L. reuteri*, *S. thermophilus*, β-galactosidase, lactic acid bacteria (LAB), permeate

Introduction

The disposal of whey remains a significant problem for the dairy industry. As whey contains 5 to 6% dissolved solids, including 3 to 5% lactose, the biological oxygen demand (BOD) is high. Generally, whey must be treated prior to discharge into the environment (Marwaha and Kennedy, 1988).

A number of applications for whey-permeate have been developed in an effort to overcome the problem of its disposal. One alternative is the use of whey as the basic medium for various fermentation processes including the production of ethanol, methane, yeast protein, xanthan gum (Fu and Tseng, 1990) or organic acids such as lactate, propionate or acetate (Mawson, 1994; Huang and Yang, 1998). Another application with a high technological and dietetic interest is the enzymatic hydrolysis of lactose, whose economic importance has been increasing ever since the 1960s (Novalin *et al.*, 2005).

The enzyme β-D-galactoside galactohydrolase (β-galactosidase, E.C. 3.2.1.23, trivially lactase) hydrolyzes lactose, the milk sugar, into two moieties glucose and galactose (Rings *et al.*, 1994). This technically and economically feasible process would also open new possibilities for the utilization of whey and whey-permeate (Zadow, 1993).

While β-galactosidase has been found in numerous biological systems, microorganisms such as yeasts, molds and bacteria still remain the only commercially exploited sources (Agrawal and Dutta, 1989). More recently, thermophilic bacteria have become an object of interest for the commercial production of β-galactosidase (Petzelbauer *et al.*, 1999). Among these, special attention has been paid to lactic acid bacteria (LAB) because of their GRAS status (Stiles and Holzapfel, 1997). Lactic acid bacteria have a long tradition of use in the food industry. Their potential uses as a source of enzymes, especially β-galactosidase, has been shown to be promising (Murad, 1998).

The aim of the current work was to optimize the growth conditions to maximize the production of β-galactosidase by some LAB strains grown on the newly modified permeate-based medium.

Experimental

Materials and Methods

Organisms. Seven LAB strains were obtained from the Department of Dairy Microbiology, National Research Centre. The obtained strains were *Lactobacillus reuteri*, *Lactobacillus acidophilus*, *Lactococcus lactis*

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subsp., *lactis* biovar *diacetilactis, Lactobacillus bulgaricus (241), Streptococcus thermophilus, Lactococcus lactis* subsp. *lactis* and *Lactobacillus casei.* The obtained strains were maintained and activated in sterile litmus milk, stored in a refrigerator at 4°C and tested for their ability to produce β-galactosidase.

β-Galactosidase production media and growth conditions

Synthetic media. Production of β-galactosidase by lactococci and lactobacilli cultures was examined in M17 synthetic broth medium (Difco) and MRS synthetic broth medium, respectively. The media were inoculated with freshly activated 1% inoculum and incubated at 37°C for 24 hrs according to Vasiljevic and Jelen (2001) and Herreros *et al.* (2003).

Permeate-based medium. The milk permeate, which is considered as a waste during the production of cheese by ultrafiltration, was obtained from a cheese production factory located in Mansoura City (Nile Delta) and used for the preparation of the permeate-based medium. In the following tests, permeate-based medium components and environmental conditions were investigated for optimum conditions leading to maximize β-galactosidase production as mentioned by Murad (1998).The pH of permeate-based media in the experiments was adjusted to 6.5 by 1N NaOH or 1N HCl and then sterilized for 30 minutes at 110°C. The media were inoculated with 1% inoculum of the freshly activated strains (24 hrs) and then incubated at for 24 hrs 37°C.

Effect of media composition and environmental conditions on the production of β-galactosidase

The previously mentioned permeate-based medium was employed for improvement of β-galactosidase production by varying its components qualitatively and quantitatively under variable environmental conditions as follows:

Effect of lactose concentration. Different concentrations of lactose in permeate medium (2, 4, 6, 8, 10 and 12%) were prepared either by diluting or adding lactose (Difco) to the medium.

Effect of potassium phosphate. Different concentrations of KH_2PO_4: K_2HPO_4 mixtures were added to permeate medium at a ratio concentration of 0.5:0.5, 0.5:1.0 and 0.5:1.5 g/l, respectively, compared with the control.

Effect of initial pH. The influence of different initial pH values was examined. The pH values were adjusted at 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 using either NaOH or 1 N HCl.

Effect of nitrogen source. Various nitrogen sources were used separately at an equivalent concentration of 0.33 g N/l media (Murad, 1998). The nitrogen sources included 4 inorganic forms [$(NH_4)_2PO_4$, $(NH_4)_2SO_4$, NH_4Cl and $NaNO_3$] and 4 organic sources (yeast extract, beef extract, peptone and tryptone).

Effect of nitrogen concentration. The optimum nitrogen source chosen was tested for its most suitable concentration ranging from 0.165 to 0.990 g N/l.

Effect of incubation temperature. The effect of incubation temperature was studied. The incubation temperatures ranged from 25 to 45°C (with 5°C increments), except for 37°C (the control), using electric chamber incubators.

Enzyme activity assay. β-galactosidase was assayed according to the method of Lederberg (1950) as described by Sanchez and Hardisson (1979). The method was strictly applied, except for the centrifugation speed which was used for separation of the bacterial cells and the sonicated cell debris at 4°C. The centrifugal speeds used were modified to 8,000 rpm/10 min and 15,000 rpm/20 min, respectively using Sigma 2K15 centrifuge.

Statistical analysis. The significances of the results were determined by the analysis of variance (ANOVA) evaluated by Duncan's multiple range tests (at 0.05), using COSTAT software, product of Cohort software Inc., Berkley, California, (Duncan, 1955).

Results and Discussion

Production of β-galactosidase by selected LAB strains. As shown in Fig. 1, β-galactosidase production showed no significant differences between the 7 tested strains, ranging from 9.3 to 10.4 U/ml under the given experimental conditions. According to the obtained results, *S. thermophilus* and *L. reuteri* (representing lactococci and lactobacilli, respectively) were chosen on the basis of the best mean β-galactosidase activity of 10.01 and 10.44 U/ml, respectively for further studies on permeate-based medium.

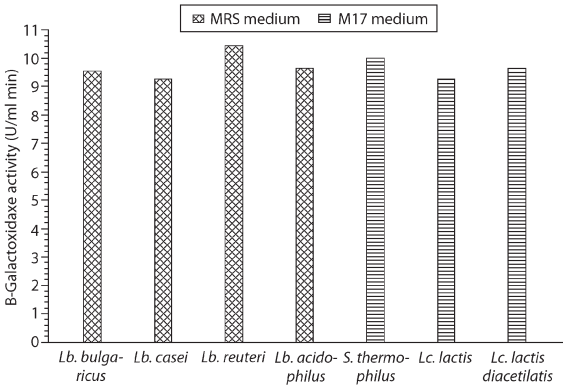

Fig 1. β-galactosidase production by LAB strains M17 and MRS media expressed as enzyme activity (U/ml)

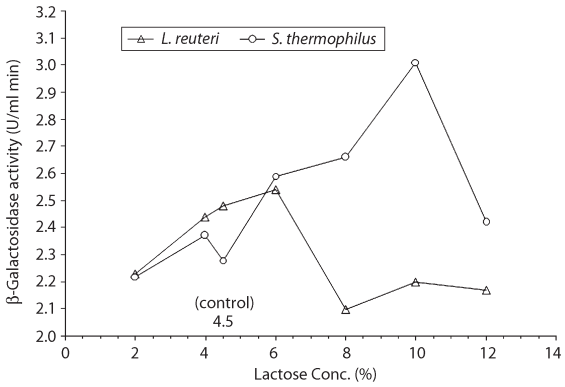

Fig 2. Effect of lactose concentration in permeate-based medium on the production of β-galactosidase.

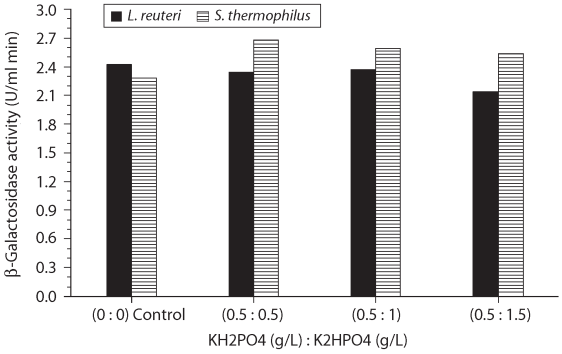

Fig 3. Effect of KH_2PO_4:K_2HPO_4 ratio (g/l) in permeate-based medium on the production of β-galactosidase.

Effect of lactose concentration. The data shown in Fig. 2 indicate that the production of β-galactosidase by *L. reuteri* and *S. thermophilus* strains increased with the increasing of lactose concentration (up to 6%), reaching 2.54 and 2.59 U/ml, respectively. Further increasing lactose concentration up to 10% decreased the *L. reuteri* enzyme production dramatically, while it increased the *S. thermophilus* enzyme production (3.01 U/ml). Hasan and Durr (1974) stated that lactose induced the synthesis of β-galactosidase.

As a fact, the amounts of carbon source in the medium may affect the expression of β-galactosidase by microorganisms (Fiedurek and Szczodrak, 1994; Inchaurrondo *et al.*, 1998). The β-galactosidase activity increased as the concentration of lactose in the medium was increased up to 4.0%. Further increasing the lactose content resulted in the reduction of β-galactosidase activity. A similar phenomenon was observed by Fiedurek and Szczodrak (1994) who investigated the biosynthesis of β-galactosidase by *Kluyveromyces fragilis*.

Inchaurrondo *et al.* (1998) clarified that the decreased β-galactosidase activity in the medium containing 5% or more lactose might be attributed to the increased concentration of internally released glucose which represses the biosynthesis of β-galactosidase by the test organism. Furthermore, it was demonstrated that 4% lactose was sufficient to induce the highest expression of β-galactosidase under the tested conditions.

This gives an indication about the efficiency of *S. thermophilus* to consume higher concentrations of lactose (10%) than *L. reuteri* (6%), indicated by the highest production of β-galactosidase. Besides, the upper limit of lactose concentration that the two strains can produce β-galactosidase efficiently couldn't be exceeded due to the accumulation of glucose by-products intracellularly, as discussed before.

Effect of potassium phosphate. The data shown in Fig. 3 illustrate the effect of potassium phosphate (acidic and alkaline form) ratio on the β-galactosidase production by both strains. In case of *L. reuteri* there was no significant difference in β-galactosidase production magnitude for the control and the phosphate buffer treatments (KH_2PO_4:K_2HPO_4, g/L) at ratios 0.5:0.5 and 0.5:1.0 (2.38 U/ml, in average). Increasing the alkaline phosphate form of potassium decreased the production level significantly at the ratio of 0.5:1.5. Apparently, there is no need to add potassium phosphate buffer because the control gave similar level of β-galactosidase production, but there is always a great need for the buffer to capture any possible excess in organic acid by-product (lactic acid), specially when the permeate sources are varied.

The production of β-galactosidase by *S. thermophilus* showed a significant difference between the control test and the whole added phosphate buffer forms ratios. At 0.5:1.0 and 0.5:1.5 ratios (KH_2PO_4:K_2HPO_4, g/L) the β-galactosidase production mean increased by 12% compared with the control, but at 0.5:0.5 ratio the increase of β-galactosidase production was 17.5% (2.68 U/ml), proving its dominancy over the control and other treatments.

These data agree with what Ramana Rao and Dutta (1977) discussed in their work on *S. thermophilus* production for β-galactosidase that was obviously stimulated by the monobasic phosphate buffer form more than di- and tri-basic forms, while in the case of *K. fragilis* maximum β-galactosidase production was achieved by addition of KH_2PO_4:K_2HPO_4 buffer at a ratio of 1:3, as stated by Fiedurek and Szczodrak later (1994). Murad (1998) tested the β-galactosidase production level from *L. bulgaricus* using different KH_2PO_4:K_2HPO_4 buffer ratios at 1:2 and 1:3 and found that at the latter ratio the organism produced maximum β-galactosidase than at the former. Hsu *et al.* (2005) found that the best buffer ratio at which the *Bifidobacterium* produced maximum β-galactosidase was 1:3 (KH_2PO_4:K_2HPO_4).

Effect of initial pH. The obtained data showed no effect of various initial pH values on *L. reuteri* production of β-galactosidase, except that there was an obvious decline in the enzyme production at the alkaline border beginning at pH 8 and above. This proved the capability of *L. reuteri* to produce β-galactosidase at maximum level (3.21 U/ml, in average) through a wide range of initial pH (5.0 to 7.5). Maximum β-galactosidase production by *S. thermophilus* was obtained at pH 6.5 (2.77 U/ml). The experimental results revealed that the increase in the enzyme production was gradually increased at pH 5 up to 6.5, followed by significant decrease in the production at pH 7 up to 8.5.

The effect of the initial pH of the medium on enzyme production by *S. thermophilus* was studied over a pH range of 4.0 to 9.0 by Ramana Rao and Dutta (1977) and they reported that maximum enzyme production was observed between pH 6.5 and 7.5. Sridhar and Dutta (1991) worked on the production of β-galactosidase from *Streptococcus cremoris* on whey. They reported that the optimum pH was between 6.5 and 7. The results presented by Murad (1998) showed that the highest enzyme production by *L. bulgaricus* was obtained at pH 5, while, Hsu *et al.* (2005) reported the optimum initial pH for β-galactosidase production by *Bifidobacterium* sp. to be 6.5. It is obvious that there is a great similarity in the effect of optimum initial pH (6.5) on the production of β-galactosidase by both strains (*L. reuteri* and *S. thermophilus*) and those reported previously by Ramana Rao and Dutta (1977), Sridhar and Dutta (1991) and Hsu *et al.* (2005).

The effect of nitrogen source. The observed results revealed that among the 8 nitrogen sources tested, the ammonium phosphate serving as a source for both nitrogen and phosphate, was found to be the best for maximum production of β-galactosidase by *L. reuteri* (5.74 U/ml) and *S. thermophilus* (6.83 U/ml). *L. reuteri* managed to use both organic and inorganic sources efficiently compared with the control, while, *S. thermophilus* preferred the simple nitrogen form (*i.e.* inorganic) more than the complex ones (*i.e.* organic).

This agreed with what was reported by Murad (1998), namely that the highest production of β-galactosidase by *L. bulgaricus* was obtained using $(NH_4)_2HPO_4$. Also, he mentioned that no activity was detected in the presence of $(NH_4)_2SO_4$. This fact was confirmed previously by Selim and EL-Diwany (1985) and Fiedurek and Szczodrak (1994) who reported that some salts such as $(NH_4)_2SO_4$ showed an inhibitory effect on β-galactosidase production by *K. fragilis.*

On the contrary, Ramana Rao and Dutta (1977) found that the best nitrogen source for the production of β-galactosidase by *S. thermophilus* was protease peptone, followed by ammonium sulphate, and they did not use any phosphate form of nitrogen.

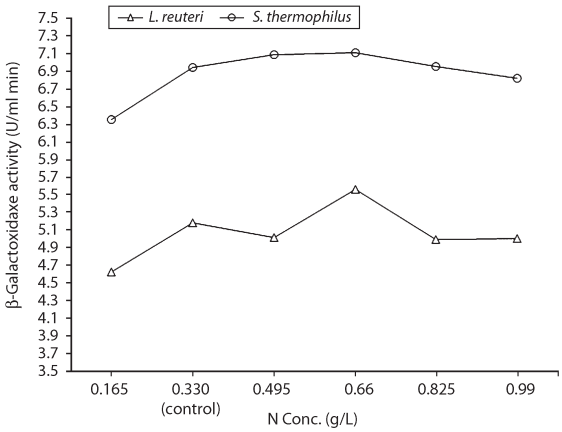

Fig 4. Effect of supplemented nitrogen concentration in permeate-based medium on the production of β-galactosidase.

These findings about the importance of nitrogen source were mentioned by Ramana Rao and Dutta (1977) and Shaikh *et al.* (1997), who found that nitrogen sources may affect the microbial biosynthesis of β-galactosidase.

Effect of nitrogen concentration. The preferred nitrogen source type (ammonium phosphate) chosen for both strains from the previous experiment was tested for its best concentration as shown in figure 4. It is clear that *S. thermophilus* production of β-galactosidase was much higher than *L. reuteri* over all the ammonium phosphate concentrations tested.

Both strains showed the same trend in response to nitrogen concentration. The β-galactosidase production significantly increased as the nitrogen concentration increased; until it reached its best level when nitrogen concentration was twice (0.66 g N/L) that of the control (0.33 g N/L), as *L. reuteri* yielded 5.56 U/ml and *S. thermophilus* gave 7.11 U/ml, then declined when it reached triple that of the control.

This trend agreed with the findings of Hsu *et al.* (2005) in their work on *Bifidobacteria*. The activity of β-galactosidase increased upon increasing the nitrogen source concentration but further increasing resulted in a sharp reduction in the activity of β-galactosidase and a reduced final population of the test organism.

Murad (1998) found that the concentration of $(NH_4)_2HPO_4$ in the growth medium of *L. bulgaricus* exhibited a profound effect on the production of β-galactosidase, as the highest activity was obtained using 0.4%. While in case of *L. reuteri* and *S. thermophilus*, the the highest β-galactosidase activity was obtained using 0.3% only.

It is worth mentioning that Jokar and Karbassi (2009) maximized β-galactosidase production by *Lactobacillus delbruekii* when grown in permeate based medium enriched with a combination of yeast extract, whey

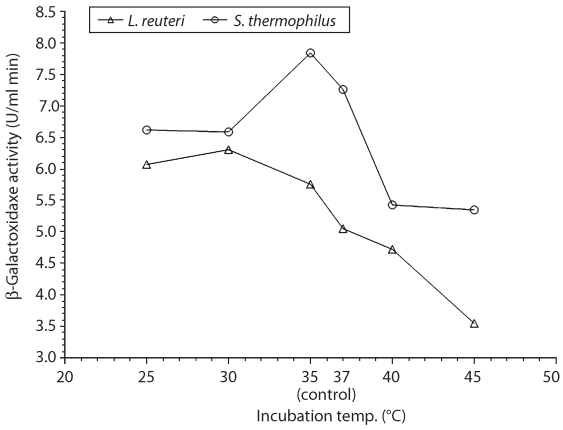

Fig 5. Effect of incubation temperature on production of β-galactosidase.

powder and wheat steep liquor as organic nitrogen sources to reach 4.924 U/ml, less than that obtained in the current study (5.56 and 7.11 U/ml) using only ammonium phosphate as an inorganic source.

Effect of incubation temperature. *L. reuteri* strain produced maximum β-galactosidase (6.31 U/ml) at 30°C followed by a significant decline in enzyme production with further increase in the incubation temperature (Fig. 5). The same trend in the production of β-galactosidase by *S. thermophilus* strain was observed with its maximum enzyme production (7.85 U/ml) at 35°C.

Hsu *et al*., (2005) stated that the activity of β-galactosidase produced by *Bifidobacteria* increased as the cultivation temperature increased from 22°C to 37°C. Further increases in the cultivation temperature led to a reduction of enzyme production accompanied by a reduction in the final viable population. These observations agree with those of Fiedurek and Szczodrak (1994) as well as Smith *et al*. (1985), which demonstrated that the highest β-galactosidase production by *B. longum* was obtained at 37 °C.

It could be concluded that the permeate (4.5% lactose) as an industrial waste can be used efficiently in the production of β-galactosidase by either *L. reuteri* or *S. thermophilus* strains (6.31 and 7.85 U/ml, respectively) when grown at initial pH 6.5, if optimized by adding KH_2PO_4:K_2HPO_4, at ratio of 0.5:0.5 g/L and ammonium phosphate at 0.66 g N/L as nitrogen source, with an incubation temperature of 30°C and 35°C, respectively.

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