BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF A β-GALACTOSIDASE FROM *BIFIDOBACTERIUM BREVE* B24

By

Sung Hun Yi

Department of Food Science and Agricultural Chemistry

Macdonald Campus of McGill University

Montreal, Quebec, Canada

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DEDICATION

TO MY PARENTS WHO SUPPORT ME WITH LOVE AND CARE

TO MY FAMILY WHO GIVE ME LOVE AND PEACE

ABSTRACT

A β-galactosidase gene from *Bifidobacterium breve* B24 which showed the higher hydrolytic and synthetic activity was cloned in *E. coli*. The complete β-galactosidase gene contained 2076 bp nucleotides and encoded 691 amino acids which had a high homology to the other *Bifidobacterium* species. This β-galactosidase was homologous to that of the *LacA* family. The *galA* gene was successfully over-expressed in *E. coli* ER2566. To observe any change in the recombinant enzyme, β-galactosidases from *Bifidobacterium breve* B24 and recombinant *E. coli* ER2566 were purified to homogeneity by ion exchange chromatography (Mono-Q) and gel-filtration chromatography (Superose-12 and Superdex 200) columns. The molecular mass of both β-galactosidases was estimated to be 75 kDa on SDS-PAGE. Activity staining on non-denaturing Native-PAGE and Superose-12 gel-filtration chromatography showed that the enzymes are composed of a dimer with a molecular mass of 150 kDa.

The optimum pHs of the native and recombinant enzymes for hydrolyzing O-nitrophenyl-β-D-galactopyranose (ONPG) were pH 6.0 and 7.0, respectively, and they were stable over the pH range of 5-8 and 6-9, respectively. The optimum temperature of both enzymes for hydrolyzing ONPG was similar at 45 ℃ and they were stable over the

temperature range of 20-45 ℃. Both enzymes were stable up to 45 ℃ during 5 h of incubation at pH 6.5. The recombinant enzyme was slightly activated by bivalent metal ions, Mg^{2+}, Mn^{2+}, and Zn^{2+} at 1 mM but strongly inhibited by Hg^{2+} and p-chloromercuribenzoic acid (PCMB). The K_m values of both native and recombinant β-galactosidases for ONPG were 2.77 and 1.82 mM, respectively, and the V_{max} values were 1.02 and 1.39 mM/min, respectively.

The two β-galactosidase activities were also tested with lactose as substrate. The optimum pH of the native and recombinant enzymes for hydrolyzing lactose was similar at pH 6.0. Both enzymes had more than 80 % of their activity in the range of pH 6-8, indicating that the enzymes were stable at neutral pH. However, the native β-galactosidase had around 40% of its activity at pH 5.0, whereas the recombinant enzyme had no activity at this pH. On the other hand, the recombinant enzyme had over 50 % of its activity at pH 9.0, while the native β-galactosidase showed lower than 5 % of its activity. The optimum temperature of both enzymes was at 45℃. The profiles of both enzyme activities were very similar except at the temperature of 10 ℃. The recombinant β-galactosidase still had around 20 % of its enzyme activity at 10 ℃, while no enzyme activity from the native enzyme was detected at this temperature. No strong activators on hydrolyzing lactose were found, but 1 mM of $HgCl_2$ or PCMB strongly inhibited the

hydrolyzing activity of the recombinant enzyme. About 47.5% of 1 M lactose was hydrolyzed in 5 h at 45 ℃, whereas 100% of 100 mM lactose was completely hydrolyzed in 5 h at 45 ℃ with 50 units of enzyme. No significant increase in hydrolytic activity was observed when over 600 mM of lactose was used.

In the presence of glucose and galactose in the reaction mixture, the recombinant β-galactosidase synthesized oligosaccharides. The total amount of oligosaccharides was increased by increasing the glucose and galactose concentration, indicating that the recombinant β-galactosidase was able to synthesize oligosaccharides without hydrolytic activity. At the highest concentration of lactose (1000 mM), 41.8 % of oligosaccharide was synthesized from lactose hydrolysates.

Lactose (4.8 %) in skim milk was almost completely hydrolyzed with 50 units of enzyme at 45 ℃ for 5 h. About 97.4 % of lactose (41.22 μg/μl) in milk was hydrolyzed to produce 46.3 % of glucose, 46.6 % of galactose, and 7.1 % of oligosaccharides. After 24 h reaction, 3.04 μg/μl, 5.75 μg/μl, 7.28 μg/μl, and 1.95 μg/μl of oligosaccharides were synthesized at 20, 37, 45, and 50 ℃, respectively. Lactose was completely hydrolyzed after 14, 12, and 6 h at 20, 37, and 45 ℃, respectively. However, at 50 ℃, lactose hydrolysis was halted and also oligosaccharides were not increased after 6 h, due to the

inactivation at 50 ℃. The K_m values of the native and recombinant enzymes on lactose were 152.08 and 95.58 mM, respectively, that were 54.9 and 52.51 times higher than ONPG. The V_{max} values on lactose by the native and recombinant enzymes were 138.95 and 189.13 mM/min, respectively.

β-Galactosidase of *Bifidobacterium breve* B24 with unique properties on high hydrolytic and transgalactosylation activity, small size of enzyme, and thermostability were studied at the biochemical and molecular levels for the first time.

RÉSUMÉ

Un gène de la β-galactosidase de *Bifidobacterium breve* B24 qui a montré l'activité hydrolytique et synthétique la plus élevée a été cloné dans *E. coli*. Le gène complet de la β-galactosidase contenait 2076 pb et codait pour 691 acides aminés qui avaient une haute homologie avec les autres espèces de *Bifidobacterium*. Cette β-galactosidase était homologue à celle de la famille *LacA*. Le gène *galA* a été surexprimé avec succès chez *E. coli* ER2566. Afin d'observer si l'enzyme recombinante a subit des changements, la β-galactosidase de *Bifidobacterium breve* B24 et de *E. coli* ER2566 recombinant a été purifiée jusqu'à homogénéité par chromatographie échangeuse d'ions (Mono-Q) et par filtration sur gel (Superose 12 et Superdex 200). Le poids moléculaire des deux β-galactosidases a été estimé à 75 kDa par SDS-PAGE. Les tests d'activité sur gel non dénaturant et par filtration sur gel Superose 12 ont montré que les enzymes sont composées d'un dimère avec un poids moléculaire de 150 kDa.

Les pH optimums des enzymes native et recombinante pour hydrolyser le O-nitrophenyl-β-D-galactopyranose (ONPG) étaient de pH 6.0 et 7.0, respectivement, et stables aux pHs de 5 à 8 et 6 à 9, respectivement. Les températures optimales des deux enzymes pour l'hydrolysation du ONPG étaient semblables à 45 °C, et stables sur l'intervalle de température de 20 à 45 °C. Les deux enzymes étaient stables jusqu'à une température de 45 °C pendant 5 h d'incubation à pH 6.5. L'enzyme recombinante a été légèrement activée par les ions de métaux bivalents Mg^{2+}, Mn^{2+} et Zn^{2+} à 1 mM, mais fortement inhibée par Hg^{2+} et PCMB. Les valeurs de K_m pour les enzymes native et

recombinante pour le ONPG étaient de 2.77 et 1.82 mM, respectivement, et les V_{max} étaient de 1.02 et 1.39 mM/min, respectivement.

Le deux activités β-galactosidase ont également été testées, utilisant le lactose comme substrat. Le pH optimum pour les enzymes native et recombinante pour l'hydrolyse du lactose était semblable, à pH 6.0. Les deux enzymes ont montré plus de 80 % de'activité dans l'intervalle de pH 6 à 8, indiquant que les enzymes étaient stables à pH neutre. Cependant, la β-galactosidase native a montré autour de 40% de son activité à pH 5.0, tandis que l'enzyme recombinante n'a eu aucune activité à ce pH. Par contre, l'enzyme recombinante a montré plus de 50 % de son activité à pH 9, tandis que la β-galactosidase native a montré moins de 5 % de son activité. La température optimale des deux enzymes était de 45 °C. Les profils d'activité enzymatique des deux enzymes étaient très semblables, excepté à la température de 10 °C. La β-galactosidase recombinante montrait autour de 20 % de son activité enzymatique à 10 °C, tandis qu'aucune activité enzymatique n'a été détectée pour l'enzyme native à cette température. Aucun activateur de l'hydrolyse du lactose n'a été trouvé, mais 1 mM de $HgCl_2$ ou de *p*-chloromercuribenzoic acid (PCMB) ont fortement inhibé l'activité de l'enzyme recombinante. Environ 47.5% de lactose 1 M a été hydrolysé en 5 h à 45 °C , tandis que 100% de lactose 100 mM a été complètement hydrolysé en 5 h à 45 °C avec 50 unités d'enzyme. Aucune augmentation significative de l'activité hydrolytique n'a été observée lorsque 600 mM plus de lactose était employé.

En présence de glucose et de galactose dans le mélange réactionnel, la β-galactosidase recombinante a synthétisé des oligosaccharides. Le montant total des oligosaccharides a augmenté en augmentant la concentration de glucose et de galactose,

indiquant que la β-galactosidase recombinante pouvait synthétiser des oligosaccharides sans activité hydrolytique. À concentration élevée de lactose (1000 mM), 41.8 % des oligosaccharides étaient synthétisés à partir d'hydrolysats de lactose.

Le lactose (4.8 %) en lait écrémé a été presque totalement hydrolysé avec 50 unités d'enzyme à 45 g/l pendant 5 h. Environ 97.4 % de lactose (41.22 g/l) dans le lait a été hydrolysé pour produire 46.3 % de glucose, 46.6 % de galactose, et 7.1 % d'oligosaccharides. Après 24 h de réaction, 3.04 g/l, 5.75 g/l, 7.28 g/l l, et 1.95 g/l d'oligosaccharides ont été synthétisés à 20, 37, 45, et 50 °C, respectivement. Le lactose a été complètement hydrolysé après 14, 12, et 6 h à 20, 37, et 45 °C, respectivement. Cependant, à 50 °C, l'hydrolyse du lactose est arrêtée, et les oligosaccharides n'ont pas augmenté après 6 h dû à l'inactivation à 50 °C. Les valeurs de K_m pour les enzymes native et recombinante sur le lactose étaient de 152.08 et 95.58 mM, respectivement, et étaient de 54.9 et 52.51 fois plus élevé que sur l'ONPG.. Les V_{max} des enzymes native et recombinante sur le lactose étaient de 138.95 et 189.13 mM/min, respectivement.

La β-galactosidase de *Bifidobacterium breve* B24, avec des propriétés uniques de forte activité hydrolytique et de transgalactosylation, d'une enzyme de faible taille, et de thermostabilité, ont été étudiés aux niveaux biochimiques et moléculaires pour la première fois.

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FORWORD

This thesis is submitted in the form of original papers suitable for journal publication. A general introduction states the rationale and the objectives of this study, and it is followed by Chapter 1, a literature review, which presents the theory and background information on this subject. The next three chapters represent the body of the thesis, each being a complete manuscript. The last section is a summary of the major conclusions. This thesis format has been approved by the Faculty of Graduate and Postdoctoral Studies, McGill University, and follows the conditions outlined in the Guidelines concerning Thesis Presentation, which are as follows:

"Candidates have the option, subject to the approval of the Department, including as part of the thesis the text of an original paper, or papers suitable for submission to learn journals for publication. In this case, the thesis must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation. Additional materials (experimental and design data as well as descriptions of equipment) must be provided sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported. The abstract, full introduction and conclusion must be included, and where more that one manuscript appears, connecting texts and common abstract, introduction and conclusions are required. A mere collection

of manuscripts is not acceptable; nor can reprints of published papers be accepted. While inclusion of manuscripts co-authored by the candidate and others is not prohibited by McGill, the candidate is warned to make an explicit statement on who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the External Examiner is made more difficult in such cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis."

Although all the works reported here is the responsibility of the candidate, the project was supervised by Dr. Byong H. Lee, Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

CONTRIBUTION OF CO-AUTHORS FOR PUBLICATIONS

Dr. Byong H. Lee, my supervisor, is the co-author on the publications presented in scientific meetings and on the manuscripts that have been published and submitted. Dr. Lee contributed in a supervisory role in this research, and he reviewed the manuscripts.

Dr. Inteaz Alli from Department of Food Science, McGill University contributed to part of this work by providing technical support and reviewing the manuscripts.

Dr Geun-Bae Kim, contributed to part of this work by helping cloning study, and he is the co-author on one of the manuscripts.

A. Part of this work has been published or submitted for publication as follows:

Yi, S. H., Kim, G. B, and Lee, B. H. 2005. Molecular characterization of β-galactosidase gene from *Bifidobacterium breve* B24 and its over-expression. (Submitted to Appl. Environ. Microbiol.)

Yi, S. H. and Lee, B. H. 2005. Purification and characterization of β-galactosidases from *Bifidobacterium breve* B24 and *E. coli* ER2566. (Submitted to Appl. Microbiol. Biotechnol.)

B. Part of this work has been presented in and submitted to scientific meetings in the form of poster presentations.

Yi, S. H. and Lee, B. H. Biochemical and molecular characterization of a β-galactosidase from *Bifidobacterium breve.* Poster presented at the 104th ASM General Meeting in New Orleans, Louisiana (May 23~27, 2004)

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Yi, S. H. and Lee, B. H. Biochemical and molecular characterization of a β-galactosidase from *Bifidobacterium breve.* Poster presented at the International Symposium on Propionibacteria and Bifidobacteria: Dairy and Probiotic Application, Saint-Malo, France. (June 2~4, 2004).

Permission to use part of this material has been obtained from the co-authors of the manuscripts listed above.

PREFACE

Claims of original research

1. This was the first study to characterize the biochemical and molecular aspects of a β-galactosidase from *Bifidobacterium breve* B24.

2. For the first time, the β-galactosidase gene from *Bifidobacterium breve* B24 was cloned and over-expressed into *Escherichia coli.*

3. For the first time, we have demonstrated that a β-galactosidase from *Bifidobacterium breve* B24 was only one of the *LacA* family proteins among bifidobacteria.

4. The native and recombinant β-galactosidases from *Bifidobacterium breve* and *Escherichia coli* were purified to homogeneity using ion exchange and gel-filtration chromatographies. The biochemical aspects of the purified both native and recombinant β-galactosidases were studied in details for the first time.

5. Before this study the transgalactosylation activity of a β-galactosidase from *Bifidobacterium breve* B24 using glucose and galactose has never been analyzed. This enzyme showed strong transgalactosylation activity.

GENERAL INTRODUCTION

Lactose is a primary carbohydrate contained in mammal's milk and dairy products (at a concentration between 5 and 10 %, depending on the source of milk) and is also called milk sugar. The consumption of lactose or lactose containing foods can cause digestion problem for almost a 70 % of the world population, as the enzyme naturally present in human intestine loses its activity during lifetime. Moreover, low solubility and sweetness of lactose lead to huge interest in industrial development of processes to hydrolyze lactose and lactose contained dairy foods. Lactose, a natural disaccharide, is consists of monosaccharides, glucose and galactose bonded by a beta-1, 4 linkages. Lactose can be hydrolyzed to monomers, glucose and galactose by enzyme (β-galactosidase) or acids. Lactose hydrolyzed milk can reduce lactose intolerance problem and lactose hydrolyzed whey syrup can be utilized in frozen desserts, confectionary, bakery, fermentation products, and beverages. The galacto-oligosaccharides can also be employed as probiotic food ingredients, humectants, and emulsifiers.

A β-galactosidase (EC 3.2.1.23, β-D-galactoside galactohydrolase), commonly known as lactase, is to be found in most of bifidobacteria and is known to catalyze not only hydrolyze β-D-galactoside linkage of lactose to produce glucose and galactose but

also has transgalactosylation activity to synthesize galacto-oligosaccharides. Both reaction activities are well characterized and applied in many food industries. Although β-galactosidases are widely distributed in nature, the most thoroughly studied β-galactosidases are obtained from *E. coli* and commercially used β-galactosidases are from mainly fungi and yeasts. The β-galactosidases with better yield, high activity, and thermostability at natural pH or active at low temperature are continuously searching due to the production cost of this enzyme.

Bifidobacteria are one of the major microflora in gastrointestinal GI tract of human. Many bifidobacteria-containing dairy and pharmaceutical products have been developed and consumed for several decades due to their promising health-promoting properties on the reduction of harmful bacteria and toxic compounds in the intestine, prevention of dental caries, reduction of total cholesterol and lipid in serum, and relief of constipation. Therefore, live probiotic bacteria, which are improving the microbial balance of the human GI tract, have been used to supplement dairy products for long time. Another way to increase the number of beneficial bacteria in the human intestine is to stimulate their growth by supplying growth factor such as oligosaccharides. Galacto-oligosaccharides, so-called prebiotics, have been shown to employ this growth-stimulating effect on probiotic bacteria, including bifidobacteria.

Due to the selectivity of these growth substrates, oligosaccharides and the health-promoting properties of bifidobacteria, considerable attention is now being focused on the transgalactosylation activity of β-galactosidase and the use of these compounds as bifidogenic factors or prebiotics.

The general objectives of this work were to study biochemical and molecular aspects of the β-galactosidase gene from *Bifidobacterium breve* B24 that was selected from 17 bifidobacteria species and to evaluate the hydrolytic and transgalactosylation activity.

The specific objectives of this research were, 1) to clone the β-galactosidase gene into *E. coli*, and analyze gene, nucleotide sequence, and amino acid sequence, as well as over-express the gene, 2) to purify and characterize the native and recombinant enzyme for biochemical characterization, and 3) to analyze hydrolytic and transgalactosylation activity in lactose and milk for the possibility of industrial applications.

CHAPTER 1

LITERATURE REVIEW: β-GALACTOSIDASE

The hydrolytic and transgalactosylation activity of β-galactosidase (EC 3.2.1.23), formation of oligosaccharides, and probiotics and prebiotics are reviewed in this chapter.

Part 1 includes a general introduction on sugars by means of mono, di, and polysaccharide. Part 2 describes lactose intolerance and related information. Part 3 is concerned about hydrolytic activity of β-galactosidase including methods of hydrolysis, mechanism, properties, source, and application. Part 4 describes types of oligosaccharides and formation mechanisms. Part 5 deals with probiotics and prebiotics. Finally part 6 discusses about improvement of enzyme activity using genetic tools. This chapter states up to date information on β-galactosidase, and the significance of this research.

1.1 SUGARS

Sugars are white crystalline carbohydrates that are soluble in water and generally have a sweet taste. They can be classified as 3 major groups like monosaccharides, disaccharides, and polysaccharides including oligosaccharides. Each group can be sub-classified based on their characteristics. Monosaccharides are simple sugars and can be classified based on the number of carbons (Table 1.1).

Table 1.1 Monosaccharide classifications based on the number of carbons.

Number of Carbons	Category Name	Examples
4	Tetrose	Erythrose, threose
5	Pentose	Arabinose, ribose, ribulose, xylose, xylulose, lyxose
6	Hexose	Allose, altrose, fructose, galactose, glucose, gulose, idose, mannose, sorbose, talose, tagatose
7	Heptose	Sedoheptulose

This slight structural difference of monosaccharide, orientation of the hydroxyl groups (-OH), makes differences in the biochemical properties, taste, and physical properties such as melting point and specific rotation.

Deoxyribose, which is missing an oxygen at position 2, is a component of deoxyribonucleic acid (DNA). The ring form of ribose is a component of ribonucleic acid (RNA)

Disaccharides are consisting of two simple sugars (Table 1.2). In almost all cases one of the sugars is glucose, with the other sugar being galactose, fructose, or another glucose. Common disaccharides are lactose, sucrose, and maltose. Lactose has a molecular structure consisting of galactose and glucose. Lactose, also known as milk sugar, occurs in the milk of mammals (4 - 6 %) in cow's milk and human milk (5 - 8 %). It is also a by-product in the manufacture of cheese. It is of interest because it is associated with lactose intolerance which is the intestinal distress caused by a deficiency of lactase. Sucrose, also called saccharose, is ordinary table sugar refined from sugar cane or sugar beets. Maltose consists of two α-D-glucose molecules with the alpha bond at carbon 1 of one molecule attached to the oxygen at carbon 4 of the second molecule.

Polysaccharides are polymers of simple sugars. Many polysaccharides, unlike sugars, are insoluble in water. Dietary fiber includes polysaccharides and oligosaccharides that are resistant to digestion and absorption in the human small intestine but which are completely or partially fermented by microorganisms in the large intestine.

Table 1.2 Disaccharide descriptions and components.

Name	Description	Component monosaccharides
sucrose	common table sugar	glucose + fructose
lactose	main sugar in milk	galactose + glucose
maltose	product of starch hydrolysis	glucose + glucose
trehalose	found in fungi	glucose + glucose

1.2 LACTOSE INTOLERANCE

Lactose intolerance is the inability to digest significant amounts of lactose, the predominant sugar of milk. This inability results from a shortage of the enzyme lactase, which is normally produced by the cells that line the small intestine. Lactose-hydrolyzed dairy products can help lactose mal-absorbers, who lack of lactase in the intestine and suffer from gastrointestinal disorders such as cramps, flatulence, and diarrhea (Littman and Hammond, 1965; Savaiano and Levitt, 1987).

The most common tests used to measure the absorption of lactose in the digestive system are the lactose tolerance test; for example, the hydrogen breath test (Michele *et al.*, 2004) measures the amount of hydrogen in a person's breath. Normally, very little hydrogen is detectable. However, undigested lactose in the colon is fermented by bacteria,

and various gases, including hydrogen, are produced. The hydrogen is absorbed from the intestines, carried through the bloodstream to the lungs, and exhaled. Raised levels of hydrogen in the breath indicate improper digestion of lactose.

1.3 HYDROLYTIC AND TRANSGALACTOSYLATION ACTIVITIES OF β-GALACTOSIDASE

1.3.1 β-Galactosidase

For the classification of each enzyme, the system has been developed to assign a name on the basis of enzyme activity. Each enzyme is allocated a four-digit EC number. The first three digits number defines the reaction and the last number is a unique serial number. Major six-category is summarized in Table 1.3.

β-Galactosidase (EC 3.2.1.23) is common name of β-D-galactoside-galactohydrolase and is also called as commercial name lactase (Llanillo, *et al.*, 1977). Lactase breaks down the β-1, 4-glycosidiclinkage of lactose and gives rise to its component monosaccharides, glucose and galactose. The hydrolysis of lactose in dairy products by lactase can be beneficial in many regards; 1) the hydrolysis of lactose

alleviates problems and improves processes for dairy products, 2) the low solubility and lack of sweetness that are often experienced in concentrated milk products and ice cream can be overcome by lactose hydrolysis, 3) cheeses that have been manufactured from hydrolyzed milk ripens more quickly than that made from normal milk (Gekas and Lopez-Leiva, 1985; Zadow, 1992), and 4) the use of lactase could also produce whey syrup sweeteners and reduce the amount of the lactose in whey, which can cause environmental pollution when discharged in large quantities (Gekas and Lopez-Leiva, 1985; Zadow, 1992; Belem and Lee, 1999).

Table 1.3 Enzyme classification systems (adapted from http://www.chem.qmul.ac.uk/ iubmb/).

Subclass	Name
EC 1	Oxidoreductases
EC 2	Transferases
EC 3	Hydrolases
EC 4	Lyases
EC 5	Isomerases
EC 6	Ligase

1.3.2 Lactose hydrolysis

Chemical hydrolysis of lactose can be performed with mineral acids or cation exchange resins. The acid hydrolysis is characterized by the relatively severe conditions

which require to completely hydrolyze lactose into glucose and galactose at high temperature under acidic conditions. Coughlin and Nickerson (1975) showed the feasibility of using a temperature range as of 50~700 ℃ with strong mineral acids (pH < 1.0) to hydrolyze lactose efficiently. The chemical hydrolysis of lactose is simple, rapid, and inexpensive unlike the enzymatic hydrolysis. However, it has undesirable features, including: 1) the denaturation of milk proteins occurs due to high temperatures and acidic conditions, 2) the presence of salts in whey causes the deactivation of the acids, thus a demineralization step is required, 3) the interaction between amino acids from proteins hydrolysis and sugars from lactose hydrolysis results in the Maillard type of browning reaction that requires a decolorization step using activated carbon, 4) cost of specific materials is required for plant construction to resist the chemically corrosive conditions, and 5) valuable essential nutrients of milk is lost and undesirable by-products are formed.

The hydrolysis of lactose using the enzyme lactase (β-galactosidase) is now the most widely used technique for the production of low-lactose dairy products. There are a number of advantages in using enzymes as industrial catalysts; 1) usually highly specific, 2) active at very low concentrations, and 3) minimal undesirable side reactions associated with chemical catalysis. The enzymatic hydrolysis of lactose depends on the properties and the characteristics of the enzyme used as they differ widely according to the enzyme

source. The enzymatic hydrolysis of lactose by β-galactosidases which are derived from microbial sources is carried out under considerably milder conditions (pH > 3.5; temperature 30 - 60 ℃).

1.3.3 Mechanism of lactose hydrolysis

Enzymatic hydrolysis of the β-1, 4-glycosidiclinkage of lactose requires two critical residues (a proton donor and a nucleophile base) at the catalytic site of β-galactosidase. The mechanism of lactose hydrolysis was first described by Wallenfels (1960) using a β-galactosidase from *E. coli*. The proposed mechanism was that the cystine and histidine residues at the catalytic site acted as proton donor and nucleophile base, respectively, during hydrolysis. This mechanism had been studied and become now clear that two glutamic acid residues (Glu482 and Glu551) found from a variety of microbial β-galactosidases were acting more likely proton donor and nucleophile base (Nisizawa and Hashimoto, 1970; Nijipels, 1981; Presnosil *et al.*, 1987). The proposed reaction mechanism is shown in Fig. 1.1.

The first step is formation of β-galactosidase-galactosyl complex and simultaneous glucose liberation. The second step is transferring a hydroxyl group to β-galactosidase-galactosyl complex. When lactose concentration is low, lactose can be more

competitive to be an acceptor; therefore galactose is formed and released from the active site. On the other hand, in a high concentration of lactose solution, lactose has more chances to bind to the β-galactosidase-galactosyl complex to lead formation of oligosaccharides. These results have been proposed and presented (Leiva and Guzman, 1995; Raymond, 1998; Rustom *et al.*, 1998; Sheu *et al.*, 1998).

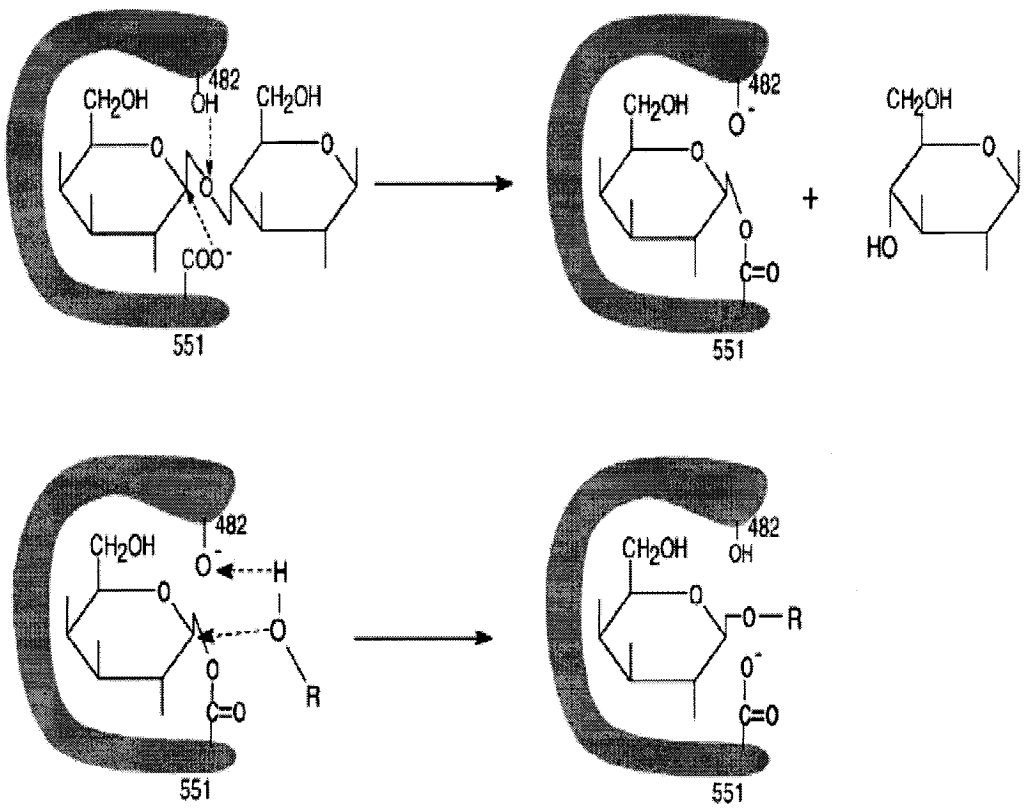


Fig. 1.1 Schematic mechanism of the lactose hydrolysis by β-galactosidase (adapted from Quinn and Xiao, 2001).

1.3.4 Detection of hydrolytic activity of β-galactosidase

A number of methods have been developed to determine β-galactosidase activity using lactose or other β-galactoside as substrates. The hydrolytic products, glucose and galactose can be determined by colorimetric method (Nikerson *et al.*, 1976) or by specific enzymatic analysis (Huber *et al.*, 1976). A chemically modified substrate, ortho-nitrophenyl-β-D-galactoside (ONPG) is commonly used for the determination of β-galactosidase activity because of its high reaction velocity and lower Km value (Richmond *et al.*, 1981). The hydrolysis of ONPG, at the nonreducing end of the β-galactosidic bond, produces galactose and *o*-nitrophenyl, which is yellow and absorbs maximally at 420 nm. One unit of the β-galactosidase activity is defined as the amount of enzyme producing 1 μmol of *o*-nitrophenyl per minute under the assay conditions. The drawback of using ONPG is that the affinity of the enzyme towards different substrates differs: therefore, ONPG units do not directly reflect the unit of the β-galactosidase on lactose. To be more precise, β-galactosidase activity should be measured under the real processing situation, such as in milk system. In addition to the substrate, the pH, temperature, and the condition of other components in the processing system can all possibly affect the performance of the enzyme. Therefore those factors should be optimized when determining the enzyme activity. Another method used for analyzing the

lactose hydrolytic activity of β-galactosidase is high-performance liquid chromatography (HPLC) with refractive index detector. This method is simple and can determine lactose, glucose, galactose, and oligosaccharide if they are present at the same time (Pirisino, 1983; Pivarnik, 1990). A rapid and economic method that can be used routinely is cryoscopic method (Palumbo *et al.*, 1995). This method measures the change of freezing point that occurs in the reaction during the reaction. The advantage of this method is that the deproteinization, which is required for other methods, is not necessary while the drawback of this method is that it cannot detect an adulteration of up to 30% of added water.

1.3.5 General properties of β-galactosidases

For the industrial applications, some parameters are very important to be considered such as optimum pH and temperature, concentration, and stability. The pH is the primary factor that affects the enzyme activity and stability. In general, β-galactosidases from fungi have optimum pH in the range of 3.0 to 5.0 and have an operational range of 2.5 to 6.0 which fits for processing acid whey, acid whey permeate, and fermented dairy products. Optimum pH and the operational pH range of β-galactosidases from yeasts are more limited than fungal β-galactosidases. Also, β-

galactosidases from fungi have higher optimum temperature for both activity and enzyme stability than those of yeasts (Mahoney, 1985). It is necessary to use high concentrations of enzyme to reduce reaction time when lactose hydrolysis is performed in mild temperature which is prone to microbial contamination.

In general, heavy metals are inhibitory, whereas potassium, sodium, magnesium, and manganese stimulate the enzyme. Some activators are more effective than others and some become inhibitory at high concentrations or in combination with other ions. Galactose has been credited as a competitive inhibitor. Effect of inhibition can be overcome by keeping the substrate concentration much higher in the vicinity of the enzyme. The efficiency of lactase enzyme can be improved by reducing or removing galactose from the system (Pivanik *et al.*, 1995). However, it has been reported that galactose had a non-competitive inhibitory effect on fungal β-galactosidase from *A. oryzae* (Shukla and Chaplin, 1993).

1.3.6 Sources of β-galactosidases

Lactase (β-Galactosidase) is widely distributed in nature and has been isolated from animals, plants, and microorganisms. Recently, the plant *Cicer arietinum* (chickpeas) has been reported to have possibility of promising application in the

hydrolysis of milk using immobilization techniques (Un *et al.*, 1999). Since the 1950s, most commercial enzymes have been produced from microbial sources since they are known to produce better yields. Presently, lactase is mostly produced from yeast, fungi and bacteria. β-Galactosidases from *Aspergillus oryzae* and *Kluyveromyces lactis* have been used widely for many years in food industries (Pivanik *et al.* 1990). For industrial use, the enzyme must be derived from sources that are under the GRAS status "generally regarded as safe" by the Food and Drug Administration (FDA). Current production seems to be restricted to the fungi *Aspergillus niger* and *Aspergillus oryzae* and the yeasts *Kluyveromyces lactis, Kluyveromyces maxianus*, and *Candida pseudotropicalis*, but the cost of crude enzyme ($300/kg) is a major hurdle to expand the lactase technology at this time. Thermostable enzymes are now more attractive to industries because of their enzymatic properties at high temperature. Operating at high temperature results in shorter processing time and reduces chances of microbial contamination. *Thermus* sp. T2 (Benevides *et al.*, 2003) and *Sterigmatomyces elviae* (Onishi and Tanaka, 1995) showed enzyme activity at 90 and 85 °C, respectively. So far no commercial cheaper recombinant lactases are on the markets.

1.3.7 Enzyme applications

The enzymatic hydrolysis can be accomplished by either free enzymes usually in a batch fermentation process, or by immobilized enzymes. An immobilized enzyme may be defined as the enzyme whose free movement has been restricted or somewhat confined to allow its use and reuse in a continuous catalytic process. The technology of enzyme immobilization has been applied successfully to the hydrolysis of lactose. Thus the inhibition of β-galactosidase by the accumulation of galactose formed during hydrolysis of lactose can be overcome by using these techniques. Many β-galactosidases have been immobilized on different types of matrix and their properties were studied (Ortega-Lopez *et al.*, 1993; Serge, 1993; Gonzalez and Doval, 1994; Meng and Charles, 1994; Carlos and Amelia, 1997; Karen, 1998; Sungur and Yildirim, 1999; Szczodrak, 1999; Eldin *et al.*, 2000; Ladero *et al.*, 2003; Benevides *et al.*, 2004).

Greenberg and Mahoney (1981) and Gekas and Lopez-Leiva (1985) gave a comprehensive survey on the different techniques of β-galactosidase immobilization stating the merits and the drawbacks of each. The properties of the enzyme and the final product specifications are the major factors that determine the exploitation of any particular immobilization technique. The enzymatic hydrolysis of lactose by β-galactosidase was found to be affected by the presence of some mineral ions naturally

occurring in milk. The most important activators are magnesium and manganese, whereas sodium and calcium have a negative effect on the activity. Also the activity of β-galactosidase was found to be hampered by phytic acid present in soybean proteins, an important finding, since milk is incorporated with vegetable proteins in many food formulations. There are contradictory reports on the effects of protein fractions in milk on the activity of β-galactosidase. Bernal and Jelen (1985) showed that the β-galactosidase activity is highest when it was used to treat lactose in whey, unlike skim milk and permeate. However, Sheth *et al.* (1988) reported that either ultrafiltration or gradual increase in protein concentration did not seem to affect the activity of β-galactosidase from *Aspergillus niger.* In the development of large-scale enzymatic manufacturing processes for lactose hydrolysis, the most important considerations are the purity, activity, non-toxicity, and the cost of the β-galactosidases.

1.4 TRANSGALACTOSYLATION ACTIVITY AND GALACTO-OLIGOSACCHARIDES

1.4.1 Galacto-oligosaccharide formation

β-Galactosidase hydrolyzes terminal, non-reducing β-D-galactose residues in β-

D-galactosides or lactose. However, some of this enzyme catalyzes both hydrolytic and reverse transgalactosylation (EC 2.4.1.22: galactosyl transferase) reaction (Reuter *et al.*, 1999; Bourne and Henrissat, 2001). The transferase activity by a β-galactosidase that produce oligosaccharides was reported in the early 1950s (Aronson, 1952; Pazur, 1953). Apart from theoretical aspects, early research was prompted by nutritional concerns about the presence of these compounds in low-lactose milk (Burvall, 1979 and 1980). Later studies were based on the need to consider oligosaccharide formation when modelling lactose hydrolysis (Presnosil, 1987). More recently, interest in the reaction has been raised by observation that oligosaccharides may have beneficial effects as 'bifidus factors'- promoting the growth of desirable intestinal microflora. Also, the transferase reaction can be used to attach galactose to other chemicals and consequently has potential applications in the production of food ingredients, pharmaceuticals and other biologically active compounds (Raymond, 1998). Lactose hydrolysis catalysed by β-galactosidases has proven to be a very complex reaction. Apart from the actual hydrolysis product, glucose and galactose, many newly formed β-glycoside, mainly di-, tri, and tetrasaccharide, occur as kinetic intermediates, derived from so-called transgalactosylation reaction (Presnosil *et al.*, 1987; Zarate and Lopez-Leiva, 1990; Nakayama and Amachi, 1999). Because transgalactosylation products (galacto-oligosaccharides) are substrate of β-galactosidases-

catalyzed hydrolysis, the composition of the product mixture changes quite significantly with progressing reaction time (Presnosil *et al.*, 1987; Smart, 1991; Boon, 1999; Nakayama and Amachi, 1999).

1.4.2 Properties of oligosaccharides

Oligosaccharides are generally defined as glycosides that contain between three and ten sugar moieties. However, interestingly, many disaccharides have similar properties to the larger polysaccharides, and are often major components of food-grade oligosaccharide products. Hence, disaccharides such as lactulose are considered as oligosaccharides (Rodney, 1998). In general, food-grade oligosaccharides are mixtures that contained different degrees of polymerized oligosaccharides. Oligosaccharides enhance quality of foods and gives health benefits, which make their use as food ingredients. The specific properties of oligosaccharides are very different depending on the formation of oligosaccharides. However, some properties are common to almost all oligosaccharides. The sweetness of the oligosaccharide is depending on structure and molecular mass of the oligosaccharides (Crittenden and Playne, 1996). Oligosaccharides are normally water soluble and mildly sweet, typically lower than sucrose and this low sweetness is useful in food production when reduced sweetness is desirable to enhance

other food flavors. Compared with mono and disaccharides, the higher molecular weight of oligosaccharides provides increased viscosity, leading to improved body and mouthfeel. They can also be used to alter the freezing temperature of frozen foods, and to control the amount of browning due to Maillard reactions in heat-processed foods. Oligosaccharides provide a high moisture-retaining capacity, preventing excessive drying, and a low water activity, which is convenient in controlling microbial contamination (Nakajima and Nishio, 1993). Although oligosaccharides possess these useful physicochemical characteristics, most of the interest in their use as food ingredients stems from their many beneficial physiological properties. Unlike starch and simple sugars, the currently available food-grade oligosaccharides are not utilized by mouth microflora to form acid or polyglucans. Hence, they are used as low-cariogenic sugar substitutes in confectionery, chewing gums, yogurts and drinks. Many oligosaccharides are not digested by humans (Tomomatsu, 1994). Oligosaccharides have recently been described as one of several 'prebiotics', which can stimulate the growth of beneficial microflora (Gibson and Roberfroid, 1995).

Worldwide, there are 12 classes of food-grade oligosaccharides currently produced commercially (Table 1.4). Both the volume and diversity of oligosaccharide products are increasing very rapidly as their functional properties become further

understood. Detailed production methods for various oligosaccharides have been reviewed by Nakajima and Nishio (1993) and Playne (1994).

1.4.3 Types of oligosaccharides and formation mechanisms

1.4.3.1 Galacto-oligosaccharides

The establishment of a bifidus microflora in the intestines of breast-fed infants has been attributed to the presence of galacto-oligosaccharides in human milk and its importance on human health has been increased (Smart, 1993).

They are produced commercially from lactose (Fig. 1.2) using the galactosyltransferase activity of β-galactosidase, which dominates lactose hydrolysis at high lactose concentrations (Smart, 1993). Major companies involved in galacto-oligosaccharide production are Yakult Honsha (Tokyo, Japan), Nissin Sugar Manufacturing Company (Tokyo, Japan) and Snow Brand Milk Products (Tokyo, Japan) (Table 1.4).

1.4.3.2 Lactulose

Of all oligosaccharides, lactulose is produced in the largest quantity. Like

galacto-oligosaccharides, it is manufactured from lactose (Fig. 1.2). An alkali isomerization process is used to convert the glucose moiety in lactose to a fructose residue (Harju, 1986). The resulting disaccharide, lactulose, is not digested by humans and promotes the preferential growth of bifidobacteria in the colon (Modler *et al.*, 1990; Tamura *et al.*, 1993). Solvay (Hannover, Germany) and Morinaga Milk Industry Co. (Kanagawa, Japan) are largest producer of lactulose (Table 1.4).

1.4.3.3 Lactosucrose

Lactosucrose is the third bifidogenic oligosaccharide that is produced using lactose as a raw material. This trisaccharide consists of a lactose molecule to which a fructose moiety is joined at the glucose residue by a β-2,1 glycosidic bond. It is manufactured from a mixture of lactose and sucrose using the transfructosylation activity of the enzyme (Fig. 1.2).

1.4.3.4 Fructo-oligosaccharides

Fructo-oligosaccharides represent one of the major classes of bifidogenic oligosaccharides in terms of their production volume. Their safety and health benefits as food ingredients have been reviewed by Spiegel *et al.* (1994). They are manufactured by

two different processes, which result in slightly different end products (Fig. 1.3). In the first method, fructo-oligosaccharides are produced from the disaccharide sucrose using the transfructosylation activity of the enzyme β-fructofuranosidase. As for the production of fructo-oligosaccharides, a high concentration of the starting material is required for efficient transglycosylation (Park and Almeida, 1991; Van Balken *et al.*, 1991; Hayashi, 1993).

Table 1.4 Production of food-grade oligosaccharides (adapted from Crittenden and Playne, 1996).

Class of oligosaccharide	Estimated production in 1995 (t)	Major manufacturers	Trade names
Galacto-oligosaccharides	15000	Yakult Honsha (Jp)[a]	Oligomate
		Nissin Sugar Manufacturing Com.(Jp)	Cup-Oligo
		Snow Brand Milk Products (Jp)	P7L and others
		Borculo Whey Products (NL)[b]	TOS-Syrup
Lactulose	20000	Morinaga Milk Industry Co. (Jp)	MLS/P/C
		Solvay (Ger)[c]	
		Milei GmbH (Ger)	
		Canlac Corporation (Can)[d]	
Lactosucrose	1600	Ensuiko Sugar Refining Co. (Jp)	Nyuka-Origo
		Hayashibara Shoji Inc. (Jp)	Newka-Oligo

Fructo-oligosaccharides	12000	Meiji Seika Kaisha (Jp)	Meioligo
		Beghin-Meiji Industries (Frn)[e]	Actilight
		Golden Technologies (USA)	NutraFlora
		Cheil Foods and Chemicals (Kor)[f]	Oligo-Sugar
		ORAFTI (Bel)[g]	Raftilose&Raftiline
		Cosucra (Bel)	Fibruline
Palatinose	5000	Mitsui Sugar Co. (Jp)	ICP/O, IOS
Glucosyl sucrose	4000	Hayashibara Shoji Inc. (Jp)	Coupling Sugar
Malto-oligosaccharides	10000	Nihon Shokuhin Kako (Jp)	Fuji-Oligo
		Hayashibara Shoji Inc. (Jp)	Tetrup
Isomalto-oligosaccharides	11000	Showa Sangyo (Jp)	Isomalto-900
		Hayashibara Shoji Inc. (Jp)	Panorup
		Nihon Shokuhin Kako (Jp)	Biotose & Panorich
Cyclodextrins	4000	Nihon Shokuhin Kako (Jp)	Celdex
		Ensuiko Surar Refining Co. (Jp)	Dexy Pearl
Gentio-oligosaccharides	400	Nihon Shokuhin Kako (Jp)	Gentose
Soybean oligosaccharides	2000	The Calpis Food Industry Co. (Jp)	Soya-oligo
Xylo-oligosaccharides	300	Suntory Ltd. (Jp)	Xylo-oligo

a) Japan
b) The Netherlands
c) Germany
d) Canada
e) France
f) Korea
g) Belgium

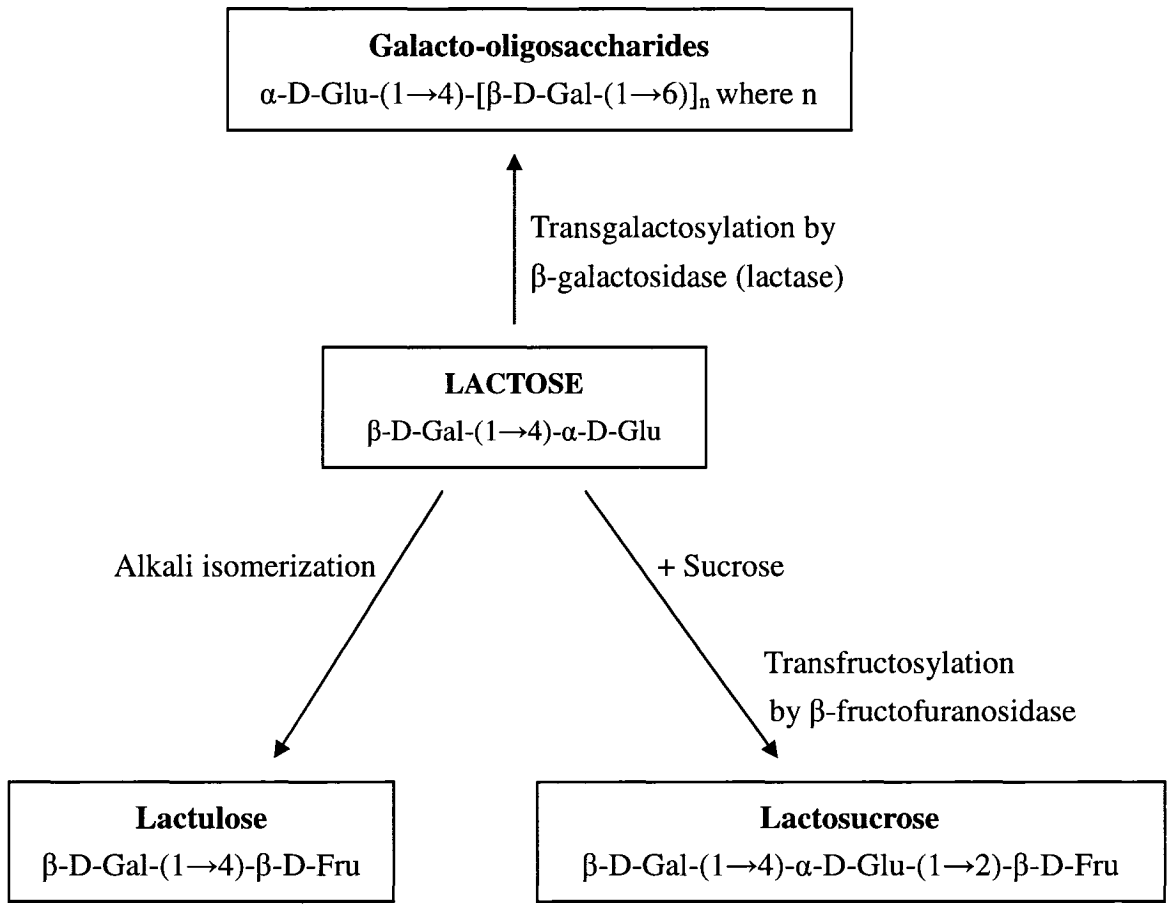


Fig. 1.2 Oligosaccharides manufactured from lactose (Gal, galactose; Glu, glucose; Fru, fructose) (adapted from Crittenden and Playne, 1996).

1.4.3.5 Palatinose (isomaltulose)

Palatinose (also referred to as isomaltulose) is produced from sucrose using an immobilized isomaltulose synthase (EC 5.4.99.11) (Fig. 1.3). This disaccharide does not promote tooth decay and is used as a low-cariogenic sweetener. It is digested in the small intestine of humans and therefore cannot act as a prebiotic. However, palatinose oligosaccharides, formed by the intermolecular dehydration of palatinose, do survive passage to the colon to stimulate the growth of bifidobacteria (Nakajima and Nishio, 1993).

1.4.3.6 Glycosyl sucrose ('Coupling Sugar')

The trisaccharide glycosyl sucrose ('Coupling Sugar') is manufactured from the disaccharides maltose and sucrose using the enzyme cyclomaltodextrin glucanotransferase (EC 2.4.1.19) (Fig. 1.3). It is produced by Hayashibara Shoji Inc. (Okayama, Japan). Glycosyl sucrose is approximately half as sweet as sucrose and, like most oligosaccharides, can be used as a substitute sweetener. The major health benefit provided by the use of this oligosaccharide in place of sucrose is the reduction of dental caries (Nakamura, 1984).

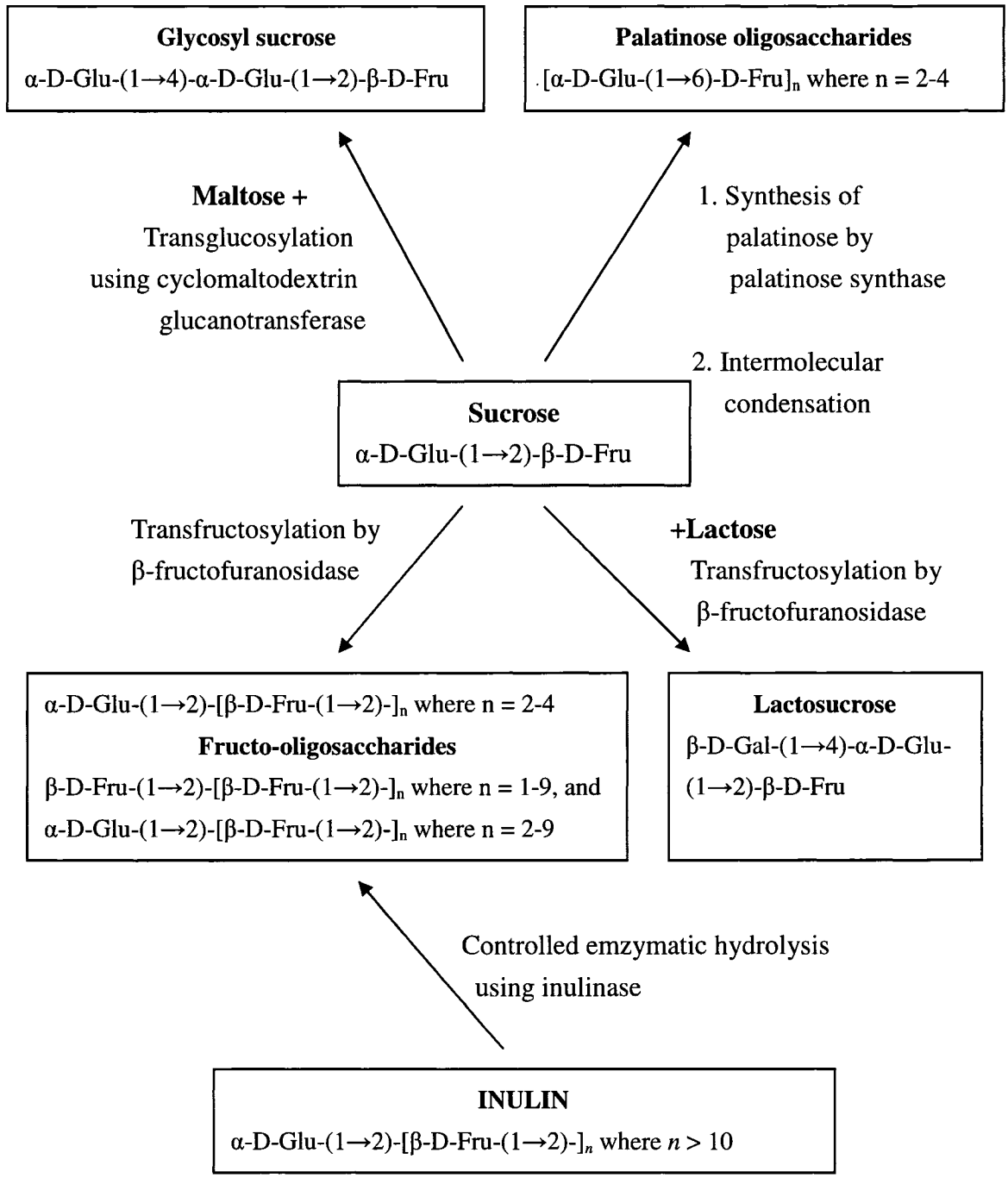


Fig. 1.3 Oligosaccharides manufactured from sucrose or inulin (Gal, galactose; Glu, glucose; Fru, fructose) (adapted from Crittenden and Playne, 1996).

1.4.3.7 Malto-oligosaccharides

Malto-oligosaccharides are not generally claimed to increase the numbers of bifidobacteria in the human colon. They are hydrolyzed and absorbed in the small intestine and do not reach the colon intact. However, Nakajima and Nisho (1993) reported that the consumption of maltotetraose-rich corn syrup has been demonstrated in human trials to reduce the levels of intestinal putrefactive bacteria such as *Clostridium perfrigens* and members of the family Enterobacteriaceae. Therefore, malto-oligosaccharides may be effective in improving colonic conditions.

1.4.3.8 Isomalto-oligosaccharides

Like malto-oligosaccharides, isomalto-oligosaccharides are produced using starch as the raw material (Fig. 1.4). However, unlike malto-oligosaccharides, there is evidence to suggest that these oligosaccharides induce a bifidogenic response (Kohmoto *et al.*, 1988; Kaneko *et al.*, 1994). Isomalto oligosaccharides consist of α-D-glucose residues linked by α-1,6 glycosidic bonds. The isomalto-oligosaccharide mixtures also contain oligosaccharides with both α-1,6 and α-1,4 linked glucose such as the trisaccharide panose. They are produced using a combination of immobilized enzymes in a two stage reactor. In the first stage, starch is liquefied using α-amylase (EC 3.2.1.1). The liquefied

starch is then processed in a second stage that involves reactions catalyzed by both β-amylase (EC 3.2.1.2) and α-glucosidase (EC 3.2.1.20). The β-amylase first hydrolyzes the liquefied starch to maltose. The transglucosidase activity of α-glucosidase then produces isomalto-oligosaccharides.

1.4.3.9 Cyclodextrins

Cyclodextrins are cyclic α-l,4 linked malto-oligosaccharides consisting of 6-12 glucose units. They are formed from starch digests by the action of cyclomaltodextrin glucanotransferase (Fig. 1.4). These oligosaccharides are capable of forming inclusion complexes with various organic compounds by incorporating them into the cavity of their cyclical structure. This can lead to desirable changes in the physical and chemical properties of the incorporated compound. Uses of cyclodextrins are stabilization of deliquescent or volatile compounds in foods, emulsification of oils and fats, protection from oxidation and photodegradation of substrates, and masking bitterness in foods and drugs (Lajos and Jozsef, 2004).

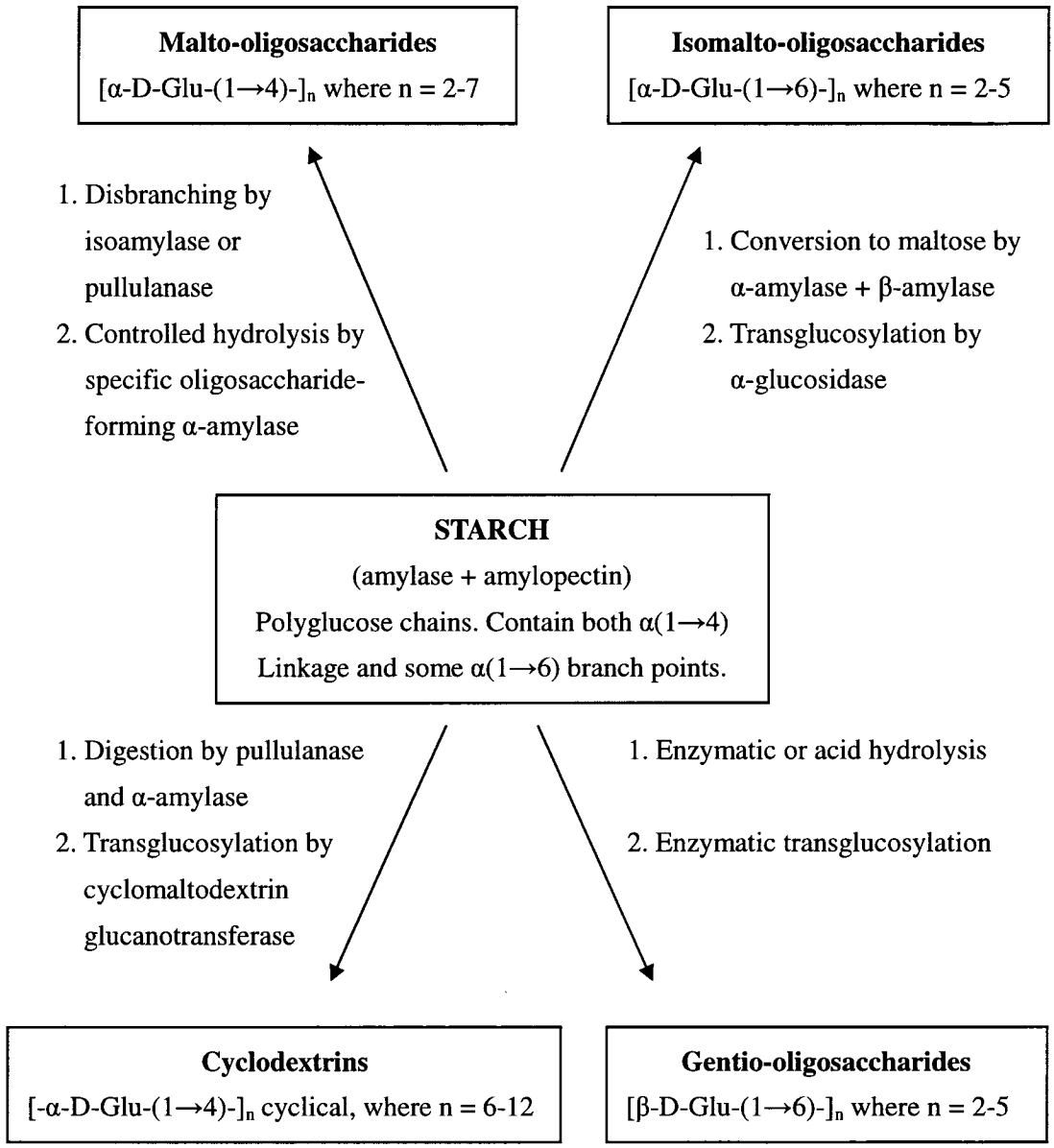


Fig. 1.4 Oligosaccharides manufactured from starch (Glu, glucose). Isomalto-oligosaccharides also contain some α(1→4) glycosidic linkages (adapted from Crittenden and Playne, 1996).

1.4.3.10 Gentio-oligosaccharides

Gentio-oligosaccharides consist of several glucose residues linked by β-1,6 glycosidic bonds. They are produced from glucose syrup by enzymatic transglucosylation. These oligosaccharides are not hydrolyzed in the stomach or small intestine, and are claimed by the manufacturer to promote the growth of bifidobacteria (Nakakuki *et al.*, 1991) and lactobacilli.

1.4.3.11 Soybean oligosaccharides

Unlike other oligosaccharides, soybean oligosaccharides are extracted directly from the raw material and do not require enzymatic manufacturing processes (Fig. 1.5). Soybean whey, a by-product from the production of soy protein isolates and concentrates, contains the oligosaccharides raffinose and stachyose, as well as sucrose, glucose and fructose. These sugars are extracted from the soybean whey and concentrated to produce soybean-oligosaccharide syrup. Although raffinose and stachyose are indigestible and producing gases (flucturance factors) in the intestine, they reach to the intestine and stimulate the growth of bifidobacteria (Oku, 1994)

1.4.3.12 Xylo-oligosaccharides

At present, xylo-oligosaccharides represent only a small proportion of the total oligosaccharide market. These oligosaccharides promote the growth of bifidobacteria in the colon (Modler, 1994) and are used predominantly in prebiotic drinks. The raw material for xylo-oligosaccharide synthesis is the polysaccharide xylan, which is extracted mainly from corncobs. The xylan is hydrolyzed to xylo-oligosaccharides by the controlled activity of the enzyme endo-1,4- β-xylanase (EC 3.2.1.8) (Fig. 1.5).

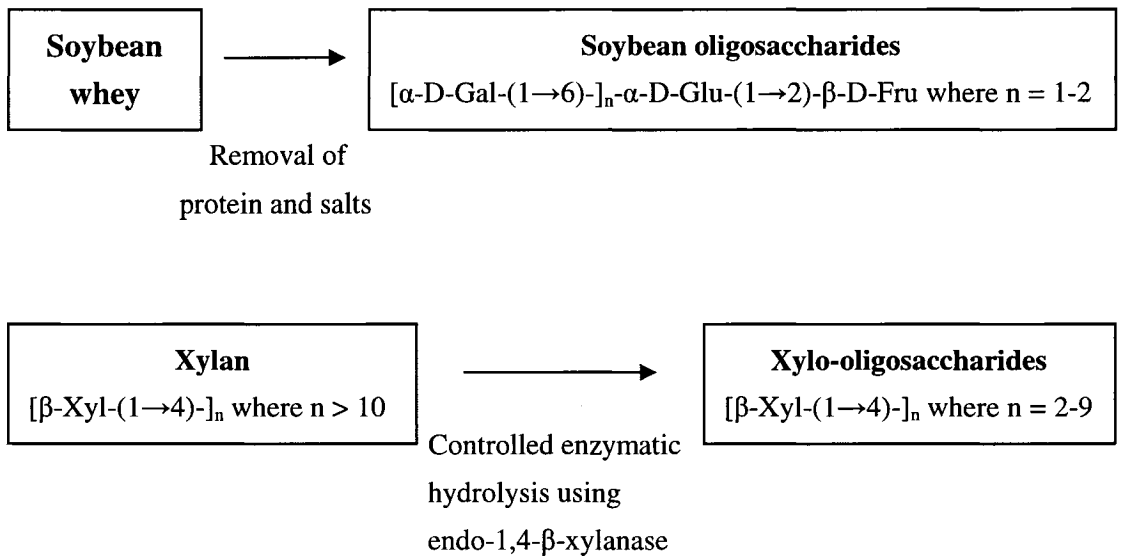


Fig. 1.5 Production of soybean and xylo-oligosaccharides (Gal, galactose; Glu, glucose; Xyl, xylose) (adapted from Crittenden and Playne, 1996).

1.4.4 Applications of oligosaccharides

The major use of oligosaccharides is in beverages. 'OligoCC', produced by The Calpis Food Industry Co., was launched in 1989 and was one of the first commercially successful functional food products. This soybean-oligosaccharide drink had estimated sales of 80 million bottles in 1989. Another example is 'Bikkle', the xylo-oligosaccharide containing beverage produced by Suntory Ltd. Increasingly, oligosaccharides are being included in probiotic yogurts and yogurt drinks to produce synbiotic products. 'Bifiel' (Yakult, Tokyo, Japan) contains galacto-oligosaccharides, whereas 'Symbalance' (Toni Milch, Zurich, Switzerland), 'Fyos' (Nutricia, Bornem, Belgium) and 'Fysiq' (Mona, Weerden, The Netherlands) all contain fructo-oligosaccharides. Oligosaccharides are also widely used in confectionery. Other current applications include desserts such as jellies and ice creams; bakery products including biscuits, breads and pastries; spreads such as jams and marmalades; and infant milk formulas. The use of oligosaccharides in the livestock industry is also increasing. An example is the fructo-oligosaccharide sold by Golden Technologies (Meiji-Coors Biotech joint venture) in the USA. Some non-food applications have also been proposed for oligosaccharides including drug delivery, cosmetics and mouth washes (Crittenden and Playne, 1996).

1.5 PROBIOTICS AND PREBIOTICS

Metchinikoff (1908) was one of the pioneers to study on probiotic effects that soured milk containing bacteria may have positive effects on intestinal health. The majority of early research was mainly focused on organisms in fermented milk products such as yogurt. However, even before 1920, researchers suggested that intestinal isolates might have better effects than non-intestinal organisms (Fuller, 1992). More recently, research efforts have concentrated on probiotics to improve health and prevent intestinal disturbances.

As probiotic bacteria, they must possess appropriate properties (Havenaar and Huis in't Veld, 1992; Lee and Salminen, 1995). The bacterium must survive the acidic conditions of the GI tract and then colonize in the intestine. There must be no pathogenic, toxic, mutagenic, or carcinogenic reaction to the organism, its fermentation products or cell components. Furthermore, the bacterium should be antagonistic towards carcinogenic and pathogenic microorganisms and it must also be genetically stable with no plasmid transfer mechanism. Finally, for an organism to be a suitable probiotic it must be easily reproducible and remain viable during processing and storage. A number of beneficial roles for probiotic strains have been reported or theorized. These benefits include: 1) re-

establishment of balanced intestinal microflora, 2) improving colonization resistance and /or prevention of diarrhea, 3) systemic reduction of serum cholesterol, 4) reduction of faecal enzymes, potential mutagens which may induce tumors, 5) metabolism of lactose and reduction of lactose intolerance, 6) enhancement of immune system response, 7) improved calcium absorption, and 8) synthesis of vitamins and predigestion of proteins (Havenaar and Huis in't Veld, 1992; Lee and Salminen, 1995; Tannock, 1995).

Because of the survivability and colonization difficulties that abound with probiotics, the prebiotic approach offers an attractive alternative. Prebiotics exploit selective enzymes production by those gut micro-organisms that may impart health benefits to the host. While some peptides, proteins and certain lipids are potential prebiotics, non-digestible carbohydrates have received the most attention. Certain carbohydrates, oligo- and poly-saccharides, occur naturally and meet the criteria of prebiotics (Gibson and Robertfroid, 1995). Some non-digestible carbohydrates have a number of functional effects on the GI tract which have been used to validate emptying, modulation of GI tract transit times, improved glucose tolerance, reduced fat and cholesterol absorption via binding of bile acids, increased volume and water carrying capacity of intestinal contents, modulation of microbial fermentation with increased short chain fatty acid (SCFA) production, decreased pH and ammonia production (Robertfroid,

1996). The combination of these effects could potentially result in improved host healths by reducing intestinal disturbances (constipation and diarrhea), cardiovascular disease and intestinal cancer.

1.6 GENETIC MODIFICATIONS

Enzyme modification can expand the diversity and develop a wide range of lactase, engineered for a particular application. Several researches have been carried out to improve the microbial strains. One interest has been the increase of thermostability of strains and the β-galactosidase to allow lactose hydrolysis prior to and during pasteurization. These enzymes give higher conversion and are less prone to microbial contamination (Gekas and Lopez-Leiva, 1985). Mutant strains of *Streptococcus thermophilus* having defective lactose transport systems having a phenotype gluS31, lacS-, sucS+, and beta gal+ are effective for use in hydrolysis of lactose (Somkuti and Steinberg, 1991). Novel methods are disclosed for the enhanced expression and secretion of lactase from filamentous fungi. The process causes enhanced production of lactase from an *Aspergillus* strain transformed with necessary DNA. The DNA sequence encoding lactase gene and the deduced amino acid sequence of the lactase have been determined (Berka *et al.*, 1994).

Important research is now being conducted to produce transgenic dairy cattle capable of producing low-lactose milk. French researchers have developed transgenic mice that expressed intestinal lactase in the mammary gland and produce low-lactose milk. A DNA construct containing the rat intestinal lactase-phlorizin hydrolase cDNA under the control of the mammary specific alpha lactalbumin promoter was introduced into the mice. Researchers reported that transgenic mice expressed the foreign lactase construct during lactation and secreted lactase into milk (Jost *et al.*, 1999).

CHAPTER 2

MOLECULAR CHARACTERIZATION OF A β-GALACTOSIDASE GENE FROM *BIFIDOBACTERIUM BREVE* B24 AND OVER-EXPRESSION STUDY

A β-galactosidase gene, *galA* from *Bifidobacterium breve* B24 was successfully cloned in *E. coli* MC1061 and its whole sequence was analyzed for the molecular characterization. The comparison of the nucleotide sequences with other microbial β-galactosidases also helped to clarify the diversity of this enzyme.

The results of this study were summarized and submitted to the journal of *Applied and Environmental Microbiology*. The manuscript entitled "Biochemical and molecular characterization of a β-galactosidase from *Bifidobacterium breve* B24" was co-authored by Sung-Hun Yi, Geun-Bae Kim, and Byong H. Lee. This project was supervised by Dr. Byong H Lee and actual experimental work and writing of manuscript were done by Sung-Hun Yi. Dr. Geun-Bae Kim, Post-Doc fellow in the Department of Food Science at McGill University was involved in the cloning study. The manuscript was edited by Dr. Byong H. Lee prior to its submission for publication.

2.1 ABSTRACT

Among 17 strains of bifidobacteria tested for β-galactosidase enzyme activity, one strain was found to be the most appropriated one for this study based on its size, characteristic, and activity. To understand this enzyme at the molecular level, the β-galactosidase gene, *galA* was screened from *Bifidobacterium breve* B24 genomic DNA library. Genomic DNA library was constructed in pBR322 plasmid vector with three different restriction enzymes *Bam*HI, *Eco*RI, and *Hind*III and subcloned into pDrive vector for the sequencing. The complete β-galactosidase gene, *galA* contained 2076 bp nucleotides and had a high homology to the other *Bifidobacterium* species. One primer set (5'-*Nde*I- and 3'-*Bam*HI-) was designed and synthesized for the PCR amplification based on *galA* sequence, and the amplified fragments were ligated into pET-36(B)+ over-expression vector for the over-expression study. pET-36(B)+ plasmid containing a β-galactosidase gene was transformed into *E. coli* ER2566, successfully induced and over-expressed under 1 mM IPTG.

2.2 INTRODUCTION

Bifidobacteria are one of the major microflora in gastrointestinal tract (GI tract) of human. Many bifidobacteria-containing dairy and pharmaceutical products have been developed and consumed for several decades due to their promising health-promoting properties, for example, reduction of harmful bacteria and toxic compounds in the intestine, prevention of dental caries, reduction of total cholesterol and lipid in serum, and relief of constipation (Lee and Salminen, 1995; Crittenden and Playne, 1996; Cherie and Glenn, 1998; Mark *et al*, 2002). Therefore, live probiotic bacteria, which are improving the microbial balance of the human GI tract, have been used to supplement dairy products for long time. Another way to increase the number of beneficial bacteria in the human intestine is to stimulate their growth by supplying growth factor such as oligosaccharides. Galacto-oligosaccharides, so-called prebiotics, have been shown to stimulate the growth of probiotic bacteria, including bifidobacteria (Tanaka *et al*, 1983; Hung and Lee, 2001).

A β-galactosidase (EC 3.2.1.23, β-D-galactoside galactohydrolase), commonly known as lactase, is found in most of bifidobacteria and is known to catalyze not only hydrolyze β-D-galactoside linkage of lactose to produce glucose and galactose but also has transgalactosylation activity to synthesize galacto-oligosaccharides. Both reaction

activities are well characterized and applied in many food industries. Lactose hydrolyzed milk can reduce lactose intolerance problem, lactose hydrolyzed whey syrup and whey permeate can be utilized in frozen desserts, confectionary, bakery, fermentation products, and beverages (Crittenden and Playne, 1996). The galacto-oligosaccharides can also be employed as probiotic food ingredients, humectants, and emulsifiers, etc (Crittenden and Playne, 1996).

Although β-galactosidases are widely distributed in nature, the most thoroughly studied β-galactosidases are obtained from *E. coli* and commercially used β-galactosidases are mainly from fungi and yeasts.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains, plasmids, and growth conditions

Bifidobacterium breve B24 was isolated from baby faces and identified by 16S rRNA gene sequence typing according to the method of Kaufmann *et al.* (1991). This strain was grown anaerobically in MRS medium (Difco, Detroit, Mich.) at 37 ℃ supplemented with 0.05 % (w/v) cysteine HCl. *E. coli* MC1061 grown in Luria-Bertani

(LB) broth at 37 ℃ was used as recipient cell in cloning experiment. *E. coli* ER2566 (BioLab) providing a T7 RNA polymerase was used as a host for the over-expression of *galA* genes from the T7 promoter. When appropriate, ampicillin (100 μg/ml) and kanamycin (40 μg/ml) were added in medium. The plasmid pBR322 was used for genomic DNA library construction. The plasmid pET36b(+) (Novagen Inc., Mississaga, ON) containing the T7 promoter was used for the over-expression of β-galactosidase. For the selection of positive clone, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranose) (Invitrogen Canada Inc.) was used as substrate.

2.3.2 Chemicals and enzymes

All restriction enzymes, T4 DNA ligase, X-gal, IPTG (Isopropyl-β-D-thiogalactopyranoside), and agarose were purchased from Roche (Laval, QC) or GibcoBRL (Burlington, ON). Bio-Rad reagent for protein assay was purchased from Bio-Rad (Mississauga, ON). O-nitrophenyl-β-D-galactopyranose (ONPG) and other chemical were purchased from Sigma (St. Louis, MO).

2.3.3 Preparation of genomic DNA library and plasmid DNA

Bifidobacterium breve B24 genomic DNA was isolated according to the method

of Meile *et al.* (2001), with some modifications. *Bifidobacterium breve* B24 cells were harvested by centrifugation (8000 g; 20 min) from 250 ml of an early stationary phase culture in MRS, washed twice with TEN buffer (100 mM NaCl; 10 mM Tris; 1 mM EDTA, pH 8.0) and incubated in lysis buffer (50 mM glucose; 25 mM Tris; 10 mM EDTA, 15 mg/ml lysozyme; 1 kU mutanolysin; 50 μg/ml RNase, pH 8.0) at 37 ℃ for 1 h. Cells were lysed with 5 % sodium dodecyl sulphate (SDS) at 65 ℃ for 15 min and the cell lysates were extracted with phenol and phenol:chloroform (1:1, v/v) extractions were performed. Genomic DNA was precipitated by absolute ethanol and washed with 70 % ethanol. DNA was dried and dissolved in TEN buffer (100 mM NaCl; 10 mM Tris; 1 mM EDTA, pH 8.0). Small-scale plasmid preparation was performed using the QIAprep Spin Miniprep kit (Qiagen, Mississauga, ON).

2.3.4 Construction of genomic DNA library and screening of positive clone

All the DNA manipulations in this study were performed according to standard procedure (Sambrook *et al.*, 1989). T4 DNA ligase and other DNA modifying enzymes were purchased from New England Biolabs Inc., Invitrogen Life Technologies, or Amersham Pharmacia Biotech. Inc. and used according to the manufactures' manuals.

The isolated chromosomal DNA from the cells was partially digested with three

different restriction enzymes, *Bam*HI, *Eco*RI, and *Hind*III and analyzed by 0.8 % agarose gel electrophoresis. DNA fragments ranging from 1.0 to 8 kb were excised on UV illuminator and purified using QIAquick Gel Extraction kit (Qiagen, Mississauga, ON) and then ligated to the linearized pBR322 plasmid that had been digested with *Bam*HI, *Eco*RI, and *Hind*III and dephosphorylated. The ligation mixture was transformed into *E. coli* MC1061 competent cells prepared by calcium chloride method (Sambrook *et al.*, 1989). The transformed *E. coli* cells were then plated on a selective medium (LB agar containing X-gal) for the detection of β-galactosidase activity.

2.3.5 DNA sequencing and sequence analysis

The nucleotide sequences were determined by AmpliTaq FS DNA polymerase fluorescent dye terminator reaction using Applied Biosystem 373 stretch automated sequencer (Applied Biosystems Inc., Foster city, Calif.). Assembly and analysis of DNA sequences were performed with DNASIS for Windows (Hitachi software). DNA homology searchs were performed with the Basic Local Alignment Search Tool (BLAST) at the website of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

2.3.6 Nucleotide sequence accession number

The *galA* gene nucleotide sequence has been deposited in the GenBank database under accession number AY691690.

2.3.7 Over-expression study

For the PCR amplification of *galA* gene, the gene was inserted in pET36b(+) over-expression vector. Two primers (5' upstream and 3' downstream), *Nde*I- (5'-GGCAT ATGAACACAACCGACGATCAG-3') and *Bam*HI (5'-CCAAGCTTTCACGTCGAGG GTGAGCG-3') were designed on the basis of *galA* gene sequence. These primers contained restriction enzyme sites at the end of *galA* gene fragment for cloning into pET36b(+) (Fig. 2.5). The PCR amplification was performed by using total chromosomal DNA from *Bifidobacterium breve* B24 and pB322B11 that contained 11 kb insert as templates for amplifying the *galA* gene. Templates DNA (100 ng each), 0.25 μM upstream and down stream primers were added to 50 μl of PCR mixture containing 200 μM of each dNTP, PCR buffer, and 2.5 units of HotStarTaq polymerase (Qiagen, Mississauga, ON). The PCR was conducted in a Perkin Elmer GeneAmp system with an initial activation step at 95 ℃ for 15 min followed by 35 cycles of a denaturation step at 94 ℃ for 1 min, an annealing step at 58 ℃ for 30 sec, and an extention step at 72 ℃ for 3

min. A final extention step of 10 min at 72 ℃ was performed to ensure complete amplification of all DNA fragments. PCR products were analyzed by electrophoresis in 0.8 % low-melting agarose gels (Gibco BRL, Burlington, ON) containing EtBr (1 μg/ml) and purified using the QIAquick PCR purification kit (Qiagen, Mississauga, ON). The amplified fragment was inserted into linearized pET36b(+) plasmid that had been digested with *Nde*I and *Bam*HI. The ligation mixture was transformed into *E. coli* ER2566 competent cells and the transformed cells were then plated on a selective LB agar containing X-gal and kanamycin for the detection of β-galactosidase activity.

Complete 2076 bp *galA* gene containing pET36b(+) vector was designated as pBT2076NB and its restriction map is shown in Fig. 2.4. The recombinant plasmid, pBT2076NB containing a blue colony was isolated from selective plate and a single colony was inoculated in 10 ml LB broth containing 40 μg/ml kanamycin at 37 ℃. The plasmid DNA was extracted using the QIAprep Spin Miniprep kit (Qiagen, Mississauga, ON) and the insert size was verified by restriction enzyme digestion on 0.8 % agarose gel electrophoresis.

2.3.8 IPTG induction of the enzyme

E. coli ER2566 provides a T7 RNA polymerase and has no β-galactosidase

activity. Previously prepared *E. coli* ER2566 cell, containing pBT2076NB plasmid was grown at 37 ℃ in 50 ml 2X YT medium (1.6 % tryptone; 1.0 % yeast extract; 0.5 % NaCl, pH 7.0) containing 40 μg/ml kanamycin until OD_{600} of 1.0 was reached. A portion (1 ml) of seed culture was inoculated in 50 ml 2X YT medium containing 40 μg/ml kanamycin with shaking at 250 rpm until OD_{600} of 1.0 was reached. Final concentration of 1 mM IPTG was added into culture for further incubation for 5 h at 37 ℃. After IPTG was added, 5 ml of culture was withdrawn at 1 h intervals and assaying the enzyme activities and growth rates. Samples (100 μl) were serially diluted and plated on LB agar plates in duplicate and incubated at 37 ℃ for 16 h. The colonies were counted and expressed as colony forming units per ml (CFU/ml). Samples (4 ml) were centrifuged at 8,000 g for 10 min and washed twice with sodium phosphate buffer (50 mM, pH 7.0) and resuspended in 500 μl of same buffer. Cells were disrupted by sonicator (550 Sonic Dismembrator, Fisher Scientific, Mississauga, ON) using a microtip with the power level at 3 for 5 min with 2 sec pulsings and 30 sec intervals under constant cooling. The disrupted cells were centrifuged (12,000 g, 15 min, 4 ℃) and the supernatants (cell free extracts) were collected and assayed for the enzyme activities.

2.3.9 Enzyme activity and protein assays

Enzyme activity was measured by a modified procedure (Craven *et al.*, 1965). Briefly, enzyme was reacted with 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) in 50 mM sodium phosphate buffer (pH 7.0) at 45 ℃ for 5 min, and reaction was stopped by adding equal volume of 1.0 M Na_2CO_3. The released *o*-nitrophenyl was quantitatively determined by measuring the optical density at 420 nm. One unit of enzyme activity was defined as the amount of enzyme liberating one μmol of *o*-nitrophenyl per min. Specific activity was defined as units per mg of protein.

Protein concentrations were determined by the Bio-Rad protein assay reagent (Bio-Rad) using bovine serum albumin (Sigma) as a standard.

2.3.10 Polyacrylamide gel electrophoresis

To determine homogeneity and subunits of purified protein, the purity of the enzyme at each purification step was examined by SDS-PAGE by the method of Laemmli (1970) using 10 % gel. To estimate their molecular mass, a broad range of Precision Plus Protein Standards (10 to 250 kDa) (Bio-Rad) was applied to the same gel and the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad). BioRad Informatix software was used for electrophoresis band analysis and over-expression level.

2.4 RESULTS

2.4.1 Cloning and characterization of β-galactosidase gene from *Bifidobacterium breve* B24

To clone β-galactosidase gene from *Bifidobacterium breve* B24, three different restriction enzymes (*Bam*HI, *Eco*RI, and *Hind*III) digested genomic DNA library (Fig 2.1) was constructed in pBR322 and screened in *E. coli* MC1061 recipient cells. Only one colony showed blue color on the selective medium containing X-gal with *Bam*HI restriction enzyme site, and it was designated as pB322B11. When insert size was analyzed on 0.8 % agarose gel electrophoresis, the size was approximately 11 kb. A restriction enzyme map of the insert DNA of pB322B11 was generated by restriction enzyme analysis (Fig. 2.2). The 11 kb insert was digested with various restriction enzymes and inserted in pBR322 vector, but none of them showed the enzyme activity (Fig. 2.3). To find out its whole sequence, a *Hind*III fragment was selected for the subcloning and sequencing based on the restriction map. About 1 kb *Hind*III fragment was excised and ligated in p-Drive vector for the sequencing (Fig. 2.4). After sequencing, 1014 bp sequence was obtained. BLAST search program (http://www.ncbi.nlm.nih.gov) with 1014 bp fragment showed high homology to the β-galactosidases of *Bifidobacterium*

longum NCC2705 (96%) (Schell *et al.*, 2002) and *Bifidobacterium infantis* HL96 (93%) (Hung *et al.*, 2001). Based on a 1014 bp *Hind*III fragment sequence, internal primers were designed for whole sequencing.

One complete open reading frame (ORF) was identified and this 2076 bp encoded 691 amino acids (Fig. 2.6). Based on its sequence homology to several published β-galactosidase genes, this complete ORF was assumed to be the structural gene, *LacA* of a β-galactosidase of *Bifidobacterium breve* B24 that was named *galA* (Fig. 2.5). The deduced protein had a 76,010 Dalton and a pI of 4.58 (http://www.embl-heidelberg.de/cgi/pi-wrapper.pl).

As shown in Fig. 2.3, subclones of p11DEE25, p11DEE157, p11DEE50, p11DHE14, p11DEH40, p11DHH10, p11DEE31, and p11DHE35 were further constructed for localizing the *galA* and for analyzing nucleotide sequence. No β-galactosidase activity was detected from those subclones and it is possible that the *galA* contained at least one *EcoR*I or *Hind*III restriction enzyme site.

Predicted promoter region sequence was 5'-TCTGCGTGACTCCCATTTGCCC ACACGTAGATATTTATTCATTAAAGGAA-3' and was located in 11 nucleotides upstream from the start codon (ATG, M; methionine). The promoter region contained putative ribosomal binding site (RBS), transcriptional start point (+1), -10 and -35 region

(Fig. 2.5). Stop codon, TAA, represented by asterisks at the end of sequence and possible transcriptional terminator (repeated inverted sequences) are also shown in Fig. 2.5.

2.4.2 Comparison of amino acid sequence

The deduced amino acid sequence of β-galactosidase gene, *galA* is shown in Fig. 2.6. The amino acid sequence homology search by BLAST analysis revealed that its multiple sequence alignment is similar to the *LacA* family proteins (Fig. 2.7). Five conserved amino acid sequences, Tryptophan-57, Glycine-83, Proline-104, Leucine-107, and Tyrosine-161 were found in all *LacA* family protein. However, overall similarity was relatively low (21 - 35 % identities, Table 2.1). Highest similarity of *galA* with *LacA* family was found to *Bacillus halodurans* C-125 from *LacA* family β-galactosidase (35% identity, Genebank accession number, NP_244568). Eleven identical amino acid residues were found in β-galactosidases from bifidobacteria. Identical amino acid residues are Glycine-23, 336, and 450, Glutamate-64 and 245, Tryptophan-98, Arginine-23, Aspartate-236, Tyrosine-272, Methionine-372, and Proline-457.

Based on the role in carbohydrate metabolism, β-galactosidases can be classified in 4 groups like *LacA*, *LacZ*, *LacY*, and *LacG* family and their number of amino acids, source, and Genebank accession numbers are summarized in Table 2.1.

In the phylogenetic analysis of a β-galactosidase from *Bifidobacterium breve* B24, *galA* was localized in unique branch indicating that a β-galactosidase from *Bifidobacterium breve* B24 was clearly distinguished from those of *LacA* family β-galactosidases (Fig. 2.9). However, *galA* formed an unique subfamily that *Bifidobacterium infantis* β-galactosidase III (Genebank accession number AAL02053), *Bifidobacterium longum* DJO10A (Genebank accession number ZP_00121008), *Bifidobacterium longum* NCC2705 β-galactosidase I (Genebank accession number NP_696337 691) were localized in the same branch (Fig. 2.10).

2.4.3 Over-expression of β-galactosidase in *E. coli* ER2566

To study the possibility of overproduction of a β-galactosidase from *Bifidobacterium breve* B24, the over-expression study was carried out with T7 RNA polymerase expression system in *E. coli* system. The target sequence, *galA* in pB322B11 was amplified using PCR and the insert was ligated in pET36b(+) and transformed into *E. coli* ER2566. The gene expression in *E. coli* ER2566 was induced by final concentration of 1 mM IPTG and its activity was analyzed by assaying the hydrolysis of ONPG. After IPTG was added, the β-galactosidase was significantly over-expressed at 2 h and more than 23.7 % (BioRad Informatix software) of total protein was observed at 3 h (Fig. 2.11).

The molecular mass of the β-galactosidase was determined on SDS-PAGE (Fig. 2.11) and apparent size was 76 kDa. It was in good agreement with the deduced size from the amino acid sequences.

The growth rate of recombinant *E. coli* ER2566 was inhibited by addition of IPTG, indicating that over-expressed enzyme may inhibit the cell division.

2.5 DISCUSSION

Chromosomal DNA digestion with *Hind*III did not cut the DNA, probably due to the high G+C content of bifidobacteria. As *Hind*III recognizes nucleotide sequence of AAGCTT, there was a low probability to meet its recognition sequence in chromosomal DNA of bifidobacteria. In fact, G+C content of β-galactosidase gene *galA* was 63.53 %. One positive clone was found from the genomic library constructed with *Bam*HI site in pBR322, which contained a 11 kb insert. A 11 kb insert was long enough to contain the whole β-galactosidase gene and was digested with various restriction enzymes for the subcloning and sequencing. Subclones of p11DEE25, p11DEE157, p11DEE50, p11DHE14, p11DEH40, p11DHH10, p11DEE31, and p11DHE35 were derived from

pB322B11 and none of them showed β-galactosidase activity. This indicated that β-galactosidase gene from *Bifidobacterium breve* B24 contained at least one of restriction enzyme that was used for constructing subclones. A derivative p11DHH10 containing a 1kb *Hind*III fragment was selected for the sequencing because of its small size and high possibility to contain β-galactosidase gene since there was no positive clone from genomic library constructed with *Hind*III restriction enzyme. DNA comparison between β-galactosidases of *B. longum* NCC2705 (Schell *et al.*, 2002) and *B. infantis* HL96 (β-gal III) (Hung *et al.*, 2001) showed the highest homology with 96 and 93 %, respectively. Whole sequencing of β-galactosidase gene was performed with the use of internal primers extension sequencing and 2076 nucleotides encoded 691 amino acids. Deduced molecular mass and pI value (http://www.embl-heidelberg.de/cgi/pi-wrapper.pl) of this sequence were 76,010 Dalton and 4.58, respectively.

Putative promoter region sequence analyzed by Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html) showed relatively high value at 0.86. Within this predicted region, -10 (Pribnow box), -35, ribosomal binding site (RBS; AGGA) as well as transcription initiation starting point were found. The components of transcriptional machinery of β-galactosidase gene, *galA* were well preserved but there was a small change in that the gene showed different -10 region

sequence "TAGATA" than typical -10 region sequence of TTGACA. Termination codon, TAA was found at the end of *galA* gene. Generally, termination of RNA synthesis occurs at specific base sequences within the DNA molecule. A hairpin structure which is one of termination factors was found 21 bp downstream of stop codon, TAA in *galA* gene. This sequence is 5'- CAAATGCGATGGCGGGCGTGTTAGCGATAACACGCCCGCC ATCGCATTTG -3'.

The sequence homology of amino acids (Thompson, 1994; http://prodes.toulouse.inra.fr/multalin/multalin.html) revealed that a β-galactosidase from *Bifidobacterium breve* B24 was the only *LacA* family member protein among bifidobacteria and it is the first observation. However, interestingly, this β-galactosidase shares less amino acid sequence homology with the *LacA* family than β-galactosidases from bifidobacteria. Five identical sequences of amino acid were found in all *LacA* family, whereas eleven identical sequences of amino acid were found in bifidobacteria species. It is thus assumed that a β-galactosidase from *Bifidobacterium breve* B24 has very different structural pattern than the *LacA* family β-galactosidase but has very closely related structural pattern to other bifidobacterial β-galactosidases. Although crystallized 3D structures of β-galactosidases from the *LacA* family and bifidobacteria have not been reported yet, it has been reported from *Thermus thermophilus* A4 (Hidaka *et al.*, 2002). The sequence similarity of amino

acids between *Bifidobacterium breve* B24 and *Thermus thermophilus* (Fig. 2.13) showed that identical amino acids found in many β-galactosidases were Glutamate-245 and Glycine-336 (Fig. 2.9). It is thus possible that these Glu-245 and Gly-336 have very important role in the catalytic site or sites due to the 3D structural location of these two amino acids or in formation of enzymes.

β-Galactosidases (lactase) can be classified in four major group depending on their mechanism. First group is *LacZ* family proteins (hydrolase) that are related to hydrolytic activity. Second group is *LacY* family proteins (permease) that are dealing with transporting substrate from outside of membrane to inside of the cells. Third group proteins are classified as *LacG* family (glycosyl hydrolase) and glycosidases (Inge *et al.*, 2000) are often regarded as this group of protein due to similar hydrolytic activity on carbohydrates. Last group is the *LacA* family proteins that they have carbohydrate transporting and metabolism properties. A β-galactosidase from *Bifidobacterium breve* B24 was the only one of *LacA* member protein.

The phylogenetic analysis of a β-galactosidase from *Bifidobacterium breve* B24 showed that diversity of enzyme was huge in the *LacA* family protein. A β-galactosidase of *Bifidobacterium breve* B24 was localized in the same branch with other bifidobacteria, indicating that their size, structure, and properties of enzymes may be similar.

The cloning of β-galactosidase gene into pET36b(+) over-expression vector has increased the expression level significantly and allows increased production of protein in gram per liter.

This study demonstrated that a β-galactosidase from *Bifidobacterium breve* B24 belongs to the *LacA* family member proteins among bifidobacteria strains. Although β-galactosidase from *Bifidobacterium breve* B24 belongs to the *LacA* family on the basis of properties of enzyme, it did not show high sequence homology of amino acid (21-35 % identities). Further studies on this enzyme are required to identify the crystallized 3D structure since it is the only one of the *LacA* family member proteins among bifidobacteria strains. The overproduction of this enzyme into yeast, *Pichia pastoris* or other expression system will be useful for industrial purpose.

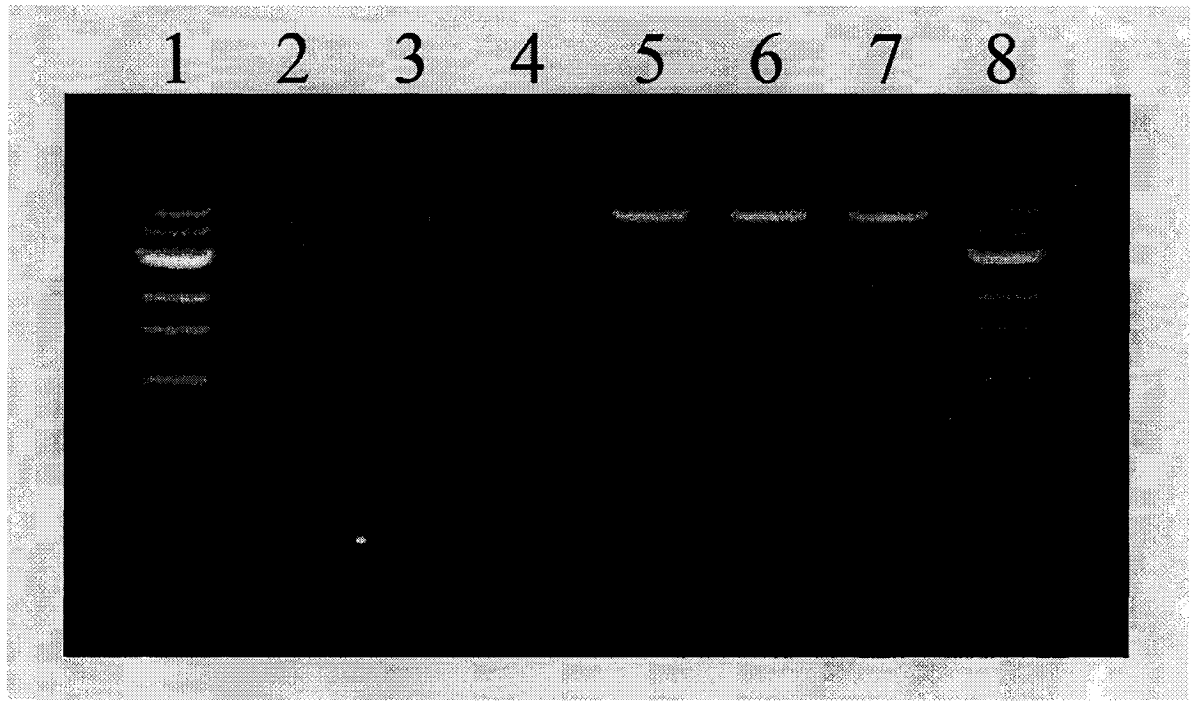
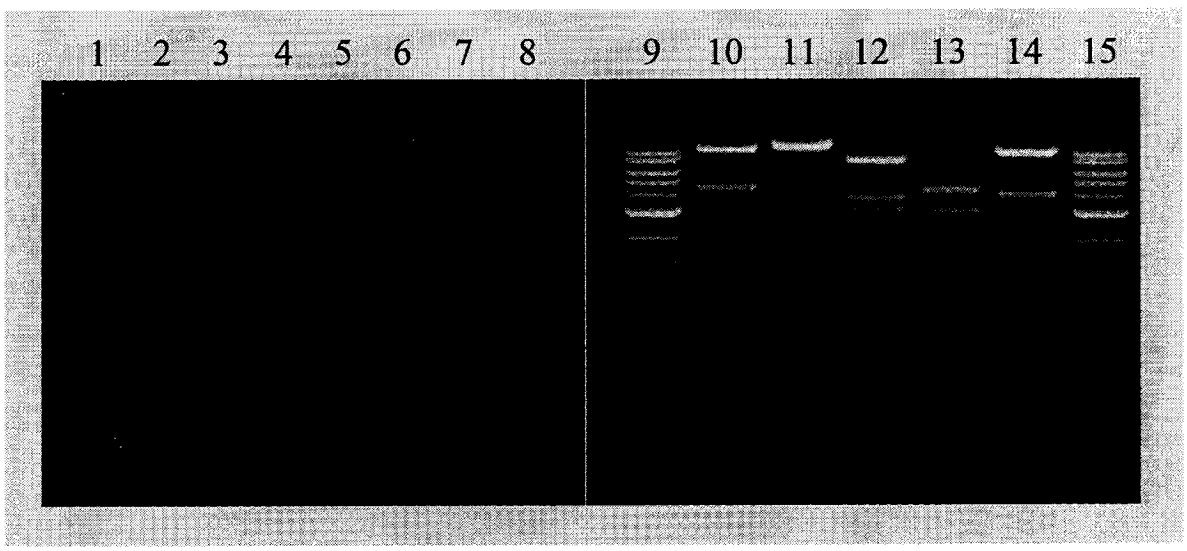


Fig. 2.1 Restriction enzyme digestion analysis of *Bifidobacterium breve* B24 chromosomal DNA and pBR322 DNA. Lane 1 and 8, 1kb size marker (NEB); lane 2 to 4, *B. breve* B24 chromosomal DNA digested with *Bam*HI, *Eco*RI, and *Hind*III, respectively; lane 5 to 7, pBR322 DNA digested with *Bam*HI, *Eco*RI, and *Hind*III, respectively.

(A)



(B)

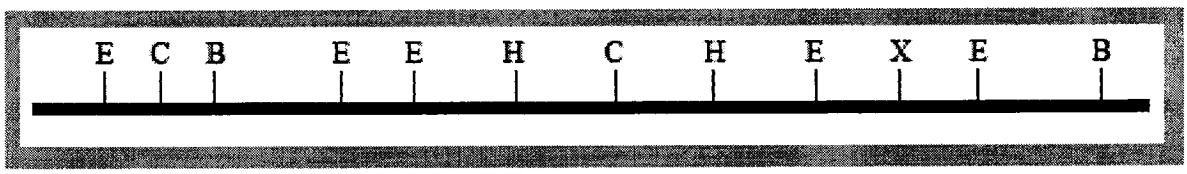


Fig. 2.2 Restriction enzyme digestion analysis of positive clone B2 (CB2) (A) and physical map of positive clone isolated (B). Lane 1, 9, and 15, 1kb size marker (NEB); lane 2 to 8 and 10 to 14, CB2 in pBR322 digested with *Bgl*II, *Kpn*I, *Not*I, *Xba*I (X), *Xho*I, *Sac*I, *Sna*BI, *Cla*I (C), *Bs*PE I, *Tth*111I, *Eco*RI (E), and *Hind*III (H), respectively.

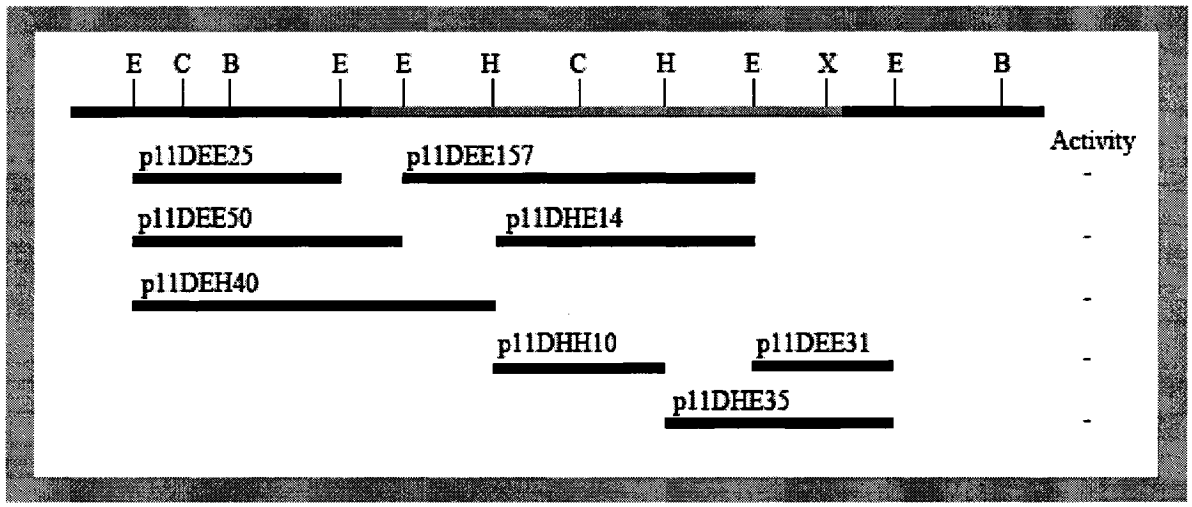


Fig. 2.3 Restriction enzyme map and β-galactosidase activity of derivatives of 11 kb insert and actual location of β-galactosidase gene *galA* is indicated in gray box. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; X, *Xba*I.

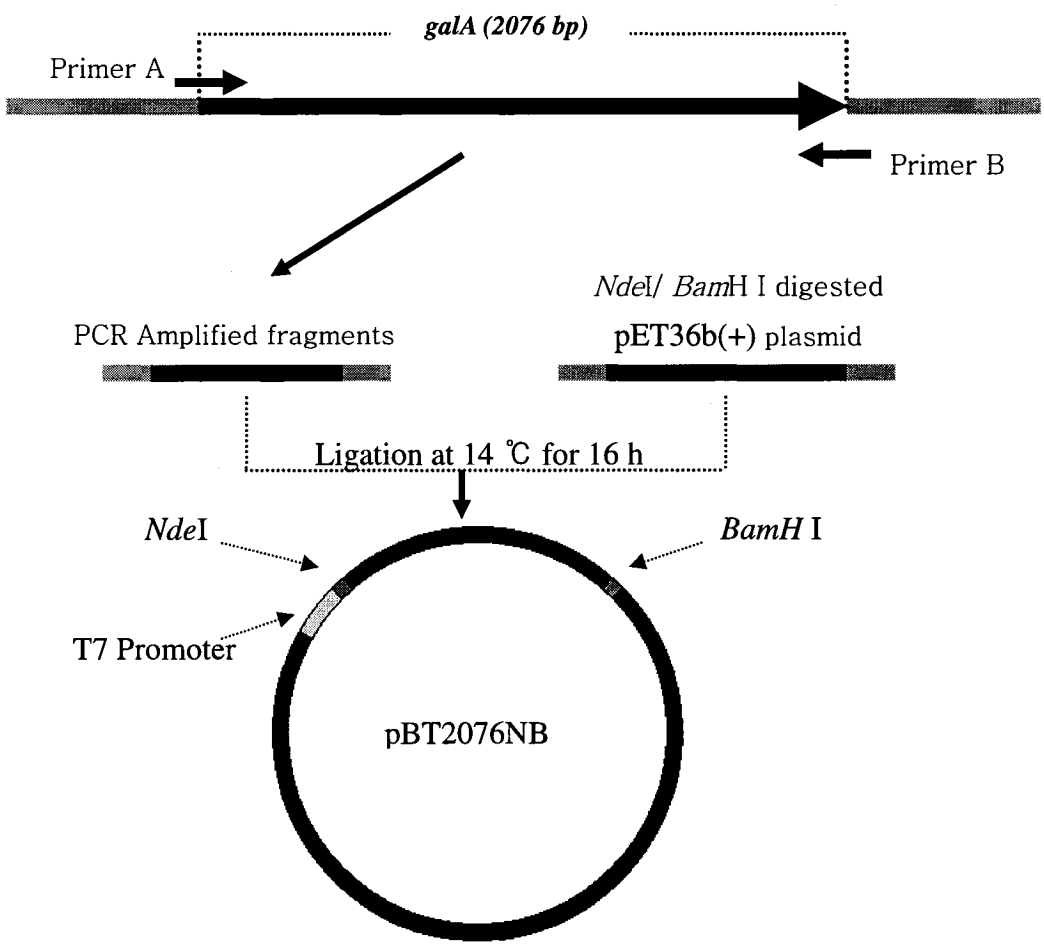


Fig. 2.4 The schematic diagram of constructing pBT2076NB plasmid for over-expression of *gal*A gene in *E. coli* ER2566. Primer A, 5'-GGCATATGAACACAA CCGACGATCAG-3'; Primer B, 5'-CCAAGCTTTCACGTCGAGGGTGAGCG-3'.

ATCGCGTTCCTGTGCCTGCAGAAGTACTGGCAGTCCGGCCCTGCCGCAGGCGCCGTCAAG
GAGTAACCGTACTTCGGCTCCCTTCTGGTGGAGGGAGCCGAGATAAAGGCATAACCACTA
ACAGGAAGGAGGCGTCTATGCAGATATTCGCATTGTTGAGAATCGTGCAGGCGCCTCCTT
GTGTA**TCTGCGTGACTCCCATTTGCCCACACGTAGATATTTATTCATTAAAGGAA**CAGCA

-35 **-10** +1 **RBS**

Pribnow box

M

TCATC**ATG**GAACATCGCGAATTCAAGTGGCCGCAGCCTCTTGCGGGTGGCAAGCCCCGCA
TCTGGTACGGCGGCGATTACAACCCCGACCAATGGCCTGAGGAAGTGTGGGACGAAGATG
TAGCCCTCATGCAGAAGGCCGGCGTCAACCTCGTCTCCGTAGCCATCTTCTCTTGGGCCA
AGCTTGAGCCCGAAGAAGGCGTGTATGACTTCGATTGGCTCGACCGCGTCATCGACAAGC
TCGGCAAGGCCGGCATCGCCGTCGATCTCGCCTCCGGCACCGCATCCCCGCCGATGTGGA
TGACCCAGGCCCACCCGGAGATCCTCTGGGTCGACTACCGCGGCGACGTCTGCCAGCCCG
GTGCCCGACAGCACTGGCGGGCCACCAGCCCGGTCTTCCTTGACTACGCGCTCAACCTGT
GTCGCAAGATGGCCGAGCACTACAAGGACAACCCCTATGTGGTCTCTTGGCACGTGAGCA
ACGAGTACGGCTGCCACAACCGCTTCGACTACTCCGAAGATGCTGAGCGCGCCTTCCAGA
AGTGGTGCGAGAAGAAGTACGGCACCATCGACGCCGTCAATGACGCCTGGGGCACCGCCT
TCTGGGCGCAGCGCATGAACAACTTCTCCGAGATCATCCCGCCGCGCTTCATCGGCGATG
GCAACTTCATGAACCCGGGCAAACTGCTTGACTGGAAGCGCTTCAGCTCCGACGCCCTGC
TCGACTTTTACAAGGCCGAGCGCGACGCCCTGCTCGAGATCGCCCCCAAGCCGCAGACCA
CCAACTTCATGGTCTCCGCCGGCGGTGCCGGCATCGATTACGACAAGTGGGGTTACGACG
TGGACTTCGTGTCCAACGATCACTACTTCACTCCTGGCGAAGCTCACTTCGACGAGCTGG
CCTACTCGGCCTCCCTGTGCGACGGCATCGCCCGCAAGAATCCGTGGTTCCTCATGGAGC
ACTCCAGCTCCGCCGTCAACTGGCGTCCGATCAACTACCGCGTCGAACCCGGCGAACTGG
TGCGTGACTCCCTGGCCCACCTGGCCATGGGCGCCGACGCCATCTGCTACTTCCAGTGGC
GCCAGTCCAAGGCCGGTGCCGAGAAGTGGCACTCCTCGATGGTTCCTCACGCGGGCCCCG
ACTCCCAGATCTTCCGCGACGTGTGCGAGCTGGGTGCCGACCTCAACAAGCTTGCTGACG
AGGGCCTGCTGAGCACCAAGCTGGTCAAGTCCAAGGTCGCCGTCGTCTTCGATTACGAGT
CCCAGTGGGCTACGGAACACACCGCCACGCCTACTCAGGAGGTACGCCATTGGACCGAGC
CGCTCGCGTGGTTCCGCGCGCTGGCGGACAACGGTCTGACCGCAGACGTGGTGCCGGTTC
GCGGTCCCTGGGACGAGTACGAAGCCGTCGTGCTGCCGAGCCTGACCATCCTGTCTGAAG
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ACACCGGTCTGGTGGACGACAAGGATCACGTCTGGCTGGGTGGCTACCCCGGCTCCATCC
GTGACGTGGTGGGCGTGCGCGTTGAGGAATTCGCCCCGATGGGCAACGACTTCCCCGGTG
CCATGGACCACCTCGACTTGGACAACGGGACCGTGGCGCACGATTTCGCCGACGTGATCA
CCTCCGTGGCCGATACCGCTCACGTGGTCGCTGCATTTAAGGCCGATAAGTGGACCGGTT

TCGACGGCGCTCCCGCCATCACCATCAACGACTTCGGCGACGGCAAGGCCGCATACGTCG
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AAACTGGCGAGAACCACTTCGTGTTCCTGTTCAACCGCACCCACGATGTGGCGATTGTGG
ACGTGGACGGCGAGCCGCTGGTCGCCTCGCTGGCTCAGGTCAACGAATCCGAGCATACGG
CGGCCATTCAGCCGAACGGTGTGCTGGTGGTAAAGCTG**TAA**AGAGGTCTATTTATAGCGA
*

TTCAAATGCGATGGCGGGCGTGTTAGCGATAACACGCCCGCCATCGCATTTGCGATTTGT

TTTGAGATGTGTATACTAAAAGATGTTATCGCAACCATAGCGAAAGGAATGGCTTCATAG
AGCCACTTCTCAGTGTCGAGGAGAAATCAATAATTGACACTAGGTAACACGCAATGATTG
CGAGCGACAAAACAGTTTGAATCGTTCTAGACGATTCGAGGAAGGAGAACAGAACGATGA
AGTTCACTACCGCCAAGAAGGCCGTTGCCCTCACCGGTGCGGTTGCCATGCTGGTTTCCG
TTACCGCCTGCGGCTCTGATAGCGGCAAGTCCAGCCAGCCGGCTCAGGATTCC

Fig. 2.5 The nucleotide sequence of *galA* and its transcriptional region. Predicted promoter region sequence is indicated in gray shadow at the upstream of start codon (ATG, M;Methionine). The putative ribosomal binding site (RBS), transcriptional start point (+1), -10, and -35 region are represented by highlight. Stop codon (TAA) is represented by asterisks at the end of sequence and possible transcriptional terminator (inverted repeate sequences) is located from 21 bp downstream of the termination codon and is also indicated by arrows. GeneBank accession number is AY691690.

```
ATGGAACATCGCGAATTCAAGTGGCCGCAGCCTCTTGCGGGTGGCAAGCCCCGCATCTGG    60
M  E  H  R  E  F  K  W  P  Q  P  L  A  G  G  K  P  R  I  W   20

TACGGCGGCGATTACAACCCCGACCAATGGCCTGAGGAAGTGTGGGACGAAGATGTAGCC  120
Y  G  G  D  Y  N  P  D  Q  W  P  E  E  V  W  D  E  D  V  A   40

CTCATGCAGAAGGCCGGCGTCAACCTCGTCTCCGTAGCCATCTTCTCTTGGGCCAAGCTT  180
L  M  Q  K  A  G  V  N  L  V  S  V  A  I  F  S  W  A  K  L   60

GAGCCCGAAGAAGGCGTGTATGACTTCGATTGGCTCGACCGCGTCATCGACAAGCTCGGC  240
E  P  E  E  G  V  Y  D  F  D  W  L  D  R  V  I  D  K  L  G   80

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K  A  G  I  A  V  D  L  A  S  G  T  A  S  P  P  M  W  M  T  100

CAGGCCCACCCGGAGATCCTCTGGGTCGACTACCGCGGCGACGTCTGCCAGCCCGGTGCC  360
 Q  A  H  P  E  I  L  W  V  D  Y  R  G  D  V  C  Q  P  G  A  120

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TTCATGAACCCGGGCAAACTGCTTGACTGGAAGCGCTTCAGCTCCGACGCCCTGCTCGAC  720
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TCGGCCTCCCTGTGCGACGGCATCGCCCGCAAGAATCCGTGGTTCCTCATGGAGCACTCC  960
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GACTCCCTGGCCCACCTGGCCATGGGCGCCGACGCCATCTGCTACTTCCAGTGGCGCCAG 1080
 D  S  L  A  H  L  A  M  G  A  D  A  I  C  Y  F  Q  W  R  Q  360

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CAGATCTTCCGCGACGTGTGCGAGCTGGGTGCCGACCTCAACAAGCTTGCTGACGAGGGC 1200
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CTGCTGAGCACCAAGCTGGTCAAGTCCAAGGTCGCCGTCGTCTTCGATTACGAGTCCCAG 1260
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CCCTGGGACGAGTACGAAGCCGTCGTGCTGCCGAGCCTGACCATCCTGTCTGAAGAGACC 1440
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GGTCTGGTGGACGACAAGGATCACGTCTGGCTGGGTGGCTACCCCGGCTCCATCCGTGAC 1560
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ATTCAGCCGAACGGTGTGCTGGTGGTAAAGCTGTAA                        2076
 I  Q  P  N  G  V  L  V  V  K  L  -                           691
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Fig. 2.6 The amino acid sequence deduced from the gene. The deduced amino acid sequence is shown by the nucleotide sequence.

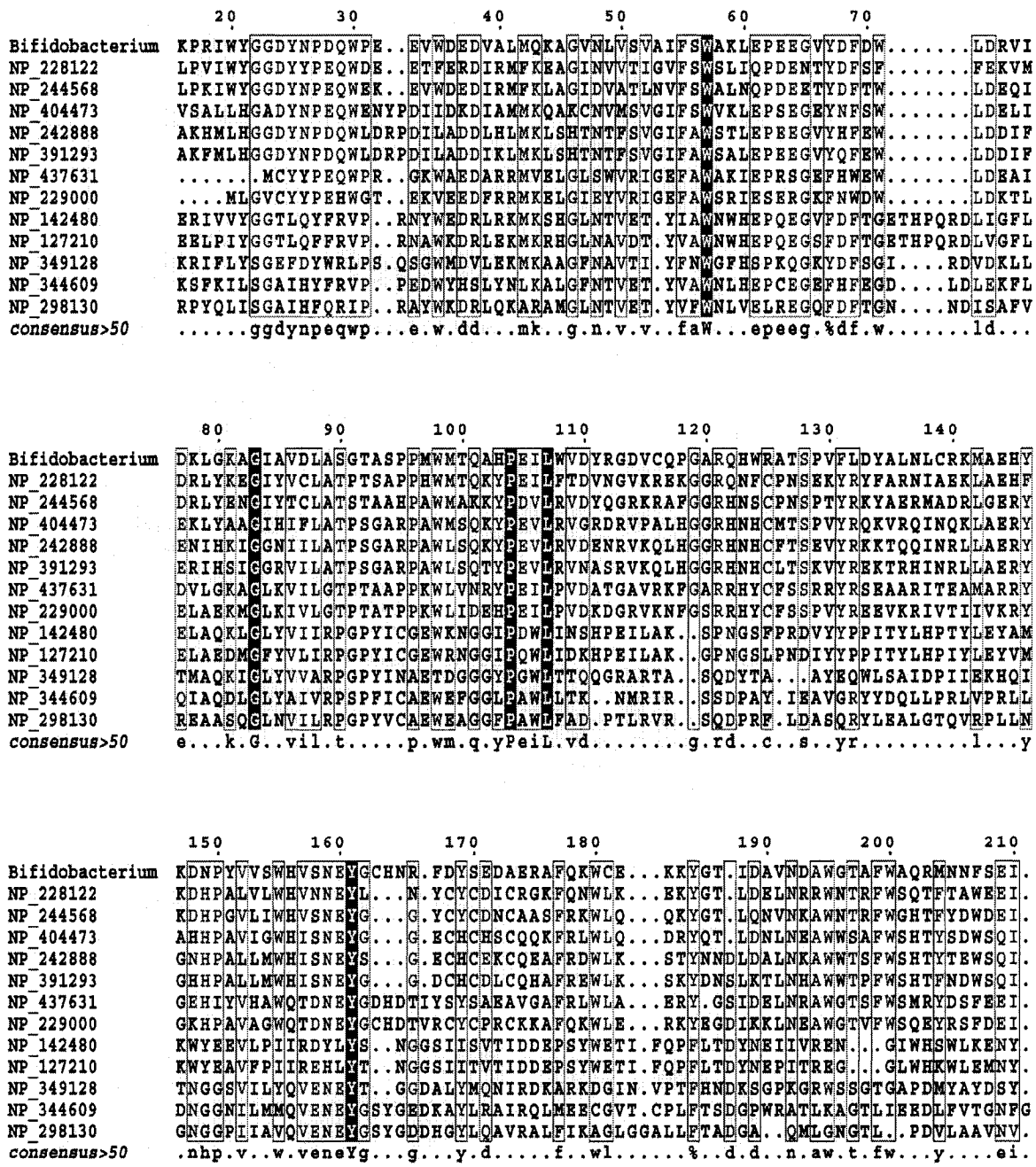

Fig 2.7 Multiple sequence alignment of *galA* of *Bifidobacterium breve* B24 with other *LacA* family β-galactosidases from various bacteria. Genebank assession numbers of *LacA* family are indicated in the picture.

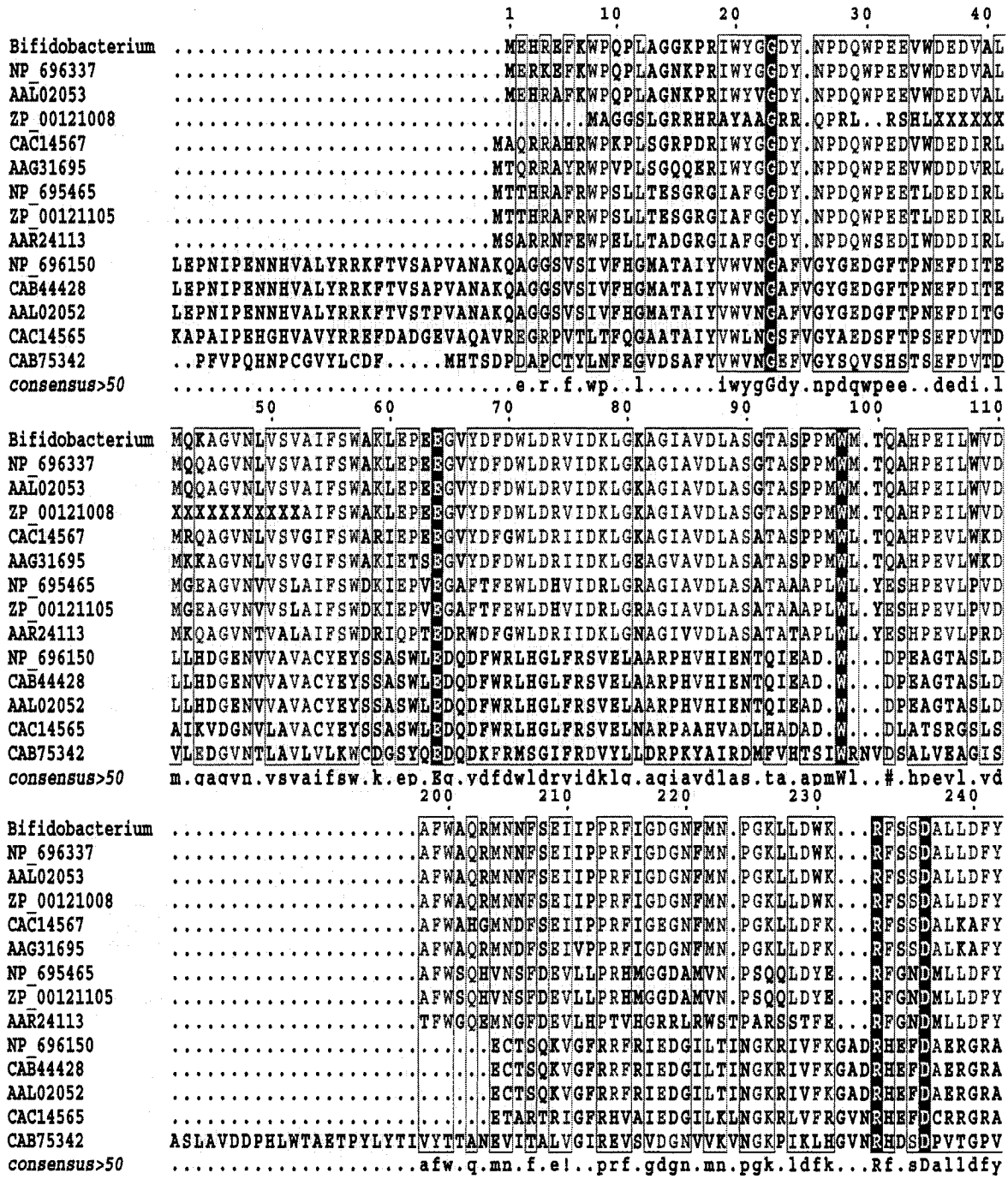

Fig 2.8 Continued on next page...

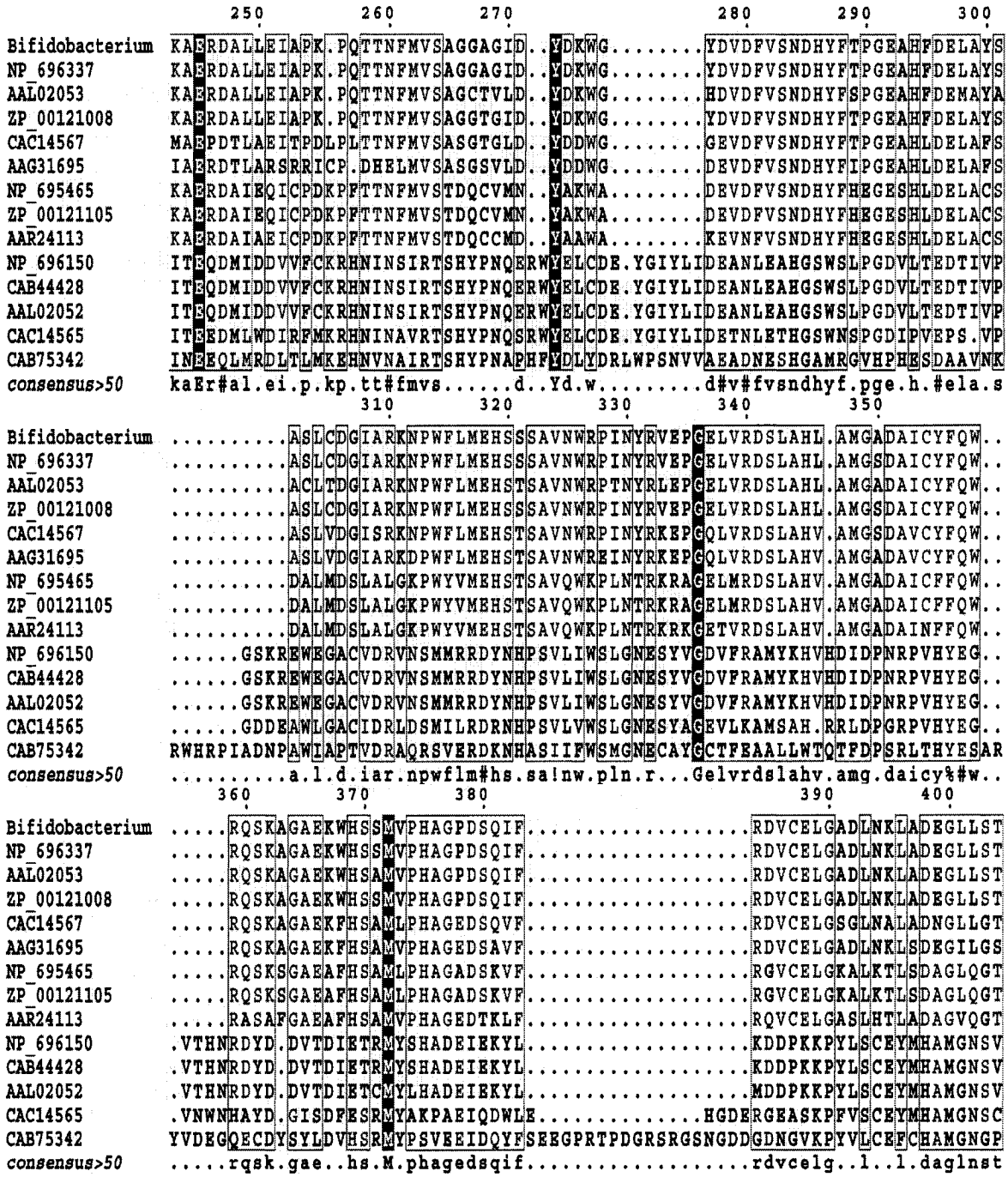

Fig 2.8 Continued on next page...

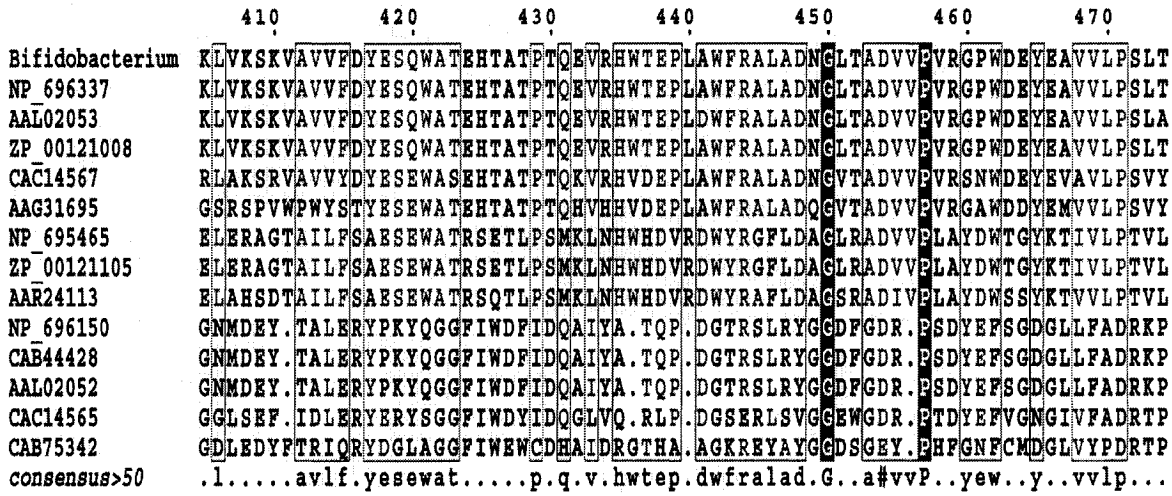

Fig 2.8 Multiple sequence alignment of *galA* of *Bifidobacterium breve* B24 with other β-galactosidases from bifidobacteria. Genebank assession numbers of bifidobacteria are indicated in the picture.

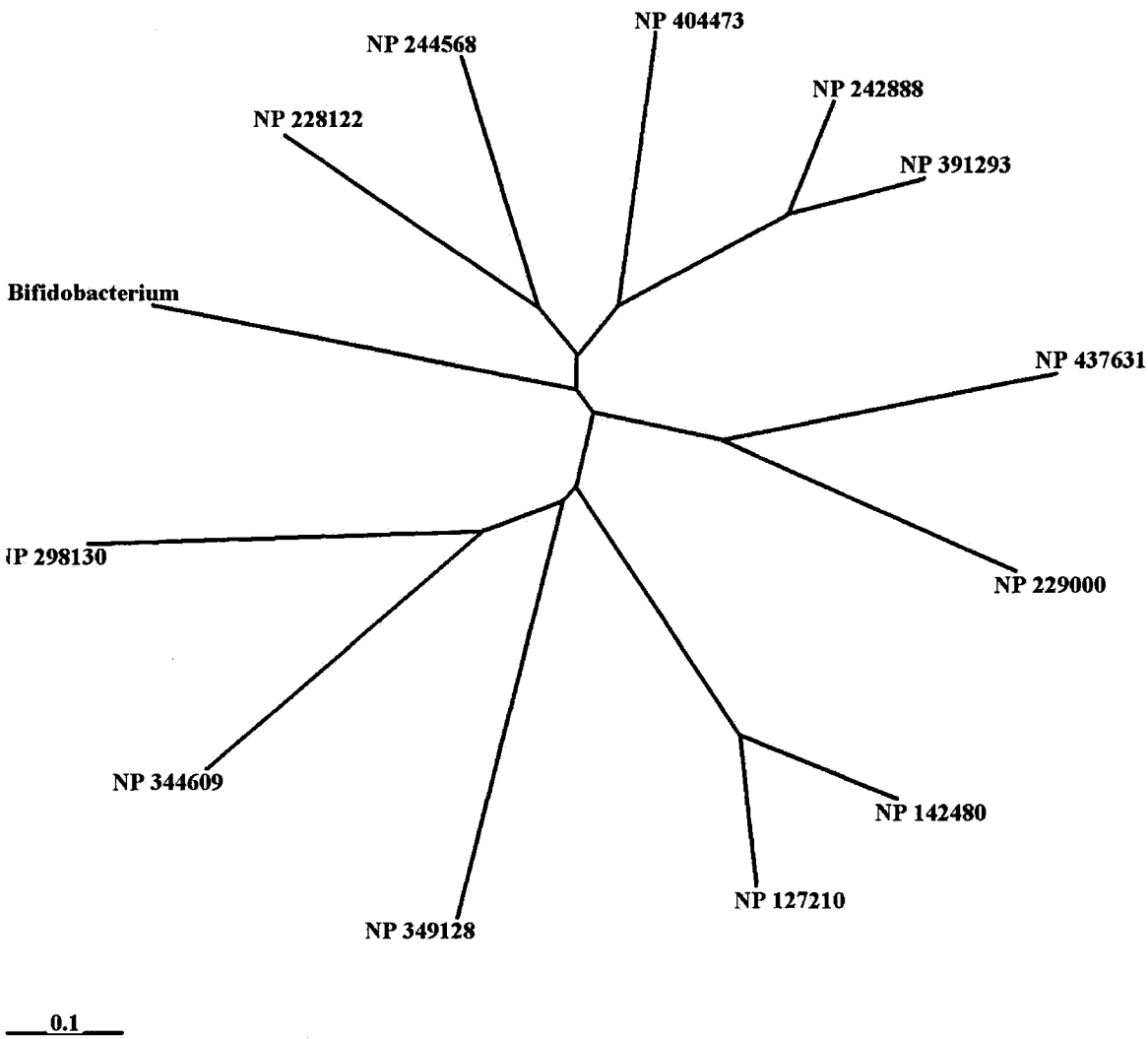

Fig. 2.9 Phylogenetic tree of *galA* of *Bifidobacterium breve* B24 with other β-galactosidases from *LacA* family β-galactosidases from various bacteria. Genebank assession numbers for the published strains are follows: Bifidobacterium, *Bifidobacterium breve* B24; NP_298130, *Xylella fastidiosa* 9a5c; NP_344609, *Streptococcus pneumoniae* TIGR4; NP_349128, *Clostridium acetobutylicum* ATCC 824; NP_127210, *Pyrococcus abyss*i GE5; NP_142480, *Pyrococcus horikoshii* OT3; NP_229000, *Thermotoga maritima* MSB8; NP_437631, *Sinorhizobium meliloti* 1021, NP_391293, *Bacillus subtilis* subsp; NP_242888, *Bacillus halodurans* C-125; NP_404473, *Yersinia pestis* CO92; NP_244568, *Bacillus halodurans* C-125 NP_228122, *Thermotoga maritima* MSB8.

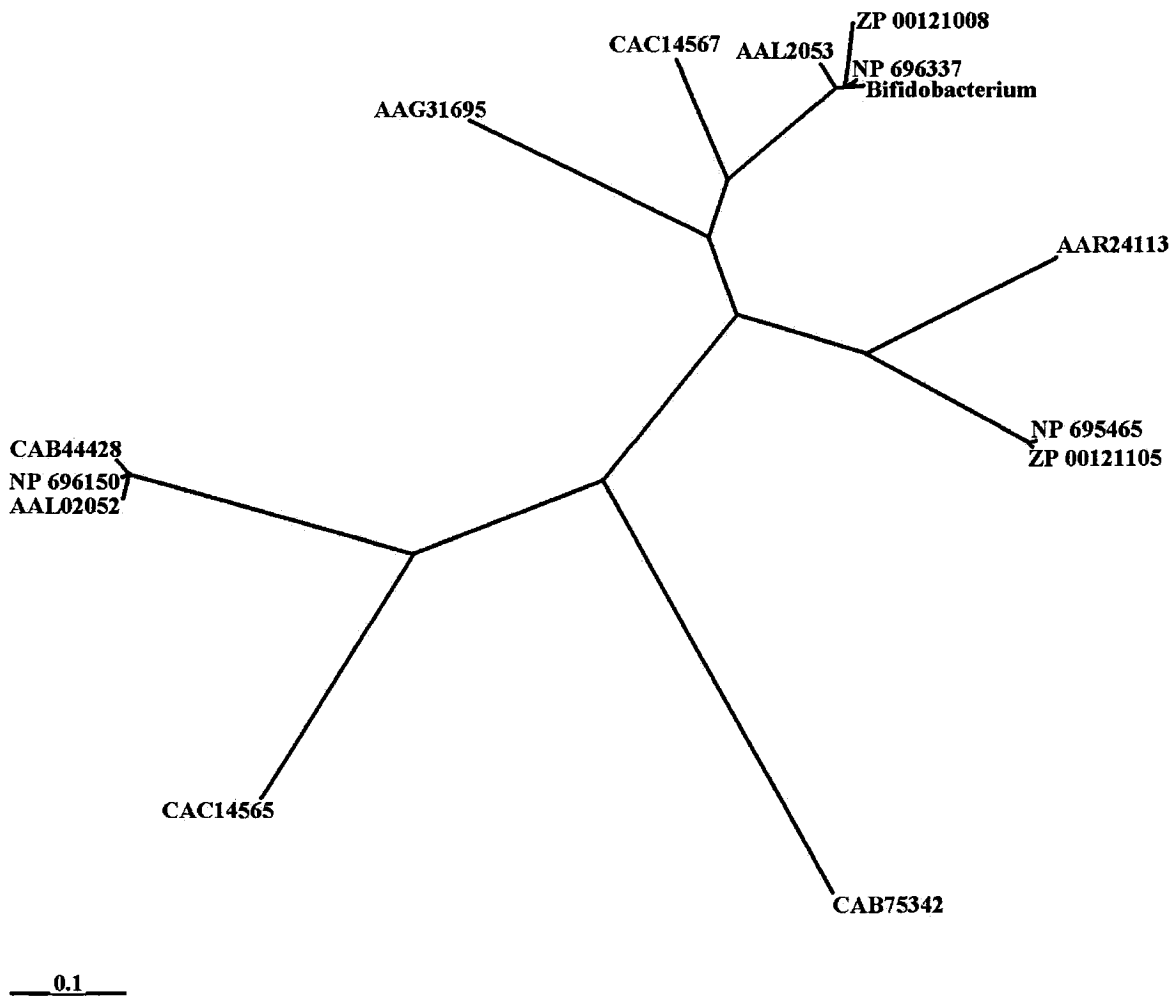

Fig. 2.10 Phylogenetic tree of *galA* of *Bifidobacterium breve* B24 with other β-galactosidases from bifidobacteria. Genebank assession numbers are indicated in the picture. Genebank assession numbers for the published strains are follows: Bifidobacterium, *Bifidobacterium breve* B24; NP_696337, *Bifidobacterium longum* NCC2705 (β-galI); ZP_00121008, *Bifidobacterium longum* DJO10A; AAL02053, *Bifidobacterium infantis* (β-galIII); CAC14567, *Bifidobacterium infantis*; AAG31695, *Bifidobacterium adolescentis*; CAB44428, *Bifidobacterium longum*; NP_696150, *Bifidobacterium longum* NCC2705 (*LacZ*); AAL02052, *Bifidobacterium infantis*; CAC14565, *Bifidobacterium bifidum*; CAB75342, *Bifidobacterium bifidum*; AAR24113, *Bifidobacterium adolescentis*.

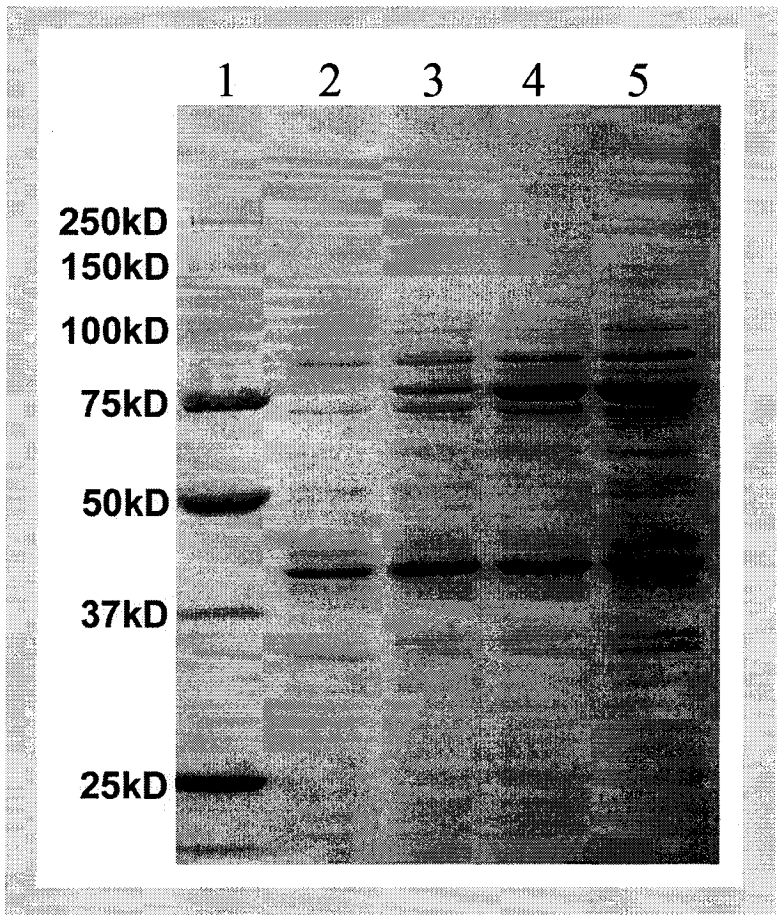

Fig. 2.11 SDS-PAGE analysis of the time course over-expression of β-galactosidase in *E. coli* ER2566. Lane 1, molecular size marker (size are indicated in picture); lane 2, no induction; lane 3 to 5, after 1, 2, and 3 h induction, respectively.

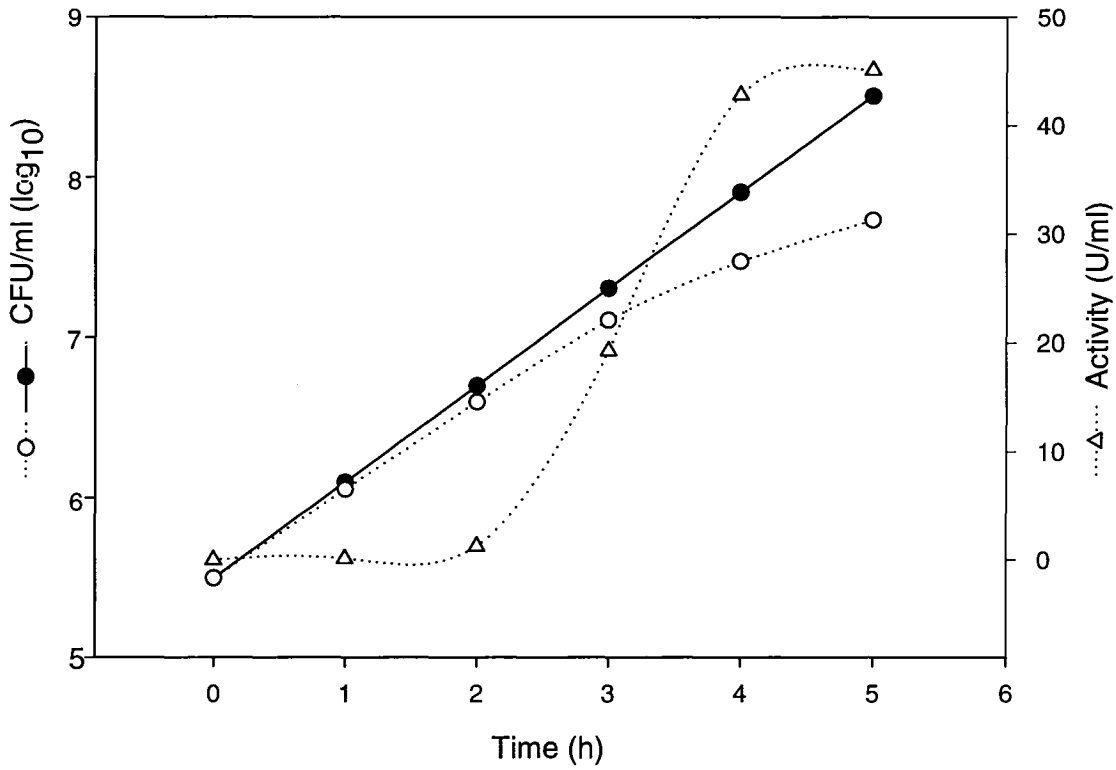

Fig. 2.12 Effect of IPTG on growth of *E. coli* ER2566 (○, with IPTG; ●, without IPTG) and the over-expressed β-galactosidase activity (△).

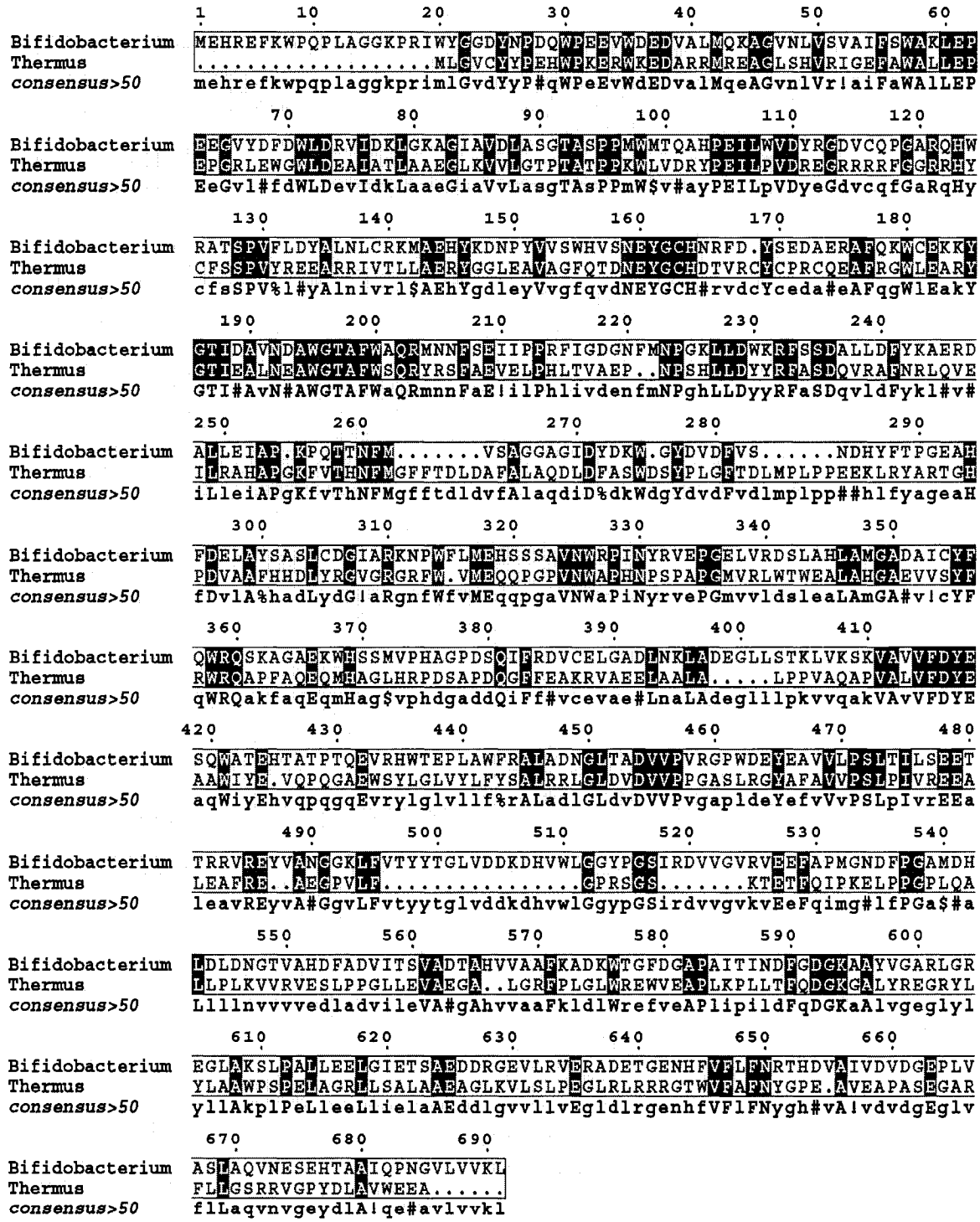

Fig. 2.13 Amino acid sequence comparison of *Bifidobacteriun breve* B24 and *Thermus thermophilus* A4. Each blocked regions are identically matched amino acids and 98% of 691 amino acids of *Bifidobacteriun breve* B24 was aligned.

Table 2.1 Classification of β-galactosidases on the basis of enzyme properties.

Family	Source	Genebank Accession #	Number of amino acids (identity; %)
Lac A	*Clostridium acetobutylicum* ATCC 824	NP_349128	982 (33)
	Bacillus halodurans C-125	NP_242888	689 (32)
	Bacillus halodurans C-125	NP_244568	672 (35)
	Bacillus subtilis subsp. subtilis str. 168	NP_391293	687 (31)
	Pyrococcus abyssi GE5	NP_127210	787 (21)
	Pyrococcus horikoshii OT3	NP_142480	778 (22)
	Sinorhizobium meliloti 1021	NP_437631	646 (29)
	Streptococcus pneumoniae TIGR4	NP_344609	595
	Thermotoga maritima MSB8	NP_228122	672 (34)
	Thermotoga maritima MSB8	NP_229000	649 (30)
	Xylella fastidiosa 9a5c	NP_298130	612
	Yersinia pestis CO92	NP_404473	686 (33)
LacZ	*Streptococcus pyogenes* M1 GAS	NP_269647	1168
	Bacillus halodurans	Q9K9C6	1014
	Escherichia coli O157:H7	NP_308424	1024
	Escherichia coli O157:H7	NP_311985	1042
	Lactococcus lactis subsp. Lactis	NP_268137	996
	Sinorhizobium meliloti 1021	NP_436544	755
	Sinorhizobium meliloti 1021	NP_386031	831
	Streptococcus pneumoniae TIGR4	NP_345155	2233
	Thermotoga maritima MSB8	NP_228998	1087
	Yersinia pestis CO92	NP_405234	1060
	Escherichia coli	P00722	1024
LacY	*Escherichia coli*	1PV6_A	417
	Escherichia coli	P16552	425
	Klebsiella oxytoca	P18817	416
	Escherichia coli	P30000	415
LacG	*Zea mays*	1HXJA	507
	Lactococcus lactis	1PBGA	468
	Sulfolobus acidocaldarius	P14288	491

CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF β-GALACTOSIDASES FROM *BIFIDOBACTERIUM BREVE* B24 AND *E. COLI* ER2566

β-galactosidases of *Bifidobacterium breve* B24 and recombinant *E. coli* ER2566 were purified to homogeneity for further characterization of activators, inhibitors, hydrolytic activity, and kinetics on ONPG.

The results of this study were summarized and submitted to the journal of *Applied Microbiology and Biotechnology*. The manuscript entitled "Purification and characterization of β-galactosidases from *Bifidobacterium breve* B24 and *E. coli* ER2566." was co-authored by Sung-Hun Yi, and Byong H. Lee. This project was supervised by Dr. Byong H. Lee and actual experimental work and writing of manuscript were done by Sung-Hun Yi. The manuscript was edited by Dr. Byong H. Lee prior to its submission for publication.

3.1 ABSTRACT

After a β-galactosidase gene of *Bifidobacterium breve* B24 was over-expressed in *E. coli* under the control of T7 promoter, β-galactosidases from *Bifidobacterium breve* B24 and *E. coli* ER2566 were purified to homogeneity by ion exchange (Mono-Q) and gel-filtration chromatography (Superose 12 and Superdex 200) columns to observe any change in their characteristics. The molecular mass of β-galactosidase was estimated to be 75 kDa on SDS-PAGE. Activity staining on non-denaturing Native-PAGE and Superose-12 gel-filtration chromatography showed that the enzymes are composed of a dimer with a molecular mass of 150 kDa. The optimum pHs of native and recombinant enzymes for hydrolyzing ONPG were pH 6 and 7.0, respectively and the enzymes stable were over the pH range of 5-8 and 6-9, respectively. The optimum temperatures of the both enzymes for hydrolyzing ONPG were similar at 45 ℃ and the enzymes were stable over the temperature range of 20-45 ℃. The both enzymes were stable up to 45℃ during 5 h of incubation at pH 6.5. The recombinant enzyme was slightly activated by bivalent metal ions, Mg^{2+}, Mn^{2+}, and Zn^{2+} at 1 mM but strongly inhibited by Hg^{2+} and PCMB. The K_m values of both native and recombinant β-galactosidases for ONPG were 2.77 and 1.82 mM, respectively and the V_{max} values were 1.02 and 1.39 mM/min, respectively.

3.2 INTRODUCTION

Many bifidobacteria-containing dairy and pharmaceutical products have been developed and consumed for several decades due to their promising health-promoting properties. A β-galactosidase (EC 3.2.1.23), commonly known as lactase is to be found in most of bifidobacteria strains and it hydrolyzes lactose to produce glucose and galactose. β-galactosidases are widely distributed in nature. The most extensive study has been with β-galactosidases from *E. coli* and its reaction mechanisms (Kuby and Lardy, 1953; Sinnot, 1978; Bader *et al.*, 1988; Ring *et al.*, 1988) and three dimensional structures (Jacobson *et al.*, 1994) were already reported. However, most industrial β-galactosidases are originated mainly from fungi and yeasts. These enzymatic hydrolysis activities overcome some disadvantages of lactose or lactose derivatives on low solubility, utilization, and sweetness of lactose compared with galactose and glucose.

Most bifidobacteria contain very high activity and multiple β-galactosidases (Roy *et al.*, 1994; Hung and Lee, 2002), which have been less studied than those of other microorganisms. Two β-galactosidase isozymes from *Bifidobacterium infantis* HL96 were cloned characterized, but the size was too big to be secreted (Hung and Lee, 1998). Thus, this study was to investigate a small size of β-galactosidase from *Bifidobacterium* species

with both high hydrolytic and transgalactosylation activities.

3.3 MATERIALS AND METHODS

3.3.1 Chemical and reagents

Unless otherwise specified, all chemicals used in this work were analytical reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO.). Cell culture media were purchased from Difco Laboratories (Detroit, MI).

3.3.2 Preparation of β-galactosidases

E. coli ER2566 cells containing a plasmid pBT2076NB were inoculated in 10 ml 2X YT medium (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl, 40 μg/ml kanamycin, pH 7.0) and incubated at 37 ℃ for 14 h. A portion (1 ml) of seed culture was inoculated in 100 ml 2X YT medium (40 μg/ml kanamycin) with shaking at 250 rpm until OD_{600} of 1.0 was reached. Final concentration of 1 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) was added into culture for further incubation for 5 h at 37 ℃. The over-expression level of the recombinant was calculated by gel scanning with BioRad Informatix software (Bio-Rad, Mississauga, ON)

To prepare a native β-galactosidase from *Bifidobacterium breve* B24, cells were inoculated in 500 ml of MRS broth (5% lactose) and incubated anaerobically for 24 h at 37 ℃.

Cells were centrifuged at 8,000 g for 20 min and washed twice with sodium phosphate buffer (50 mM, pH 7.0) and resuspended in 10 ml of the same buffer. Cells were disrupted by sonicator sonicator (550 Sonic Dismembrator, Fisher Scientific, Mississauga, ON) using a macrotip with the power level at 6 for 10 min for *E. coli* ER2566 and 20 min for *Bifidobacterium breve* B24 with 2 sec pulsing and 30 sec intervals under constant cooling. The disrupted cells were centrifuged (12,000 g, 20 min, 4 ℃) and the supernatants (cell free extracts) were used for purification.

3.3.3 Enzyme activity and protein assays

Enzyme activity was measured by a modified Craven procedure (Craven *et al.*, 1965). Briefly, enzyme was reacted with 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) in 50 mM sodium phosphate buffer (pH 7.0) at 45 ℃ for 5 min, and reaction was stopped by adding equal volume of 1.0 M Na_2CO_3. The released *o*-nitrophenyl was quantitatively determined by measuring the optical density at 420 nm. One unit of enzyme activity was defined as the amount of enzyme liberating one μmol of

o-nitrophenyl per minute. Specific activity was defined as units per mg of protein.

Protein concentrations were determined by the Bio-Rad protein assay reagent (Bio-Rad, Mississauga, ON) using bovine serum albumin (Sigma) as a standard.

3.3.4 Enzyme purification

3.3.4.1 Anion-exchange chromatography

The cell free extract (CFE) was directly applied to an anionic-exchange column (Mono Q HR 5/5, Amersham Pharmacia Biotech Inc.) using the FPLC system (Amersham Pharmacia Biotech Inc.) equilibrated with buffer A (50 mM bis-Tris propane buffer, pH 6.5). Elution was performed with a linear gradient of 1 M sodium chloride in buffer A at a flow rate of 0.5 ml/min, and 1 ml fractions were collected. After several runs of an Anion-exchange chromatography, fractions exhibiting enzyme activity were pooled for gel-filtration.

3.3.4.2 Superose 12 gel-filtration chromatography

The active fractions from anion-exchange chromatography were pooled, desalted, and further concentrated using the Ultrafree-15 centrifugal filter unit (30 MWCO, Millipore). The pooled active fraction was applied to a gel filtration column (Superose-12

HR 10/30, Amersham Pharmacia Biotech Inc.) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride. Elution was performed at a flow rate of 0.25 ml/min, and 1 ml fractions were collected. After several runs of chromatography, fractions exhibiting enzyme activity were pooled and stored at 4 ℃ for further analysis.

3.3.4.3 Superdex 200 gel-filtration chromatography

The active fractions from Superose-12 chromatography were pooled and further concentrated using the Ultrafree-15 centrifugal filter unit (30 MWCO, Millipore). The pooled active fraction was applied to second gel filtration column (Superdex 200 HR 10/30, Amersham Pharmacia Biotech Inc.) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride. Elution was performed at a flow rate of 0.25 ml/min, and 1 ml fractions were collected. After several runs of chromatography, fractions containing enzyme activity were pooled and stored at 4 ℃ for further SDS-PAGE analysis.

3.3.5 Gel electrophoresis and activity staining

3.3.5.1 SDS-PAGE analysis

To determine homogeneity and subunits of purified protein, the purity of the enzyme at each purification step was examined by SDS-PAGE by the method of Laemmli (1970) using 10 % gel. To estimate the molecular mass, a broad range of Precision Plus

Protein Standards (10 to 250 kDa) (Bio-Rad Laboratories) was applied to the same gel and the gel was stained with Coomassie Brilliant Blue R-250.

3.3.5.2 Native-PAGE analysis for activity staining

Samples were loaded in a nondenaturing 10% (w/v) acrylamide gel and electrophoresis was carried out following method of Laemmli (1970) without SDS. An agarose (0.2%, GibcoBRL Inc.) in 50 mM sodium phosphate buffer (pH 7.0) solution was boiled for 30 sec and cooled at 50 ℃ in water bath. Final concentration of 4 mg/ml of X-gal was added and mixed completely. After Native-PAGE electrophoresis was carried out, X-gal agarose solution was poured on PAGE gel and waited for solidification of agarose gel. The gel was incubated in 37 ℃ until blue bands were appeared.

3.3.6 Determination of native molecular mass of the purified β-galactosidase

The native molecular mass was determined by Superose 12 gel-filtration chromatography (Superose 12 HR 10/30) using combination of standard protein of LMW and HMW gel filtration calibration kits (catalase, 232 kDa; aldolase, 158 kDa; albumin, 67 kDa; ovalbumin, 43.0 kDa) purchased from Amersham Pharmacia Biotech Inc. The mobile phase used was 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM

sodium chloride at a flow rate of 0.25 ml/min.

3.3.7 Characterization of β-galactosidase

3.3.7.1 Effect of pH and temperature

Three buffer systems, citrate buffer (50 mM, pH 3- 6), sodium phosphate buffer (50 mM, pH 5-9), and sodium carbonate buffer (50 mM, pH 9-11) were used to determine the optimum pH for enzyme activity. ONPG (4 mg/ml) was dissolved in three different buffers and then used as substrate solution. To determine the pH stability, enzymes were pre-incubated in different pHs at 18 ℃ for 3 h and then substrate solutions were added and incubated for 10 min to determine the residual activity. The effect of temperature was measured by incubating the enzymes and substrates in 50 mM sodium phosphate buffer in the range of 10 to 65 ℃ for 10 min. The thermal stability was determined by incubating enzyme at the desired temperature, and the residual activity was measured at 45 ℃.

3.3.7.2 Effect of inhibitors and activators

Enzymes were incubated with 1, 10, and 100 mM of divalent metal ions and chemicals in 50 mM sodium phosphate buffer containing 4 mg/ml ONPG at 45 ℃ for 10 min. The enzyme activity without effectors was used as control.

3.3.7.3 Enzyme kinetics

The kinetic constants (K_m and V_{max}) were determined with ONPG concentrations ranging from 0.1 to 10 mM in sodium phosphate buffer (50 mM) at 45℃. The reaction was stopped by adding same volume of Na_2CO_3 (1 M). The Lineweaver-Burk plot was constructed by using a least-square, best-fit Michaelis-Menten equation (Lineweaver and Burk, 1934) and the kinetic constants were computed from the slope and intercept of the regression line.

3.4 RESULTS

3.4.1 Purification of β-galactosidases

To investigate the biochemical characteristics of both β-galactosidases of *Bifidobacterium breve* B24 and recombinant *E. coli* ER2566, a full length *galA* was over-expressed by T7 RNA polymerase expression system in *E. coli* ER2566. After 5 h induction, the over-expression level of β-galactosidase was about 30 % (BioRad Informatix software) out of total protein in *E. coli* ER2566. Both β-galactosidases were purified by FPLC system equipped with ion exchange (Mono Q) and gel-filtration (Superose 12 and Superdex 200) columns. Overall purification results are summarized in

Table 3.1. The specific activities of the purified native and recombinant enzymes were 1099.62 and 8073.60 units/mg, respectively. The native and recombinant enzymes were significantly purified, but the purifications were only 23.18 and 26.56 fold (Fig. 3.2). The homogeneity of both native and recombinant enzymes during purification steps are shown in Figs. 3.1 and 3.2.

3.4.2 Activity staining of β-galactosidases

For the determination of molecular mass of β-galactosidases, activity staining was performed on non-denaturing gel. As shown in Fig. 3.3, native and recombinant enzymes have the same molecular mass of 150 kDa, indicating that this recombinant enzyme was originated from a β-galactosidase gene of native strain. During the incubation for the activity staining, molecular marker proteins were diffused into agarose gel that was hard to identify their location.

3.4.3 Effect of pH and temperature on enzyme activity

While the optimum pH of native enzyme was pH 6.0, the recombinant enzyme was pH 7.0 (Fig. 3.4). Native enzyme was stable at pH 6 for longer than 3 h, whereas the recombinant enzyme was stable at pH 7 but with a half of its activity (Fig. 3.5). The

optimum temperatures of both enzymes were at 45 ℃ (Fig. 3.6). Both enzymes were stable at the temperature ranging between 10 - 45 ℃ up to 5 h or more. The activities of both enzymes were significantly decreased when temperature was higher than 45 ℃ within 1 h (Figs. 3.7 and 3.8).

3.4.4 Activators and inhibitors

As shown in Table 3. 2, in most cases, no major differences were found in both activities. However, at a final concentration of 1 mM of $MgCl_2$, $MnCl_2$, and $ZnCl_2$, the enzyme activity of the recombinant was significantly increased. The both enzyme activities were completely inhibited in the presence of $HgCl_2$ and *p*-chloromercuribenzoic acid (PCMB) at any concentration.

3.4.5 Enzyme kinetics

The kinetic constant (K_m) and maximum reaction velocity (V_{max}) of native and recombinant enzymes with ONPG were determined from Lineweaver Burk plot. The K_m values of wild and recombinant enzymes were 2.77 and 1.82 mM, respectively and the V_{max} values were 1.02 and 1.39 mM/min, respectively (Fig. 3.10).

3.5 DISCUSSION

In this study, β-galactosidases from *Bifidobacterium breve* B24 and the recombinant *E. coli* ER2566 were successfully purified. The recombinant β-galactosidase expressed in *E. coli* ER2566 appeared to have the same enzyme activity as that of *Bifidobacterium breve* B24. Many β-galactosidases were purified and characterized from *Bacillus megaterium* (Shaw *et al.*, 1998), *Bifidobacterium infantis* (Hung and Lee, 2002), *Kluyveromyces lactis* (Karen *et al.*, 1998), *Pyrococcus woesei* (Slawomir *et al.*, 2000), *Streptococcus pneumoniae* (Dorothea *et al.*, 2000), *Sterigmatomyces elviae* (Onishi *et al.*, 1995), *Sulfolobus solfataricus* (Moracci *et al.*, 1992) and *Thermus* sp. (Alejandro *et al.*, 1998; Benevides *et al.*, 2004). Although bifidobacteria are well studied and used in many dairy food industries, few β-galactosidases were purified and characterized from bifidobacteria and especially β-galactosidases from *Bifidobacterium breve* have not been studied.

The homogeneities of purified β-galactosidases from *Bifidobacterium breve* B24 and the recombinant β-galactosidase were examined by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) (Figs. 3.1 and 3.2). The molecular mass was determined by SDS-PAGE as well as Superose 12 gel-filtration chromatography (Fig. 3.9). Estimated

molecular mass of the purified β-galactosidase was around 75 kDa on SDS-PAGE and 148 kDa on gel-filtration chromatography. This value was very similar to the deduced molecular mass from amino acid sequence analysis. The purified β-galactosidase from *Bifidobacterium breve* B24 was relatively smaller than other β-galactosidases of *Thumus aquaticus* (>700 Kda; Berger *et al.*, 1996), *Kluyveromyces lactis* (114 kDa; Cavaille and Combes, 1995), *Streptococcus thermophilus* 11F (116 kDa; Smart and Richardson, 1987), *Aspergillus oryzae* (110 kDa; Ogushi *et al.*, 1980), *Sterigmatomyces elviae* CBS8119 (86 kDa; Onishi and Tanaka, 1995), *Lactococcus lactis* ssp. *lactis* ATCC7962 (115 kDa; Lee *et al.,* 1997). The other β-galactosidases reported are three β-galactosidases (620 kDa; hexamer, 236 kDa; dimer, 180 kDa; monomer) from *Bifidobacterium bifidum* DSM20215 (Peter *et al.*, 2001), one β-galactosidase (140 kDa, dimer) from *Bifidobacterium infantis* DSM20088 (Peter *et al.*, 2001) and two β-galactosidases from *Bifidobacterium infantis* HL96 (113 and 76, heterodimer) (Hung *et al.*, 2001).

Activity staining was performed to determine the native molecular mass (Fig. 3.3). A β-galactosidase from *Bifidobacterium breve* B24 and the recombinant β-galactosidase was located in the same location with 150 kDa, indicating that the recombinant β-galactosidase had similar biochemical characteristics to that of native enzyme.

Comparison of the molecular mass from SDS-PAGE and Superose 12 gel-filtration chromatography with activity staining on Native-PAGE indicated that the β-galactosidase was consisted of two 75 kDa subunits.

The optimum pHs for both enzymes were different by 1 pH unit. The optimum pHs of native enzyme and the recombinant enzyme were pH 6 and 7, respectively (Fig. 3.4). The structure of protein is very important to have the maximal enzyme activity and some changes such as subunits conformation and amino acid sequence can affect the enzyme activity. Differences in optimal pH between β-galactosidases produced by recombinant *E. coli* ER2566 and *Bifidobacterium breve* B24 may be caused by 1) posttranslational changes of the enzyme after cloning and/or 2) mutation during PCR amplification of *galA* gene that occurred to yield slightly different formation of protein. Similar observation in change of thermostability was reported by Slawomir *et al.* (1998).

However, surprisingly, the recombinant β-galactosidase showed significant activity at pH 9.0 (42 %), whereas the native enzyme was inactive at the same pH. The opposite result was observed at acidic pH region in that the native β-galactosidase showed enzyme activity at pH 5.0 (38 %), whereas the recombinant enzyme was almost inactive at the same pH and similar phenomenon was reported by Benevides *et al.* (2004). The native β-galactosidase was stable in the ranges of pH 5-7, while the recombinant β-

galactosidase was stable in range of pH 6-9 (Fig. 3.5).

The analysis of the optimum temperature on both enzymes revealed that both enzymes presented the maximal activity at 45 ℃ (Fig. 3.6). In both cases, the activity was rapidly decreased above 50 ℃ within 60 min. (Figs. 3.7 and 3.8), most likely due to thermal denaturation effect. The relationship between thermal stability and enzyme structure or substrate is closely linked (Moracci *et al.*, 1992; Claire and Gregory, 2001; Kintia and Fox, 1996). When the thermostable enzyme is cloned and expressed in mesophilic hosts, usually thermal stability is retained, indicating that thermostability is genetically encoded. It is possible that changing amino acid sequence, mutation, and other biochemical techniques can be applied to increase thermal stability. In both cases, more than 90 % of their activities were kept for 5 h below 45 ℃, and thus the thermal stability of recombinant enzyme was transferred from genetic information from *Bifidobacterium breve* B24.

No significant activators on the enzyme activity of both enzymes were found. However, the recombinant enzyme was significantly enhanced by addition of 1 mM of $MgCl_2$, $MnCl_2$, and $ZnCl_2$ with different rates. Both enzyme activities were inhibited in the presence of $HgCl_2$ and *p*-chloromercuribenzoic acid (PCMB) at any concentration (Table 3.2). This indicated the possible involvement of functional sulfhydryl group at or

near the active site.

Various methods to increase lactose hydrolysis have been reported. Ultrasound-enhanced lactose hydrolysis in milk by β-galactosidase and whole-cell was reported by Wang *et al.* (1996). A classical chemical mutagenesis protocol using ethyl methanesulfonate (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used to produce food grade β-galactosidase (Ibrahim and O'Sullivan, 2000).

While the calculated K_m and V_{max} values of the native enzyme were 2.77 mM and 1.02 mM/min, those of the recombinant one were 1.82 mM and 1.4 mM/min, respectively.

After studying biochemical properties of β-galactosidase from *Bifidobacterium breve* B24, we found some interesting properties such as smaller size, high activity, thermal stability, and neutral pH. This enzyme can easily be over-expressed and prepared in gram scale per liter with relatively high purity. Further studies on the over-expression in a yeast system will be useful to investigate the possibility of scale up and industrial application.

Table 3.1 Summary of the purification steps of β-galactosidases.

Source of enzyme	Purification	Total Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Yield (%)
Native B24	CFE [a]	228.12	10,820	47.43	1	100
	Mono Q [b]	31.65	9,012	284.74	6.00	83.29
	S 12 [c]	6.56	5,696	868.29	18.30	52.64
	S 200 [d]	2.64	2,903	1,099.62	23.18	26.83
Recombinant *E. coli*	CFE	148.52	45,134	303.89	1	100
	Mono Q	18.76	31,239	1665.2	5.47	69.21
	S 12	3.17	21,223	6,694.94	22.03	47.02
	S 200	1.25	10,092	8,073.60	26.56	22.36

a) Cell free extract
b) Anion-exchange column
c) Superose 12 gel-filtration column
d) Superdex 200 gel-filtration column

Table 3.2 Inhibitors and activators on β-galactosidase activity using ONPG as substrate.

Chemicals	Relative activity (%)[a]						
	Native β-galactosidase				Recombinant β-galactosidase		
	Concentration (mM)				Concentration (mM)		
	0[b]	1	10	100	1	10	100
$CaCl_2$	100	101.47 ± 3.96	92.50 ± 3.35	35.17 ± 1.31	72.57 ± 2.17	69.87 ± 1.78	0.09 ± 0.01
$CoCO_3$	100	100.46 ± 3.35	93.91 ± 1.97	86.08 ± 2.04	122.87 ± 5.92	108.66 ± 3.32	103.97 ± 3.23
$CrCl_3$	100	112.84 ± 4.51	101.60 ± 4.42	9.68 ± 0.10	97.01 ± 2.97	46.14 ± 2.07	0.09 ± 0.01
$CuSO_4$	100	21.52 ± 0.72	16.10 ± 0.41	0.04 ± 0.00	31.36 ± 1.09	0.52 ± 0.03	0.09 ± 0.00
$Fe_2(SO_4)_3$	100	117.12 ± 3.33	85.28 ± 2.08	11.42 ± 0.05	86.35 ± 2.84	20.42 ± 0.13	0.09 ± 0.01
$HgCl_2$	100	0.04 ± 0.01	0.046 ± 0.01	0.04 ± 0.01	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.01
KCl	100	15.56 ± 0.56	87.68 ± 1.02	51.96 ± 0.86	108.81 ± 1.73	100.42 ± 1.51	92.04 ± 1.45
LiCl	100	71.49 ± 2.19	100.00 ± 1.02	74.57 ± 2.74	119.61 ± 1.52	104.12 ± 1.62	90.90 ± 1.52
$MgCl_2$	100	14.29 ± 0.26	111.17 ± 2.04	101.27 ± 3.12	148.31 ± 2.32	135.38 ± 2.30	132.11 ± 2.20
$MnCl_2$	100	29.35 ± 1.11	115.78 ± 3.43	11.88 ± 0.10	155.42 ± 3.55	141.92 ± 3.48	0.09 ± 0.00
NaCl	100	69.89 ± 1.95	94.84 ± 2.27	89.83 ± 4.06	106.82 ± 3.02	94.74 ± 2.78	88.06 ± 2.31
$NiCl_2$	100	18.44 ± 0.12	63.40 ± 0.48	22.92 ± 1.19	112.22 ± 1.23	92.46 ± 0.84	40.74 ± 0.52
$ZnCl_2$	100	115.98 ± 4.24	114.38 ± 3.39	0.04 ± 0.00	153.14 ± 6.07	131.97 ± 3.67	0.09 ± 0.00
EDTA	100	68.25 ± 1.56	67.25 ± 2.01	0.00 ± 0	75.89 ± 1.86	73.56 ± 2.04	70.76 ± 1.95
Urea	100	66.55 ± 1.92	61.20 ± 1.19	55.26 ± 1.05	76.03 ± 2.31	70.25 ± 2.20	67.70 ± 1.97
Cys-HCl	100	59.66 ± 1.67	0.00 ± 0	0.00 ± 0	69.20 ± 1.70	0.00 ± 0	0.00 ± 0
PCMB	100	0.00 ± 0	0.00 ± 0	0.00 ± 0	0.00 ± 0	0.00 ± 0	0.00 ± 0
Me-Ethanol	100	70.20 ± 2.15	71.25 ± 3.52	73.22 ± 3.65	74.81 ± 3.87	78.25 ± 3.91	81.32 ± 3.98

a) Activity was measured in triplicates.

b) The activity without chemicals was used as a control.

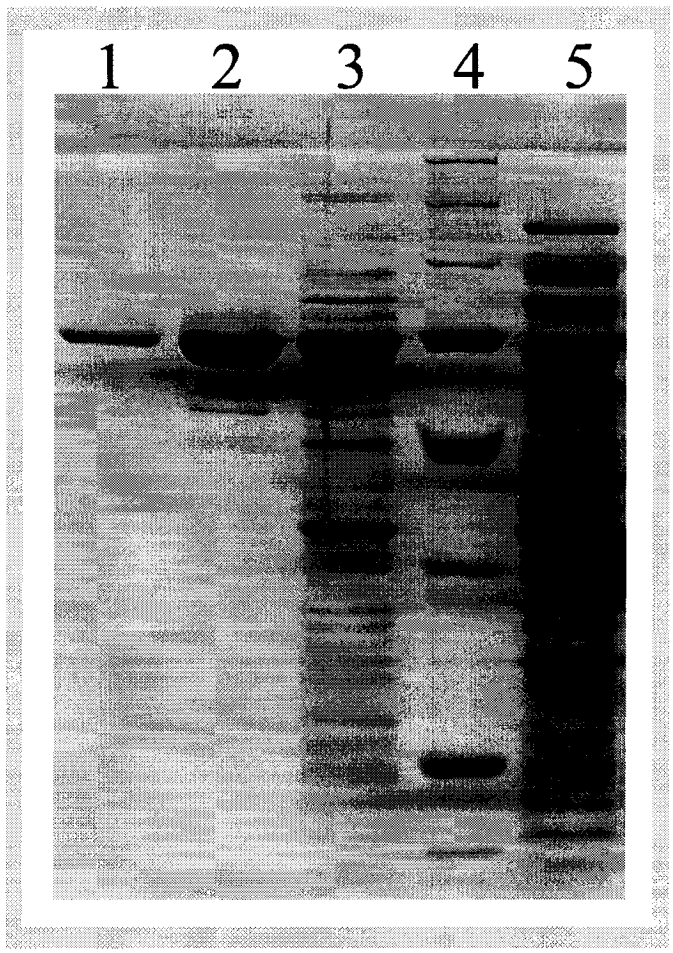

Fig. 3.1 SDS-PAGE analysis on the fractions from *B. breve* B24 during purification steps. Lane 1, purified β-galactosidase after Superdex 200 gel-filtration; lane 2, active fraction after Superpose12 gel-filtration; lane 3, active fraction after Mono Q; lane 4, molecular size markers (from top; 250, 150, 100, 75, 50, 37, 25, and 20 kDa); lane 5, *Bifidobacterium breve* B24 cell free extract.

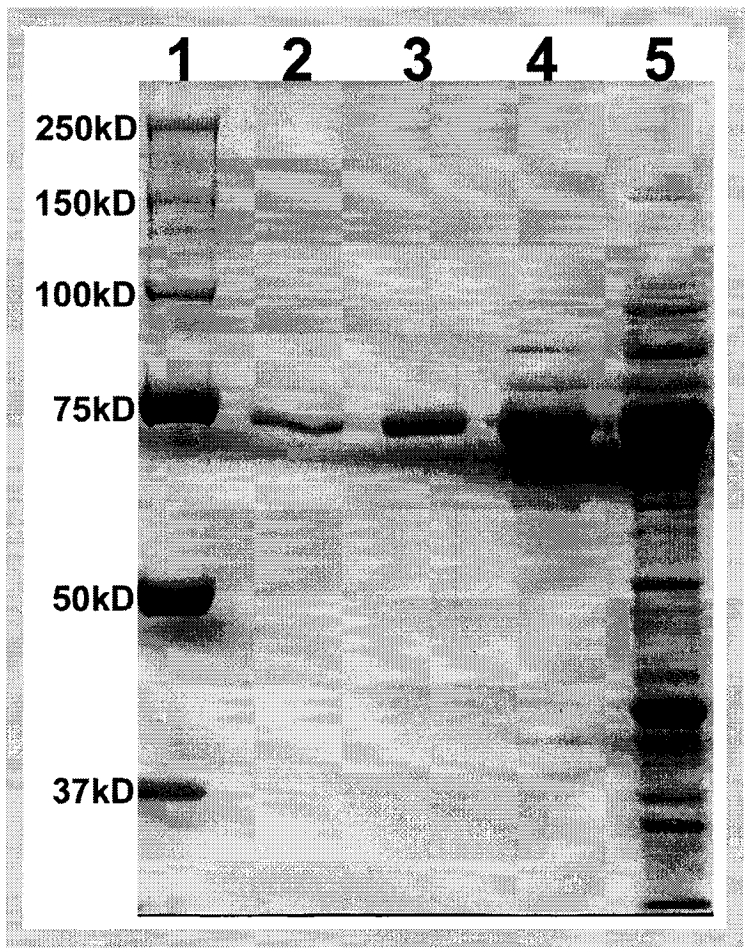

Fig. 3.2 SDS-PAGE analysis on the fractions from the recombinant *E. coli* ER2566 during purification steps. Lane 1, molecular size markers (sizes are indicated on the picture); lane 2, purified β-galactosidase after Superdex 200 gel-filtration; lane 3, active fraction after Superose 12 gel-filtration; lane 4, active fraction after Mono Q; lane 5, over-expressed recombinant *E. coli* ER2566 cell free extract.

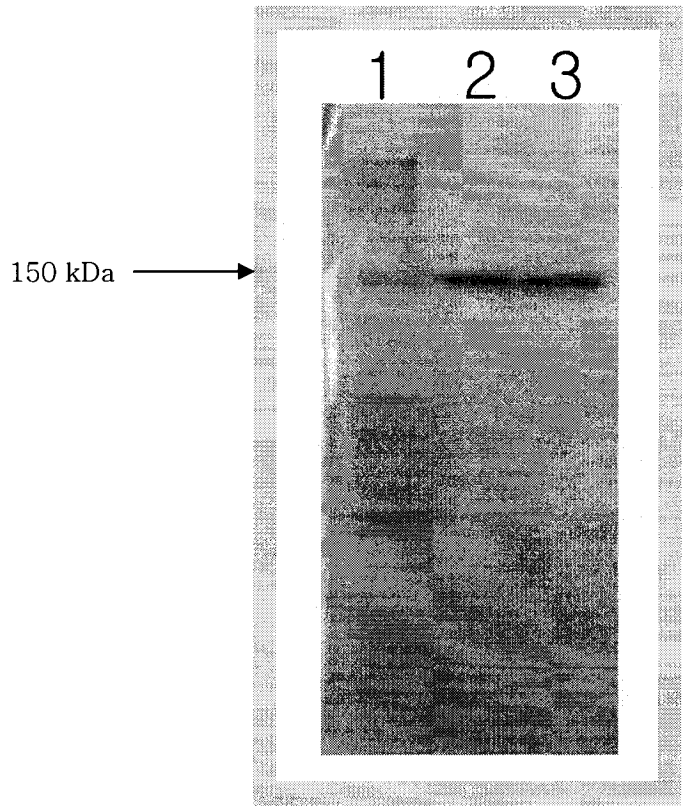

Fig. 3.3 Activity staining of β-galactosidase on non-denaturing polyacrylamide gel. Lane 1, molecular size marker; lane 2, purified β-galactosidase from *E. coli* ER2566; lane 3, purified β-galactosidase from *Bifidobacterium breve* B24.

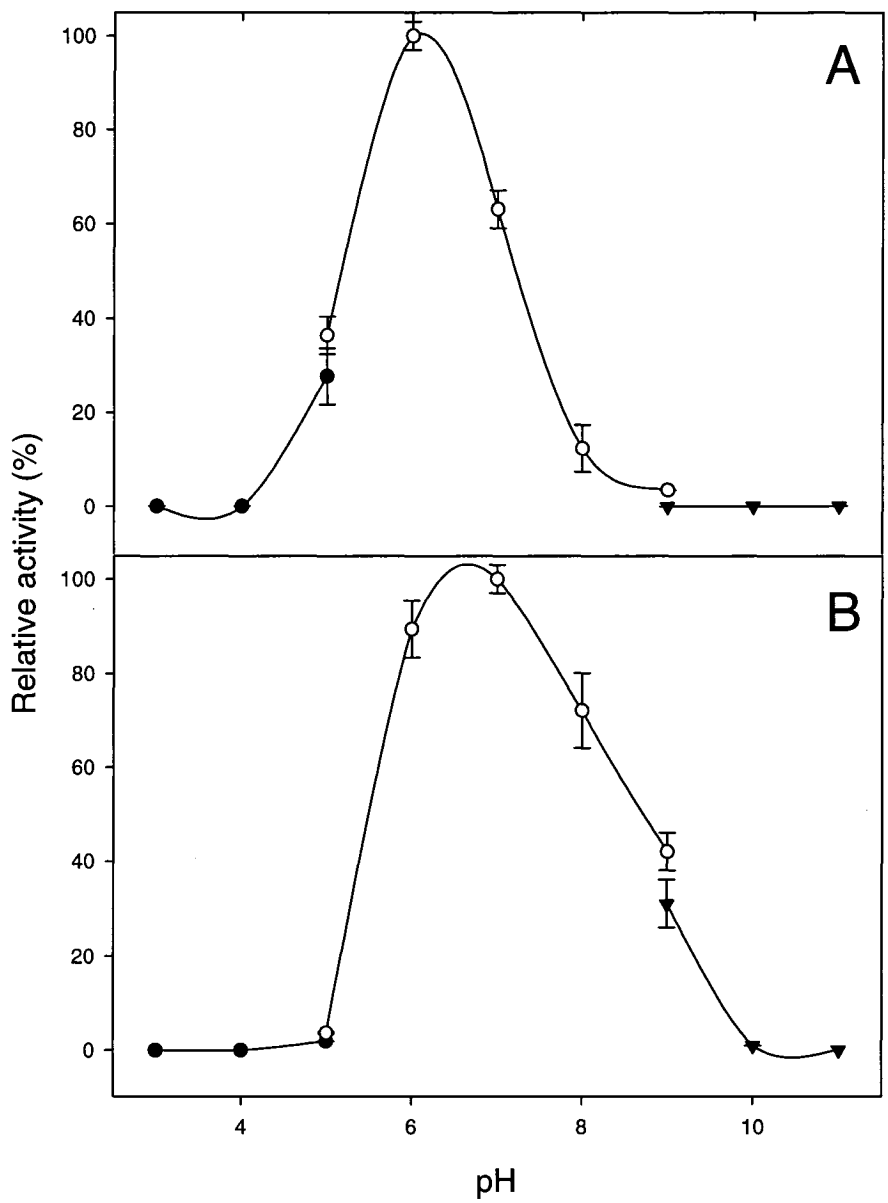

Fig. 3.4 Optimum pHs of β-galactosidases from *Bifidobacterium breve* B24 (A) and *E. coli* ER2566 (B) using ONPG. The buffers were citrate (●, 50 mM, pH 3-6), sodium phosphate (○, 50 mM, pH 5-9), and sodium carbonate (▼, 50 mM, pH 9-11). The results were means of triplicates ($P<0.05$).

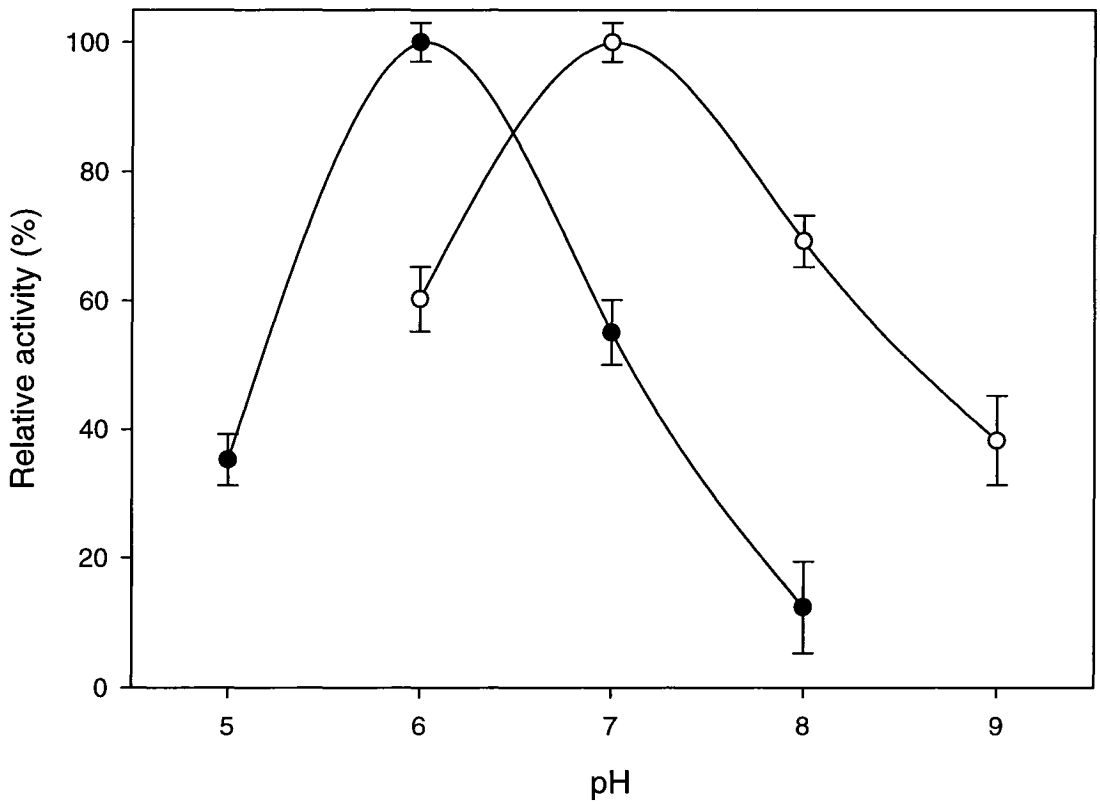

Fig. 3.5 pH stability of β-galactosidases from *Bifidobacterium breve* B24 (●) and *E. coli* ER2566(○) using ONPG. The buffer was sodium phosphate (50 mM, pH 5 - 9). The results were means of triplicates ($P<0.05$).

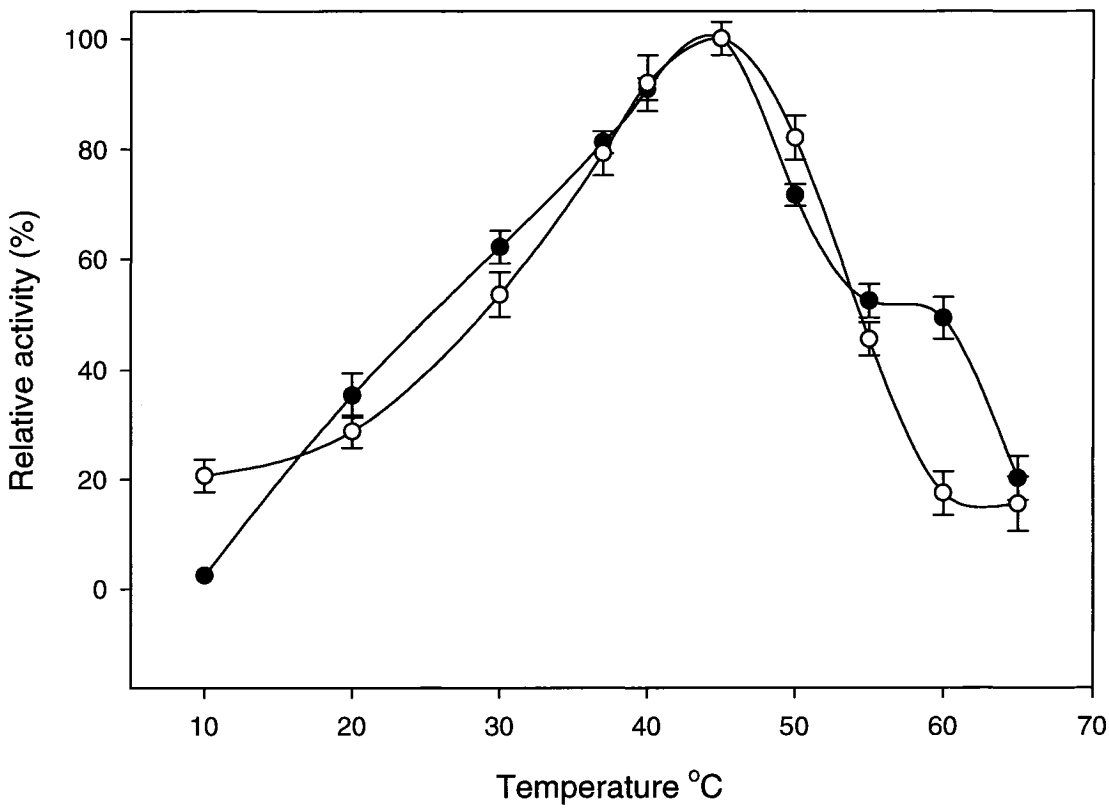

Fig. 3.6 Optimum temperature of β-galactosidases from *Bifidobacterium breve* B24 (●) and *E. coli* ER2566 (○) using ONPG. The buffers were 50 mM sodium phosphate (pH 6) for native enzyme and 50 mM sodium phosphate (pH 7) for the recombinant enzyme. The results were means of triplicates ($P<0.05$).

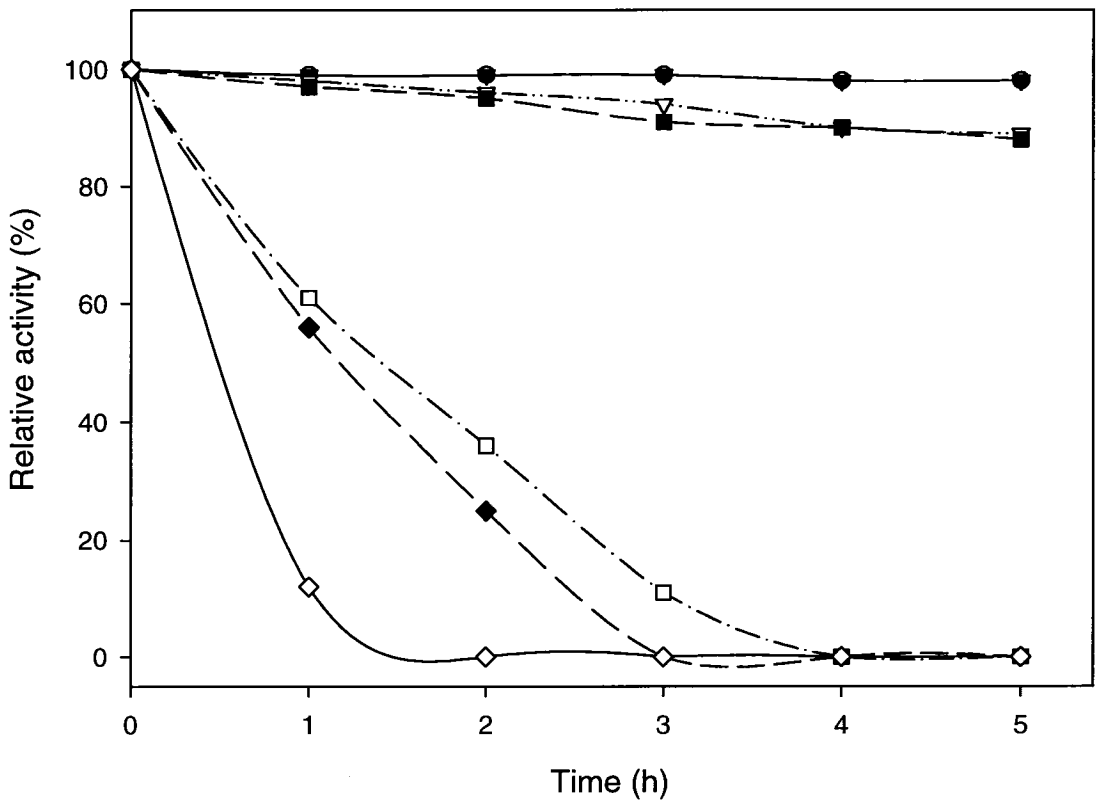

Fig. 3.7 Thermal stability of β-galactosidase from *Bifidobacterium breve* B24 using ONPG at different temperatures of 20℃ (●), 30℃ (○), 37℃ (▼), 40℃ (▽), 45℃ (■), 50℃ (□), 55℃ (◆), 60℃ (◇). The buffer was 50 mM sodium phosphate (pH 6). The results were means of triplicates ($P<0.05$).

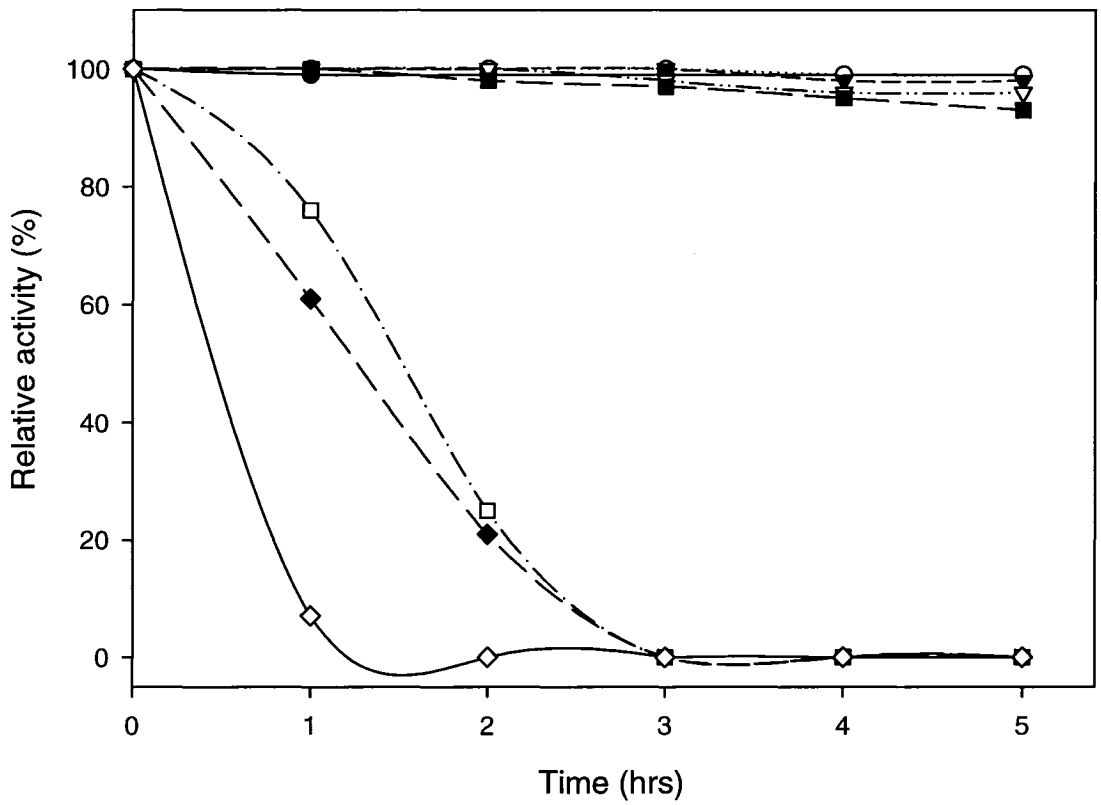

Fig. 3.8 Thermal stability of β-galactosidase from *E. coli* ER2566 using ONPG at different temperatures of 20℃ (●), 30℃ (○), 37℃ (▼), 40℃ (▽), 45℃ (■), 50℃ (□), 55℃ (◆), 60℃ (◇). The buffer was 50 mM sodium phosphate (pH 7). The results were means of triplicates ($P<0.05$).

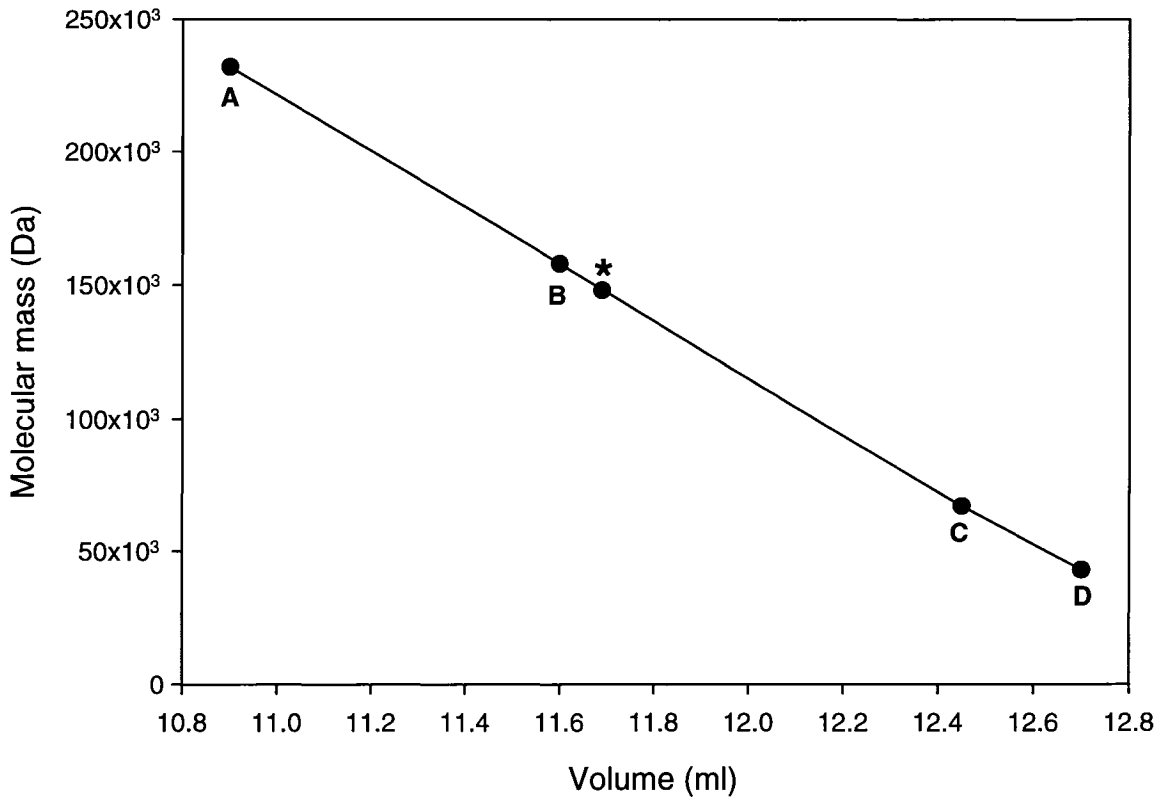

Fig 3.9 Determination of molecular mass of β-galactosidase by gel filtration. A, catalase (232 kDa); B, aldolase (158 kDa); C, albumin (67 kDa); D, ovalbumin (43.0 kDa); *, purified native and recombinant β-galactosidases (75.6 kDa).

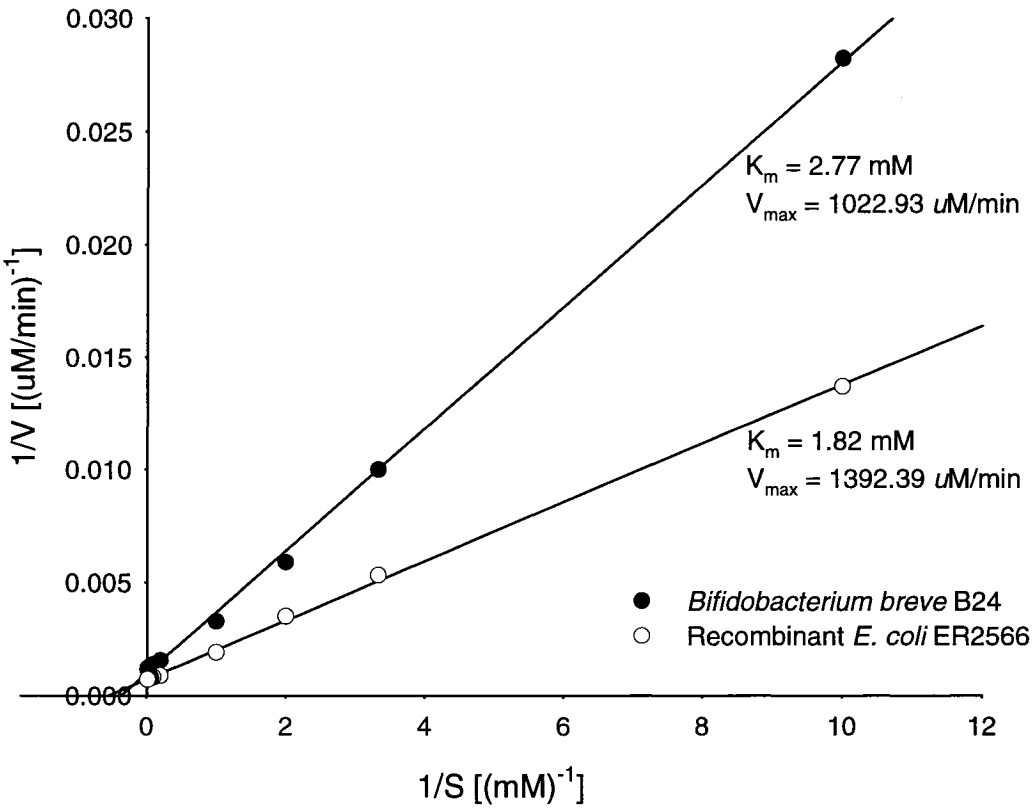

Fig. 3.10 Lineweaver-Burk plot of the β-galactosidases activity using ONPG.

CHAPTER 4

HYDROLYTIC AND TRANSGALACTOSYL ACTIVITIES BY β-GALACTOSIDASES FROM *BIFIDOBACTERIUM BREVE* B24 AND RECOMBINANT *E. COLI* ER2566

Both β-galactosidases from *Bifidobacterium breve* B24 and recombinant *E. coli* ER2566 were tested on the hydrolytic and transgalactosylation activity using lactose and milk as substrates. The formation mechanism of galacto-oligosaccharides using this enzyme was proven with the production of oligosaccharides in the presence of glucose and galactose alone without lactose.

This project was supervised by Dr. Byong H. Lee and actual experimental work and writing of manuscript were done by Sung-Hun Yi. The manuscript was edited by Dr. Byong H. Lee and this publication will be submitted to *Applied Microbiology and Biotechnology.*

4.1 ABSTRACT

Two β-galactosidases from *Bifidobacterium breve* B24 and recombinant *E. coli* ER2566 were extracted and purified to study their characteristics on lactose as substrate.

The optimum pHs of the native and the recombinant enzyme for hydrolyzing lactose were similar at pH 6. Both enzymes retained more than 80 % of their activity in the ranges of pH 6-8. However, β-galactosidase from *Bifidobacterium breve* B24 had about 40% of its activity on lactose at pH 5.0, whereas the recombinant enzyme showed no activity at this pH. On the other hand, the recombinant enzyme had over 50 % of its activity at pH 9, while native enzyme had lower than 5 % of its activity. The optimum temperatures of both enzymes were at 45℃ and both enzymes showed very similar profiles except at 10 ℃. The recombinant β-galactosidase showed 20 % of its activity at 10 ℃, while no enzyme activity was found from of β-galactosidase from *Bifidobacterium breve* B24 at 10 ℃.

No strong activators on hydrolyzing lactose were observed but 1 mM of $HgCl_2$ or 1 mM of *p*-chloromercuribenzoic acid (PCMB) inhibited hydrolytic activity of the recombinant one completely.

About 47.5% of 1 M lactose was hydrolyzed in 5 h at 45 ℃, whereas 100% of

100 mM lactose was completely hydrolyzed with 50 units of enzyme at the same conditions. No significant increase in hydrolytic activity was observed when lactose concentration was higher than 600 mM. In the presence of glucose and galactose in the reaction mixture, the recombinant β-galactosidase could synthesize oligosaccharides. The yield of oligosaccharides was significantly increased by increasing glucose and galactose concentration, indicating that the recombinant β-galactosidase was able to synthesize oligosaccharides without hydrolytic activity. At the concentration of 1 M lactose, 41.8 % of oligosaccharide was synthesized from lactose hydrolysates.

Lactose (4.8 %) in skim milk was almost completely hydrolyzed with 50 units of enzyme at 45 ℃ for 5 h. About 97.4 % of lactose (41.22 μg/μl) in milk was hydrolyzed to produce 46.3 % of glucose, 46.6 % of galactose, and 7.1 % of oligosaccharides. After 24 h reaction, 3.04 μg/μl, 5.75 μg/μl, 7.28 μg/μl, and 1.95 μg/μl of oligosaccharides were synthesized at 20, 37, 45, and 50 ℃, respectively. Lactose was completely hydrolyzed after 14, 12, and 6 h at 20, 37, 45 ℃, respectively. However, at 50 ℃, lactose was still present and also oligosaccharides were not increased after 6 h due to the inactivation at 50 ℃.

The K_m values of the native and recombinant enzymes on lactose were 152.08 and 95.58 mM, respectively that were 54.9 and 52.51 times higher than ONPG. The

maximum reaction velocity (V_{max}) values on lactose by the native and recombinant enzymes were 138.95 and 189.13 mM/min, respectively.

4.2 INTRODUCTION

Lactose utilization is limited in food products because of its low solubility, sweetness, and lactose intolerance. Lactose hydrolysates have more nutritional and technological values than lactose itself. The resulting sugars are sweeter, easily fermentable and thus directly absorbed from the intestine. These phenomena led to the development of low-lactose or lactose-free milk and the production of sweeteners from hydrolyzed lactose whey. The hydrolytic activity of β-galactosidase has been studied extensively over the last several decades and used widely for production of lactose-free milk. However, a β-galactosidase has not only hydrolytic activity, but also has synthetic activity (transferase) and this synthetic activity is so called transgalactosylaction. The transferase activity of β-galactosidase that produces oligosaccharides containing galactose during lactose hydrolysis was reported in the early 1950s (Aronson, 1952; Pazur, 1953). Early research was mainly focused on nutritional concerns about the presence of oligosaccharide in low lactose milk (Burvall *et al.*, 1979). Recently, interest

in the reaction of β-galactosidase has been focused on the formation of galacto-oligosaccharides from lactose. When galacto-oligosaccharides are taken orally, these promote the growth of *Bifidobacterium* species in the large intestine. Therefore, galacto-oligosaccharides are helpful for the maintenance of human health. Oligosaccharides are found as major components of many natural products such as plant cells or milk in either free or bind form, and many oligosaccharides are broken down in the upper intestine by hydrolytic enzymes. However, some oligosaccharides due to their chemical structure are resistant to digestive enzymes and pass into the large intestine. These non-digestible oligosaccharides can reach the colon and are preferentially utilized by the *Bifidobacterium* genus as carbon and energy sources, producing short chain fatty acids (SCFAs) that stimulate the bowel function and suppress the production of putrefactive substances (Rastall and Maitin, 2002). Due to the selectivity of these growth substrates and the health-promoting properties of bifidobacteria, considerable attention is now being focused on the use of these compounds as bifidogenic factors or prebiotics (Alander *et al.*, 2001). Among several classes of oligosaccharides, GOSs have attracted particular attention because they are present in human breast milk that enhances the growth of bifidobacteria in the GI tract of newly born, breast-fed infants (Gopal *et al.*, 2001). The transferase activity can also be used to attach galactose to other chemicals and consequently has potential application

in the production of food ingredients, pharmaceuticals and other biologically active compounds (Raymond, 1998). The production of galacto-oligosaccharides using various microbial β-galactosidases has been reported (Onishi and Tanaka, 1995; Berger *et al.*, 1996; Crittenden and Playne, 1996; Iwasaki *et al.*, 1996; Raymond, 1998; Lamoureux *et al.*, 2002; Hung and Lee, 2002). Oligosaccharides are also used as food ingredients to improve the quality of many foods. As food ingredients, they provide useful modifications like flavor, physical properties and humectants. Major uses are in beverages, infant milk powders, confectionary, bakery products, yogurts, and dairy desserts (Crittenden *et al.*, 1996).

Generally, the hydrolysis of lactose occurs at low lactose concentrations, but oligosaccharide production by the transgalactosylation activity increases with increasing concentration of lactose. These reactions occur simultaneously which makes the reaction mechanism very complicated.

4.3 MATERIALS AND METHODS

4.3.1 Chemical and reagents

Unless otherwise specified, all chemicals used in this work were analytical reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO.). The culture media were purchased from Difco Laboratories (Detroit, MI).

4.3.2 Preparation of β-galactosidases

E. coli ER2566 cells containing a recombinant plasmid pBT2076NB were inoculated in 10 ml 2X YT medium (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl, 40 μg/ml kanamycin, pH 7.0) and incubated at 37 ℃ for 14 h. A portion (1 ml) of seed culture was inoculated in 100 ml 2X YT medium (40 μg/ml kanamycin) with shaking at 250 rpm until OD_{600} of 1.0 was reached. Final concentration of 1 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) was added into culture for further incubation for 5 h at 37 ℃.

To prepare the native β-galactosidase from *Bifidobacterium breve* B24, cells were inoculated in 500 ml of MRS broth containing 5% lactose and incubated anaerobically for 24 h at 37 ℃.

Cells were centrifuged at 8,000 g for 20 min and washed twice with sodium phosphate buffer (50 mM, pH 7.0) and resuspended in 10 ml of the same buffer. Cells were disrupted by sonicator (550 Sonic Dismembrator, Fisher Scientific, Mississauga, ON) using a macrotip with the power level at 6 for 10 min for *E. coli* ER2566 and 20 min for *Bifidobacterium breve* B24 with 2 sec pulsing and 30 sec intervals under constant cooling. The disrupted cells were centrifuged (12,000 g, 20 min, 4 ℃) and the supernatants (cell free extracts) were used for purification.

4.3.3 Enzyme activity and protein assays

The enzyme activity measured by ONPG (*o*-nitrophenyl-β-D-galactopyranoside; Sigma) was used for comparison purpose to lactose hydrolysis. Enzyme activity was measured by a modified method of Craven *et al.* (1965). Briefly, enzyme was reacted with 4 mg/ml ONPG in 50 mM sodium phosphate buffer (pH 7.0) at 45 ℃ for 5 min, and the reaction was stopped by adding equal volume of 1.0 M Na_2CO_3. The released *o*-nitrophenyl was quantitatively determined by measuring the optical density at 420 nm. Enzyme activity on lactose was measured by HPLC analysis. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing one μmol of lactose per min under the specific condition.

Protein concentrations were determined by the Bio-Rad protein assay reagent (Bio-Rad, Mississauga, ON) using bovine serum albumin (Sigma) as a standard.

4.3.4 Sugar analysis by HPLC

The HPLC analyses were carried out in a Waters High Performance Liquid Chromatography (HPLC) (Millipore), equipped with a 600E system controller, a differential refractometer R401, a temperature control module, an autosampler (Waters 600 satellite WISP), a Millenium 2010 chromatography manager. Chromatographic separation of sugars was achieved with a cation-exchange column ION-300 (300 mm x 7.8 mm, Transgenomic Inc. CA, USA). The flow rate was adjusted to 0.25 ml/min with 0.02 N H_2SO_4 as mobile phase and column temperature was maintained at 55 ℃.

Running time was set to be 18 min. Peaks were identified by comparing retention times with sugar standards. The sugars were eluted with the sequence of oligosaccharides, lactose, glucose, and galactose. The respective peak areas were used for the quantitative analysis.

All data from HPLC chromatogram was converted to the contents of sugar using standard curve (Fig. 4.2).

4.3.5 Effect of pH and temperature

Three buffer systems, citrate (50 mM, pH 3-6), sodium phosphate (50 mM, pH 5-9), and sodium carbonate (50 mM, pH 9-11) were used to determine the optimum pH for enzyme activity. Lactose (100 mM) was dissolved in three different buffers and used as substrate solution to determine the optimum pH at 45 ℃ for 5 h.

The optimum temperature was measured by incubating the enzymes and substrates in 50 mM sodium phosphate buffer (pH 6.0) in the ranges from 10 to 65 ℃ for 5 h.

4.3.6 Effect of inhibitors and activators

The recombinant enzyme was incubated with 1 and 100 mM of divalent metal ions and chemicals in 50 mM sodium phosphate buffer (pH 6.0) containing 100 mM lactose at 45 ℃ for 5 h. The enzyme activity without effectors was used as control.

Due to similar characteristics of both native and recombinant enzymes except for optimum pH, the native enzyme was omitted in further experiments except for the study of enzyme kinetics.

4.3.7 Optimization of lactose hydrolysis and transgalactosylation

Various concentrations (100-1000 mM) of lactose dissolved in 50 mM sodium phosphate buffer (pH 6.0) were used as substrate solutions. The assay was carried out by adding enzyme (50 units) to lactose (100-1000 mM) in 50 mM sodium phosphate buffer (pH 6.0), and the reactions were performed at 45 ℃ for 5 h.

To determine the transgalactosylation activity without hydrolysis the enzyme was incubated with glucose and galactose mixture (10 to 500 mM). Glucose (1 M) and galactose (1 M) were separately dissolved in 50 mM sodium phosphate buffer (pH 6.0) and same volumes of each solution were mixed to make final concentration of 500 mM solution. By serial dilutions with 50 mM sodium phosphate buffer (pH 6.0), 10-500 mM glucose and galactose mixtures were prepared and used for analyzing transgalactosylation activity at 45 ℃ for 5 h.

After all the reactions, samples were heated to the remove residual enzyme activity, centrifuged (13,000 g; 30 min), and filtered with a 0.45 um filter (Millipore, Bedford, MA).

4.3.8 Enzyme kinetics

The K_m and V_{max} values were determined with different lactose concentrations

(1-100 mM) in 50 mM sodium phosphate buffer (pH 6.0) at 45℃. After the reactions, samples were heated immediately in boiling water for 1 min, centrifuged (13,000 g; 30 min), and filtered with a 0.45 um filter (Millipore). The Lineweaver-Burk plot was constructed by using a least-square, best-fit Michaelis-Menten equation (Lineweaver and Burk, 1934) and the kinetic constants were computed from the slope and intercept of the regression line.

4.3.9 Lactose hydrolysis in milk

Samples of commercial milk (Natrel, QC) were purchased from a store. After the reactions with the recombinant β-galactosidase, all samples were deproteinated by the modified method of Kajuhiro *et al* (1983). Briefly, the recombinant enzyme was incubated with skim milk (50 U/ml) at various conditions, and the reactions were stopped by heating in boiling water for 1 min. Portions (0.3 ml) of the sample mixture were deproteinated with 0.6 ml of 1.8 % $Ba(OH)_2 \cdot 8H_2O$ and 0.6 ml of 2% $ZnSO_4 \cdot 7H_2O$, and then centrifuged at 13,000g for 20 min. The supernatants were collected and filtered with a 0.45 μm filter (Millipore) for HPLC analysis.

4.4 RESULTS

4.4.1 Lactose hydrolysis and transgalactosylation

During lactose hydrolysis by β-galactosidases from *Bifidobacterium breve* B24 and recombinant *E. coli* ER2566, hydrolyzed glucose and galactose were bound together to form at least 3 different types of galacto-oligosaccharides. An example of HPLC chromatogram of lactose hydrolysis and transgalactosylation by the recombinant β-galactosidase is shown in Fig. 4.1. The chromatogram indicated that the β-galactosidase has both hydrolytic activity to produce glucose and galactose and transgalactosylation activity to synthesize oligosaccharides. Calculated retention times of oligosaccharides, lactose, glucose, and galactose were 8.3-10.9, 11.55, 14.05, and 15.22 min, respectively (Fig. 4.1). Due to the intensity difference in height of peaks in HPLC chromatogram, only 2 oligosaccharide peaks were visible.

4.4.2 Effect of pH and temperature on enzyme activity

The optimum pHs of *Bifidobacterium breve* B24 and the recombinant enzyme on lactose hydrolysis were at pH 6 (Fig. 4.3). Both enzymes showed more than 80% of their activity on lactose in the ranges of pH 6-8, indicating that the enzymes are stable in

neutral pH. However, native enzyme had 40% of its activity at pH 5.0, whereas the recombinant enzyme had only 5% of its activity. On the other hand, the recombinant enzyme showed over 50% of its activity at pH 9, while native β-galactosidase showed less than 5% of its activity.

The optimum temperatures of both enzymes were at 45℃ (Fig. 4.4). The profile of enzyme activities of both enzymes was very similar except at 10℃. While the recombinant β-galactosidase showed about 20 % of its enzyme activity at 10℃, none of the native enzyme activity was detected at this temperature.

4.4.3 Activators and inhibitors

As shown in Table 4.1, no significant differences were found on the hydrolytic activity by the recombinant enzyme. Generally, the recombinant enzyme activity was decreased by all divalent metal ions and chemicals except for $ZnCl_2$. The recombinant enzyme activities were also completely inhibited in the presence of $HgCl_2$ and *p*-chloromercuribenzoic acid (PCMB) at both concentrations and these results are good agreement with those of ONPG.

4.4.4 β-Galactosidases and transgalactosylation activity

In order to understand characteristics of β-galactosidases, some of enzyme properties on lactose were analyzed.

The effects of enzymes and time course on the hydrolysis of lactose are shown in Figs. 4.5 and 4.6. An enzyme (50 units) was required to hydrolyze 100 mM lactose completely within 5 h at 45 ℃.

After the optimum conditions were established, different concentrations of lactose (100-1 M) were hydrolyzed by the recombinant enzyme for 5 h and the results are shown in Fig. 4.7. About 47.5% of 1 M lactose was hydrolyzed in 5 h at 45 ℃, whereas 100% of 100 mM lactose was hydrolyzed completely in 5 h at 45 ℃. No significant increase was found in hydrolytic activity when lactose concentration was higher than 600 mM, indicating that the enzyme was saturated with lactose.

In the presence of glucose and galactose in the reaction mixture, the recombinant β-galactosidase synthesized oligosaccharides. Total amount of oligosaccharides was increased by increasing glucose and galactose concentrations in the reaction mixture (Fig. 4.8). The results indicated that the recombinant β-galactosidase was able to synthesize oligosaccharides without hydrolytic activity.

At the concentration of 1 M lactose, 41.8% of oligosaccharide was synthesized

from lactose hydrolysates (Fig. 4.9).

The hydrolytic activity of the recombinant β-galactosidase was also tested with skim milk. Lactose was almost completely hydrolyzed with 50 units of enzyme at 45 ℃ for 5 h. About 97.4 % of lactose (41.22 μg/μl) was hydrolyzed to produce 46.3 % of glucose, 46.6 % of galactose, and 7.1 % of oligosaccharides (Fig. 4.10).

The optimum temperature on the production of oligosaccharides from lactose in milk was tested with 50 units of the recombinant β-galactosidase at various temperatures (Fig 4.11). After 24 h reaction, 3.04 μg/μl, 5.75 μg/μl, 7.28 μg/μl, and 1.95 μg/μl of oligosaccharides were synthesized at 20, 37, 45, and 50 ℃, respectively. Lactose was completely hydrolyzed after 14, 12, and 6 h at 20, 37, and 45 ℃, respectively. However, lactose hydrolysis was inhibited at 50 ℃ and also oligosaccharide production was not increased after 6 h, due to the inactivation of enzyme at this temperature.

4.4.5 Enzyme kinetics

The K_m and V_{max} values of the native and recombinant enzymes with lactose were determined from Lineweaver Burk plot. The Km values of the native and recombinant enzymes were 152.08 mM and 95.58 mM, respectively that are 54.9 and 52.51 times higher than ONPG. The V_{max} values of the native and recombinant enzymes

were 138.9 and 189.1 mM/min, respectively (Fig. 4.12).

4.5 DISCUSSION

The native and recombinant β-galactosidases showed not only hydrolytic activity on lactose but also had transgalactosylation activity to produce galacto-oligosaccharides from lactose hydrolysates. As oligosaccharides enhance health promoting effects, various types of β-galactosidases have been used in food industries as free or immobilized forms. Immobilized enzymes can be used over a longer period with continuous systems. Many β-galactosidases have been immobilized on different types of matrix and their properties were studied (Gonzalez and Doval, 1993; Ortega-Lopez *et al.*, 1993; Meng and Charles, 1994; Carlos and Amelia, 1997; Karen, 1998; Sungur and Yildirim, 1999; Szczodrak, 1999; Eldin *et al.*, 2000; Ladero *et al.*, 2003; Benevides, 2004).

Some parameters are important to increase the production of oligosaccharides like pH, temperature, and substrate concentration. The rate of enzyme activity was significantly affected by pH. The conformation of an enzyme can be changed when the ionization state of its amino acid side chains is altered by changes in pH (Ou *et al.*, 2001). The optimum pHs of both native and recombinant β-galactosidases were same at pH 6.0,

but the recombinant β-galactosidase was stable at broader ranges of pH than the native β-galactosidase.

Generally, increasing temperature will increase the rate of chemical reactions and enzyme reactions also increase with temperature from the Arrhenius equation (Edwards and Hassall, 1980). The enzyme reaction rates are approximately doubled by each 10 ℃ rise in temperature (Q_{10} = 2.0, temperature coefficiency), though the Q_{10} varies from one enzyme to another depending on the activation energy of the catalytic energy. The rate of enzyme reactions increases with increasing temperature until an optimum temperature is reached. The optimum temperatures of both native and recombinant β-galactosidases were same at 45 ℃ and still retained over 60 % of their activity at 50 ℃.

At very low substrate concentration, chances of collision between enzyme and substrate are low, therefore increasing substrate concentration increases chances of collision between two molecules. However, increasing substrate concentration beyond certain point had no increasing enzyme activity. No significant increase in hydrolytic activity was observed above 600 mM lactose and this concentration is appeared to be a saturated point for the recombinant β-galactosidase. The formation of galacto-oligosaccharide is depending on initial lactose concentration and is almost insignificant

at low concentration. The relationship between initial lactose concentration and oligosaccharide production was reported by Inge *et al.*(2000) using β-glycosidase which has same role in the production of oligosaccharides. High amount of oligosaccharides were produced from high initial concentration (270 g/l) of lactose with 26 % conversation rate with β-glycosidase from *Sulfolobus solfataricus* and 32 % conversation rate with β-glycosidase from *Pyrococcus furiosus*. In both cases, 80 % of initial lactose was hydrolyzed.

The transgalactosylation activity was demonstrated with the recombinant β-galactosidase using glucose and galactose as substrates. Oligosaccharides were synthesized from glucose and galactose mixture. Production rate was increased by increasing glucose and galactose concentration up to 500 mM (Fig. 4.8). This indicates that the recombinant β-galactosidase had both lactose hydrolytic and transgalactosylation activities.

The results in Fig 4.9 showed that the concentration of galactose over 500 mM inhibited lactose hydrolysis but increased the synthesis of oligosaccharides. At this point, hydrolysis of lactose was maximal and began to synthesize oligosaccharides. The level of glucose was constant probably due to structure of oligosaccharides that incorporate more galactose than glucose. At the high concentration of lactose (1 M), about 47.5 % of

lactose was hydrolyzed and 41.8 % of oligosaccharide was synthesized. β-Galactosidase from *Aspergillus oryzae* showed over 30 % of oligosaccharide conversion rate with lactose concentration of 0.139-1.67 M at 40 ℃ within 5 h (Iwasaki *et al.*, 1996). β-Galactosidase from *Sterigmatomyces elviae* showed 40 % of oligosaccharide conversion rate with lactose concentration of 0.56 M at 60 ℃ within 48 h (Onishi and Tanaka, 1995). β-Galactosidase from *Sulfolobus solfataricus* showed over 47 % of oligosaccharide conversion rate with lactose concentration of 1 M at 75 ℃ within 72 h and 35.5 % of oligosaccharide conversion rate with lactose concentration of 1 M at 65 ℃ within 96 h (Stephan *et al.*, 1999).

When the hydrolytic and transgalactosylation activities on lactose (4.8 %) in skim milk were tested, lactose (97.39 %) was hydrolyzed to produce 46.26 % of glucose, 46.62 % of galactose, and 7.12 % of oligosaccharide at 45 ℃ for 5 h reaction (Fig. 4.10). Lactose was completely hydrolyzed after 14, 12, and 6 h at 20, 37, 45 ℃, respectively (Fig. 4.11) and thus this enzyme may be used to prepare lactose free milk. Changing parameters, mostly temperature, could change sugar content in milk. Interesting result on hydrolysis of lactose in milk was reported by Kintia and Fox (1996) that enzymatic hydrolysis of lactose in milk by β-galactosidase increased the heat stability (HCT; heat coagulation time) of milk due to the participation of glucose and galactose in the

Maillard reaction. Lactose hydrolysis in milk by immobilized β-galactosidase was reported by Rogalski *et al.* (1994) that some enzymatic characteristics such as pH stability and thermal stability were increased by immobilization on glutaraldehyde and bis-oxide using cross-linking method.

The K_m on lactose was higher than ONPG. The K_m values of the native and recombinant enzymes were 152.08 and 95.58 mM, respectively that are 54.9 and 52.51 times higher than ONPG. Both β-galactosidases had high affinity for ONPG than lactose.

In this study, the native and recombinant β-galactosidases showed some interesting properties for industrial application. The further studies on the structure of galacto-oligosaccharides, increasing thermal stability by site-directed mutagenesis, increasing substrate solubility are required to expand the knowledges on β-galactosidase and oligosaccharide for the industrial application.

Table 4.1 Effect of inhibitors and activators on recombinant β-galactosidase activity on lactose hydrolysis (100 mM, 45 ℃).

Chemicals	Relative activity (%)[a]		
	0 mM[b]	1 mM	100 mM
$CoCO_3$	100	93.06 ± 3.29	67.38 ± 2.51
$CrCl_3$	100	59.87 ± 2.01	0.00 ± 0
$CuSO_4$	100	8.02 ± 0.29	0.00 ± 0
$Fe_2(SO_4)_3$	100	78.45 ± 2.98	0.00 ± 0
$HgCl_2$	100	0.00 ± 0	0.00 ± 0
KCl	100	95.06 ± 4.07	94.19 ± 4.10
$MgCl_2$	100	96.72 ± 2.39	0.00 ± 0
$MnCl_2$	100	14.25 ± 0.62	0.00 ± 0
NaCl	100	89.44 ± 3.52	0.00 ± 0
$ZnCl_2$	100	104.47 ± 2.05	0.00 ± 0
EDTA	100	75.89 ± 3.05	70.76 ± 2.86
Urea	100	76.03 ± 2.54	67.70 ± 1.69
Cys-HCl	100	69.20 ± 1.95	0.00 ± 0
PCMB	100	0.00 ± 0	0.00 ± 0
Me-Ethanol	100	81.32 ± 4.47	74.81 ± 2.83

a) The results were means of triplicates ($P<0.05$).

b) The activity without chemicals was used as a control.

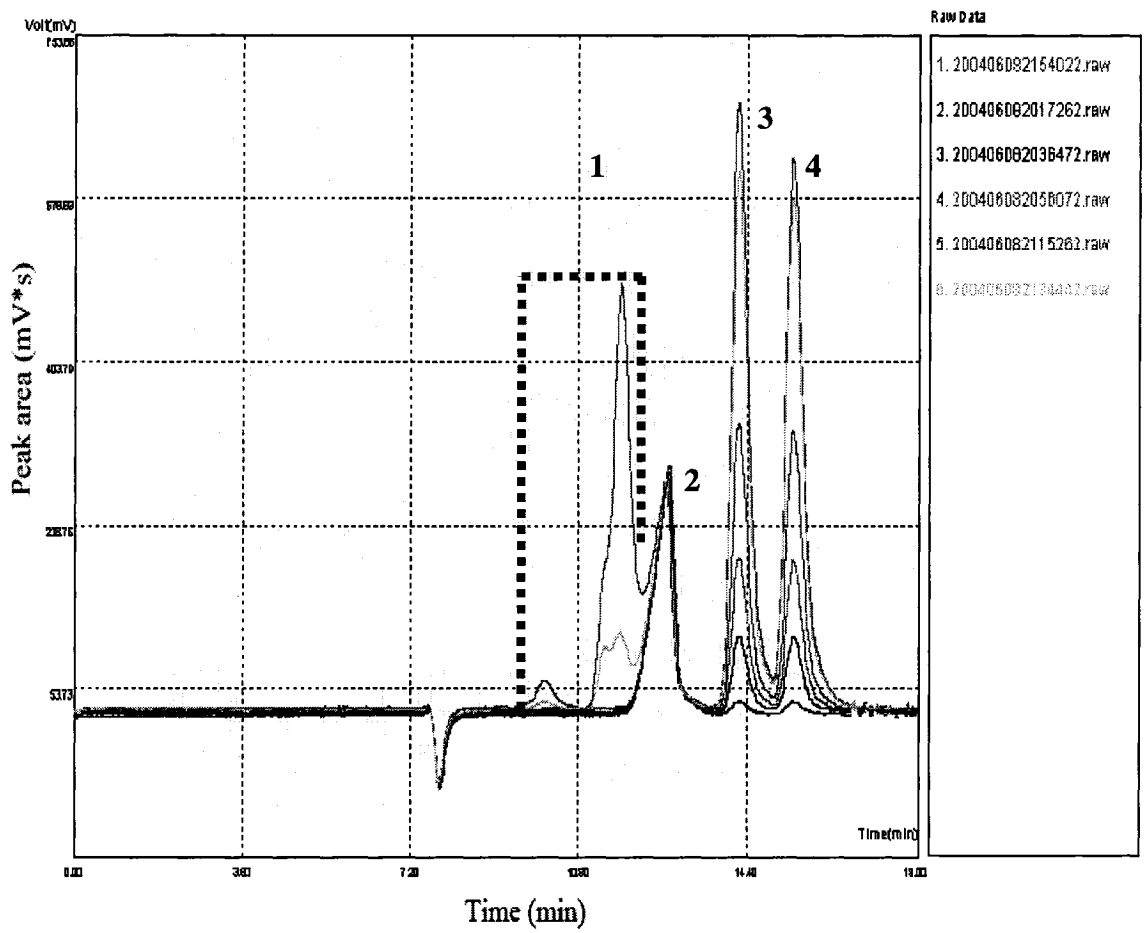

Fig. 4.1 The HPLC chromatogram from the lactose hydrolysis by β-galactosidase from *E. coli* ER2566. Peaks 1, oligosaccharide; 2, lactose; 3, glucose; 4, galactose.

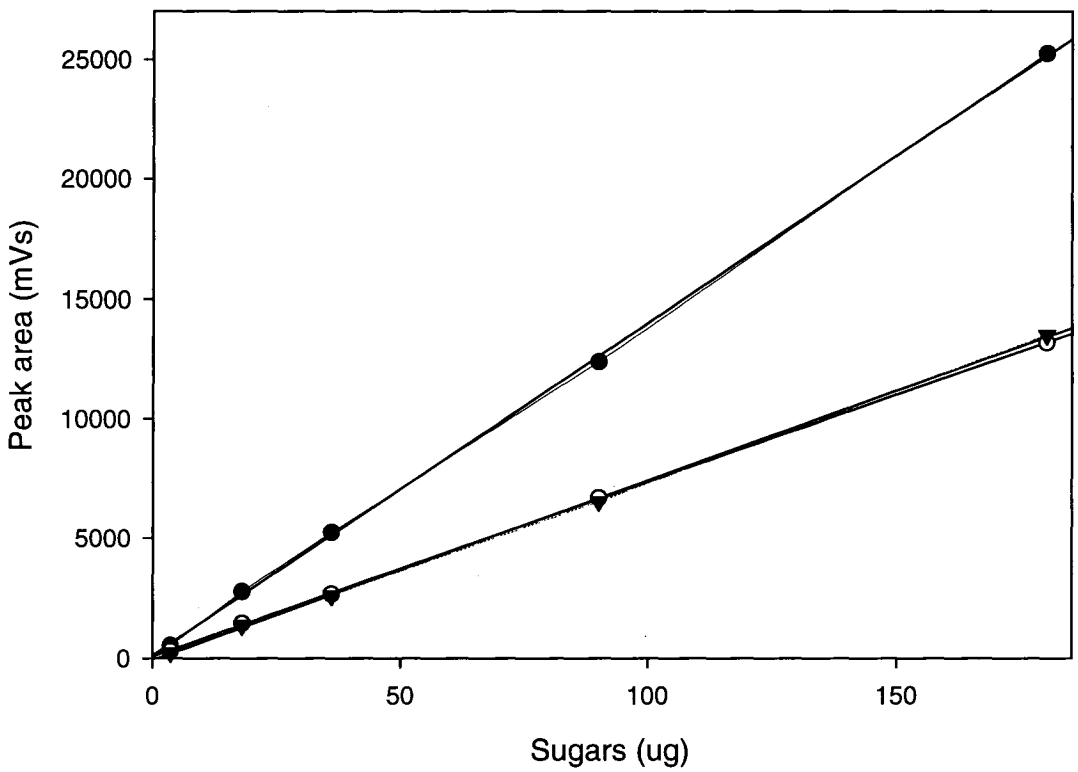

Fig. 4.2 The standard curves of lactose (●), glucose (○), and galactose (▼) for calculation of sugars content from HPLC chromatogram.

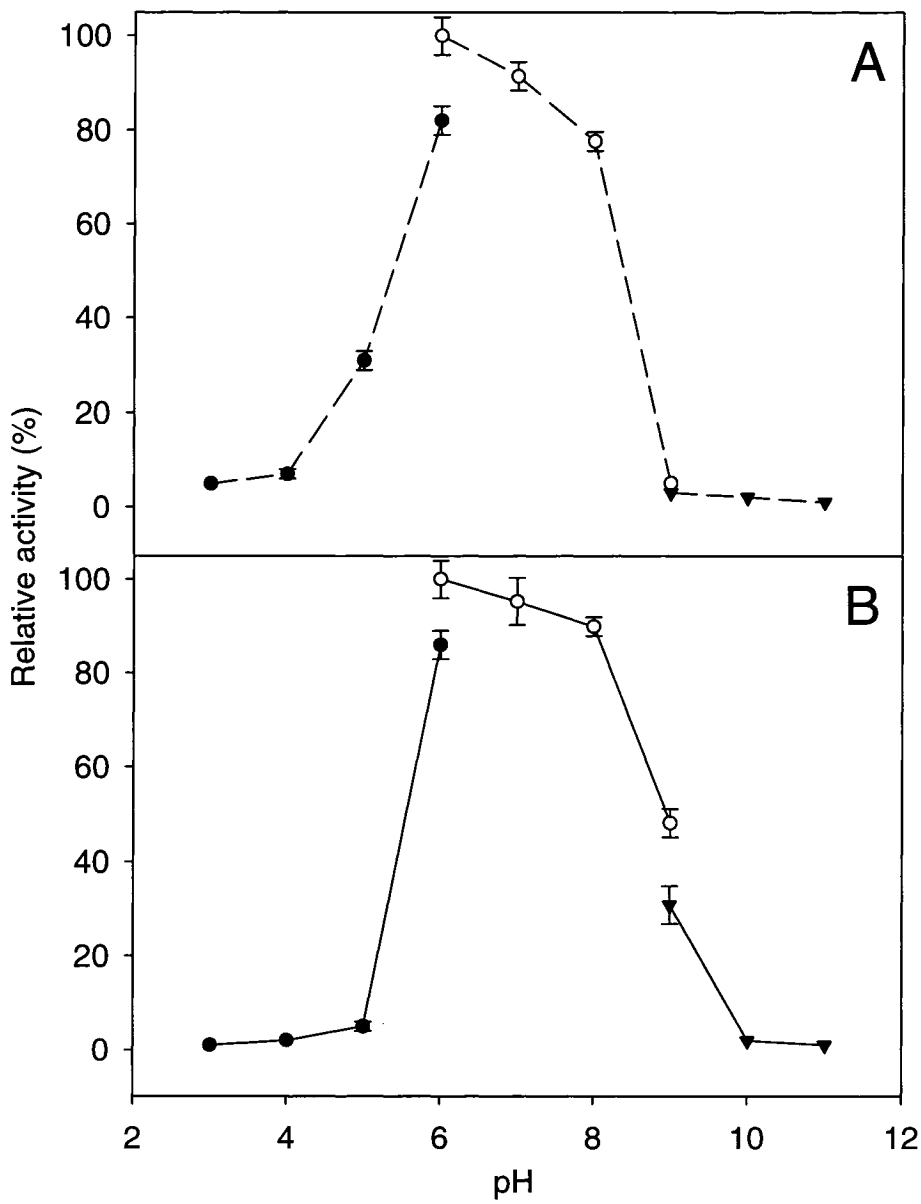

Fig. 4.3 Optimum pHs of β-galactosidases from *Bifidobacterium breve* B24 (A) and *E. coli* ER2566 (B) on lactose (100 mM, 45 ℃). The buffers used are 50 mM citrate (●, pH 3-6), 50 mM sodium phosphate (○, pH 6-9), and 50 mM sodium carbonate (▼, pH 9-11). The results were means of triplicates ($P<0.05$).

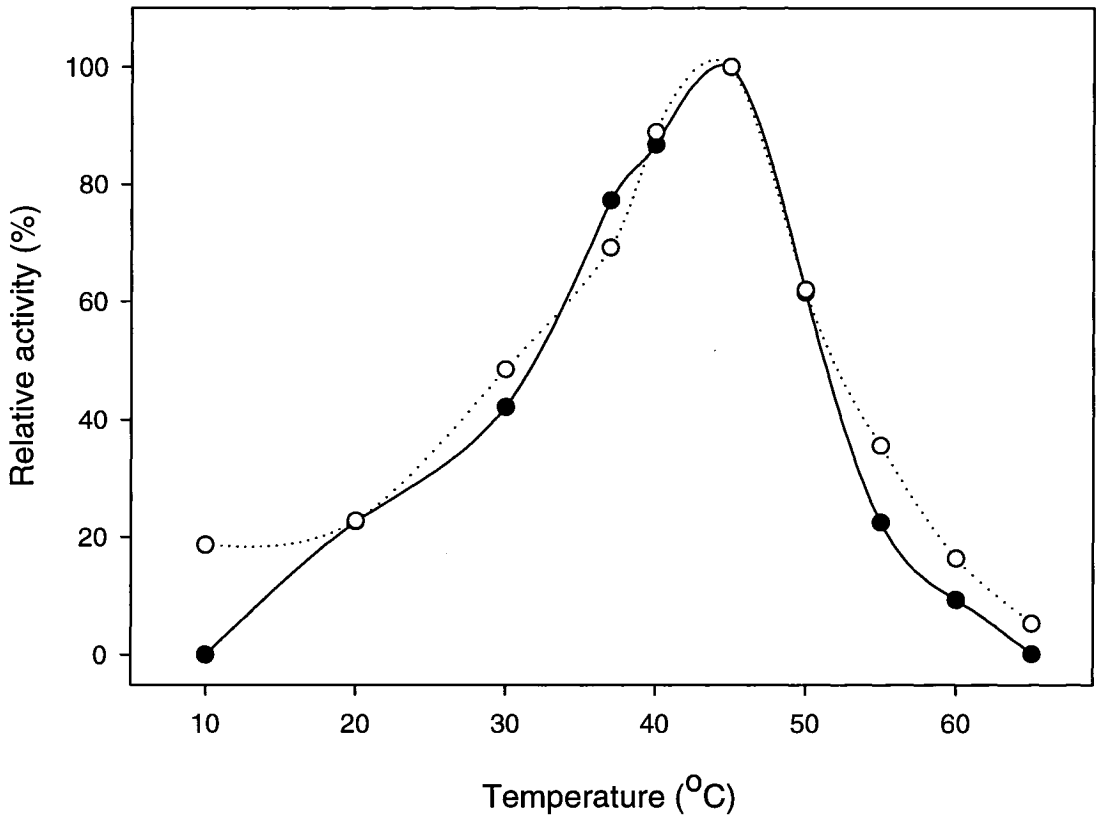

Fig. 4.4 Optimum temperatures of β-galactosidases from *Bifidobacterium breve* B24 (●) and *E. coli* ER2566(○) on lactose (100 mM, 45 ℃). The buffer used was 50 mM sodium phosphate (pH 6). The results were means of triplicates ($P<0.05$).

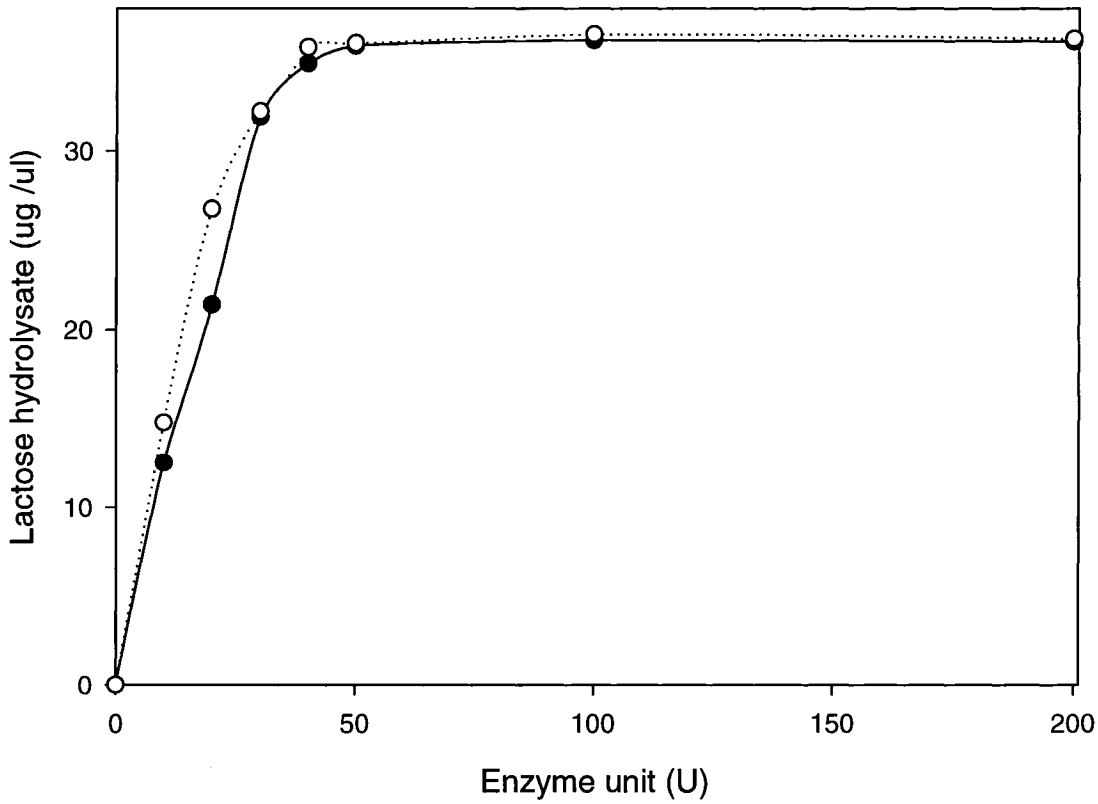

Fig. 4.5 Effect of enzyme concentration on the hydrolysis of lactose. Various units (0-200 units) of β-galactosidases from *Bifidobacterium breve* B24 (●) and *E. coli* ER2566 (○) were used. Enzymes were incubated in 100 mM lactose in 50 mM sodium phosphate buffer (pH 6.0) for 5 h at 45 ℃. The results were means of triplicates ($P<0.05$).

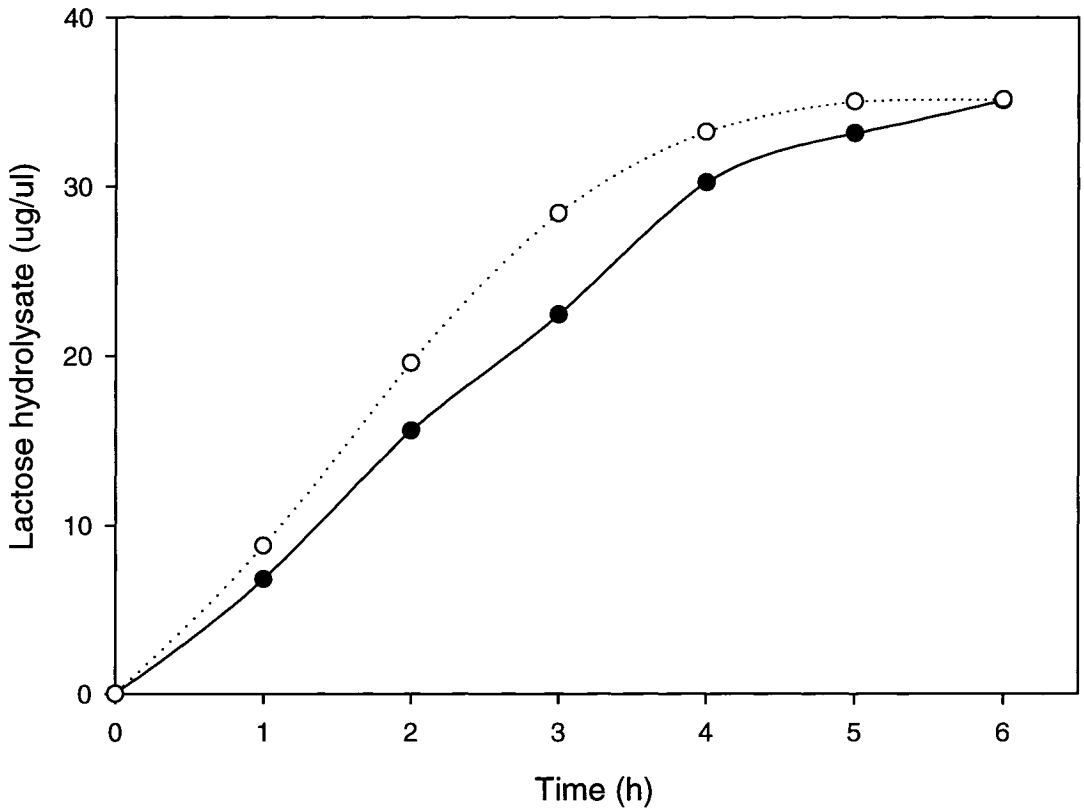

Fig. 4.6 Time course of lactose hydrolysis by β-galactosidases from *Bifidobacterium breve* B24 (●) and *E. coli* ER2566 (○). Enzymes (50 units) were reacted with 100 mM lactose in 50 mM sodium phosphate buffer (pH 6.0) at 45 ℃. The results were means of triplicates ($P<0.05$).

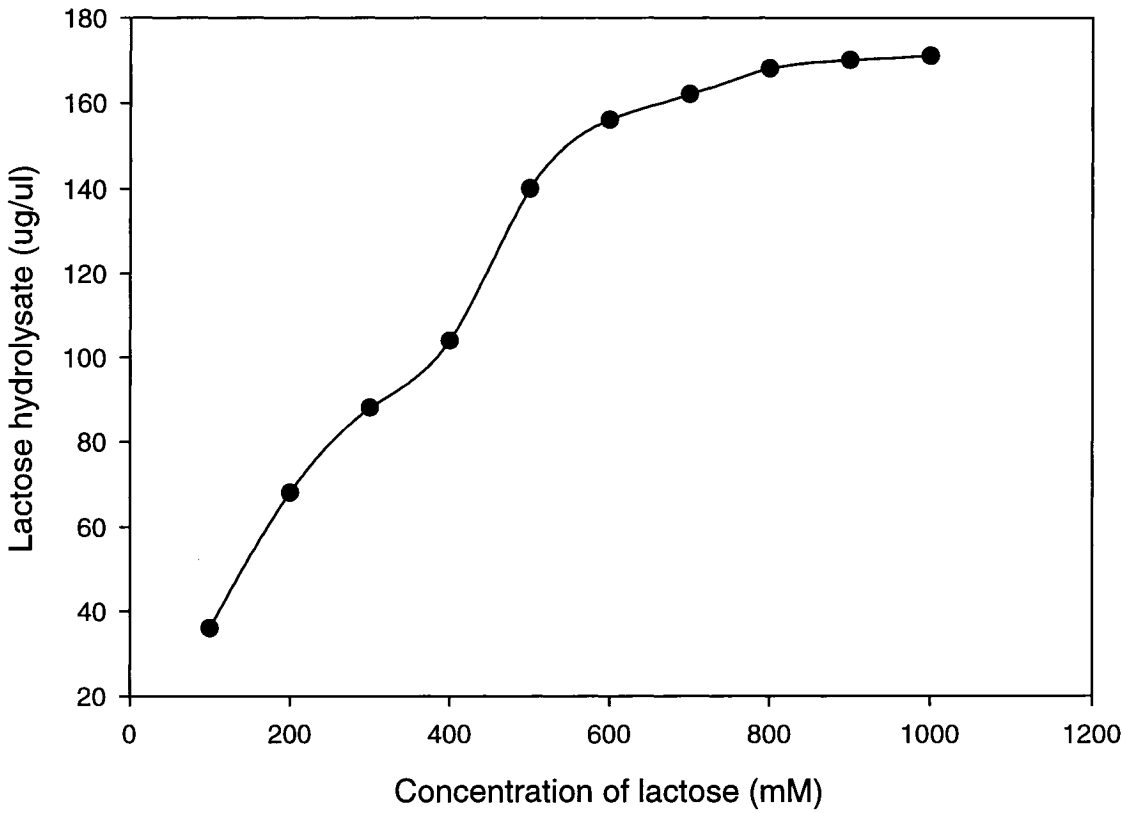

Fig. 4.7 Effect of β-galactosidase from *E. coli* ER2566 on the hydrolysis of lactose. β-Galactosidase from *E. coli* ER2566 was incubated with various concentration of lactose in 50 mM sodium phosphate buffer (pH 6.0) for 5 h at 45 ℃. The results were means of triplicates ($P<0.05$).

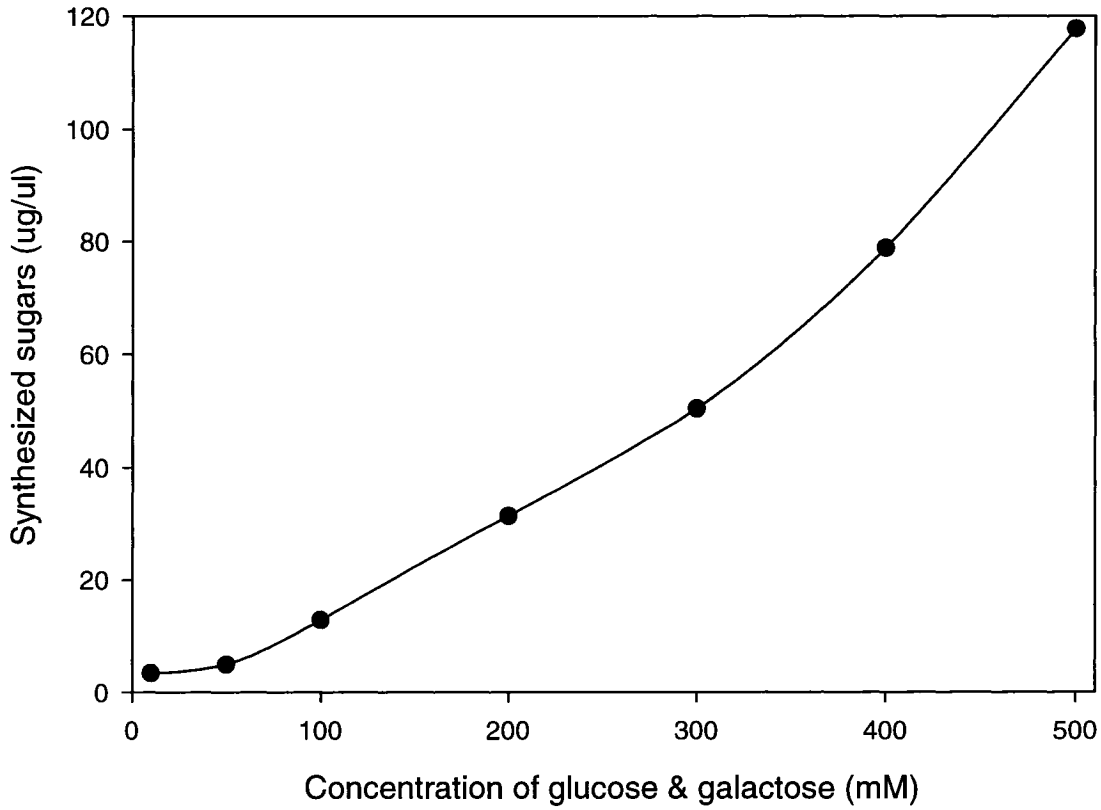

Fig. 4.8 Production of oligosaccharides from various concentrations of glucose and galactose mixture. β-Galactosidase (50 units) from *E. coli* ER2566 was used for the reaction in the presence of different concentrations of glucose and galactose in 50 mM sodium phosphate buffer (pH 6.0) for 5 h at 45 ℃. The results were means of triplicates ($P<0.05$).

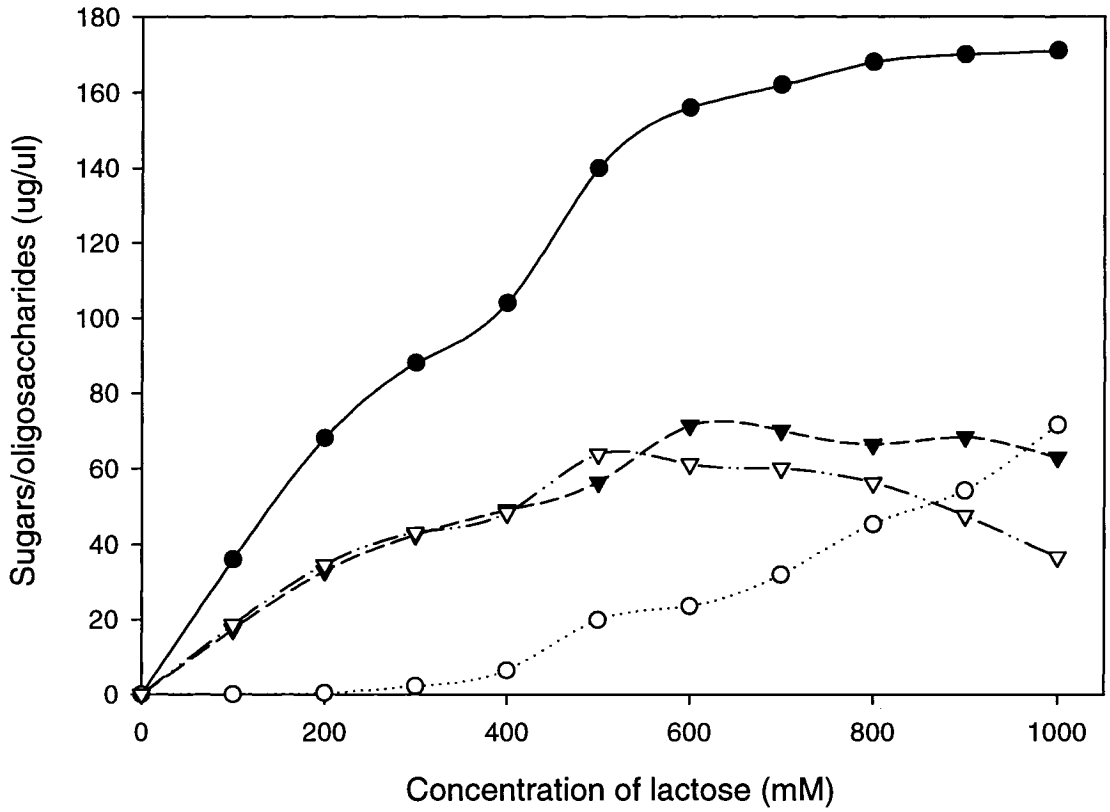

Fig. 4.9 Lactose hydrolysis and oligosaccharide production from various concentrations of lactose. β-Galactosidase (50 units) from *E. coli* ER2566 was used for reaction with various concentrations of lactose in 50 mM sodium phosphate buffer (pH 6.0) for 5 h at 45 ℃. Symbols: ●, lactose ; ▼, glucose; ▽, galactose; ○, oligosaccharide. The results were means of triplicates ($P<0.05$).

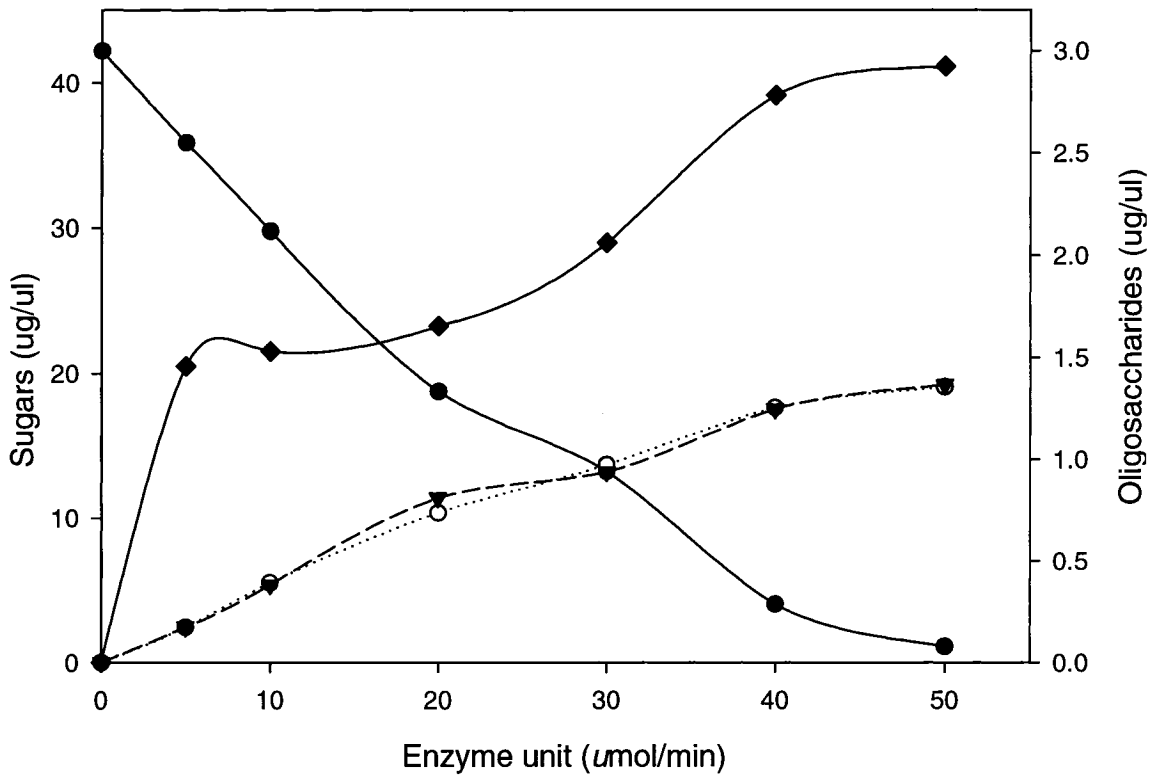

Fig. 4.10 Lactose hydrolysis and oligosaccharide production in milk. β-Galactosidase (5-50 units) from E. coli ER2566 was used for reaction for 5 h at 45 ℃. Symbols: ●, lactose ; ▼, glucose; ○, galactose; ♦, oligosaccharide. The results were means of triplicates ($P<0.05$).

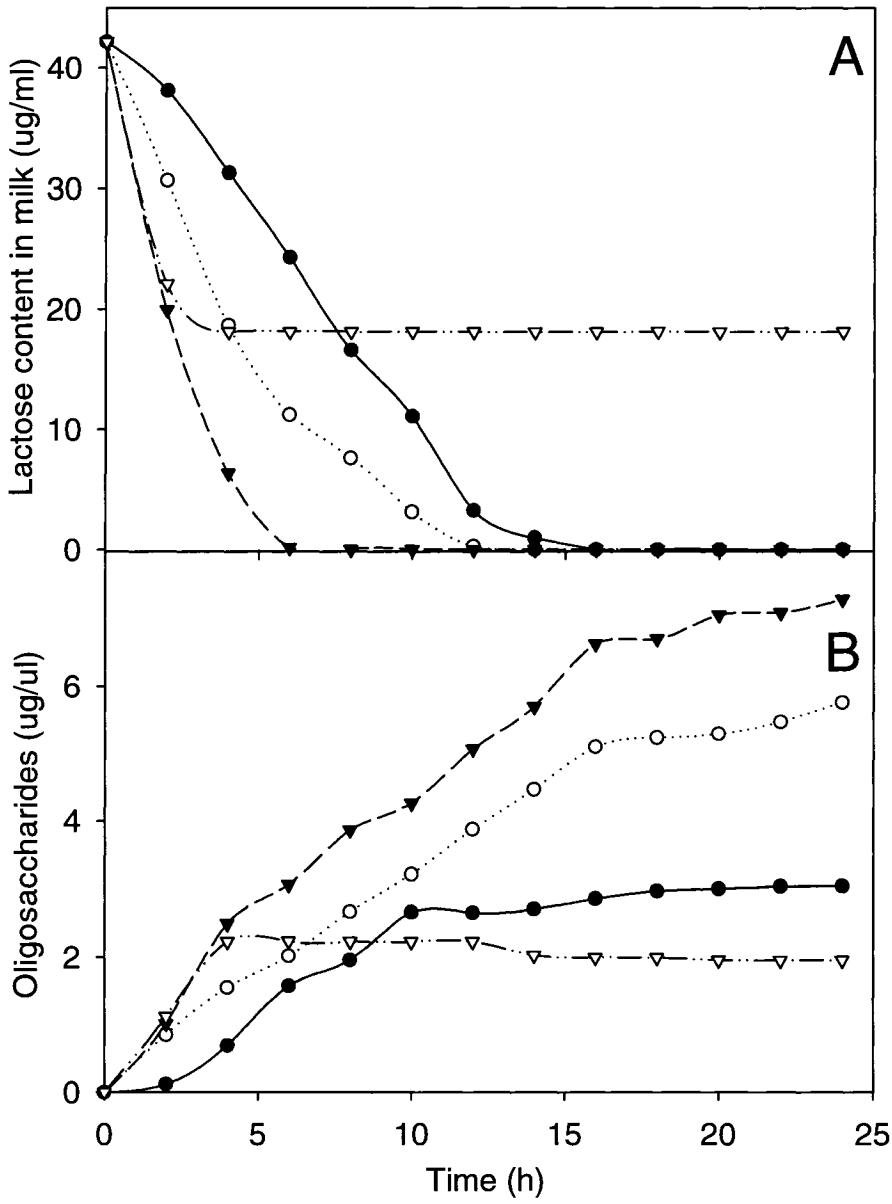

Fig. 4.11 Effect of temperature on lactose hydrolysis (A) and oligosaccharide production (B) in milk by β-galactosidase from *E. coli* ER2566. Enzyme (50 units) was reacted with milk at 20℃ (●), 37℃ (○), 45 ℃ (▼), and 50℃ (▽).The results were means of triplicates ($P<0.05$).

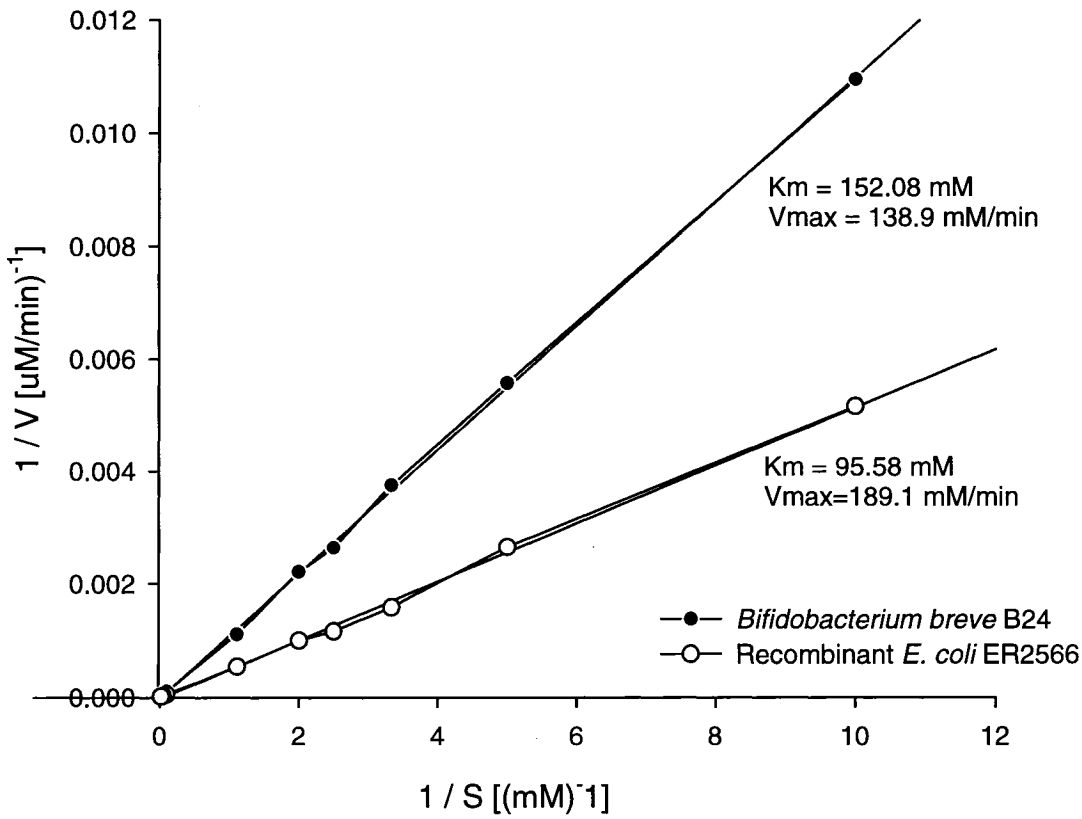

Fig. 4.12 Lineweaver-Burk plot of β-galactosidases activity using lactose.

GENERAL CONCLUSION

A 11 kb nucleotide fragment containing the gene *galA* was introduced into *E. coli* MC1061 and complete *galA* gene sequence was determined. The *galA* gene was found to be 2073 bp long, which encoded a 691 amino acid polypeptide with deduced molecular weight and pI value of 76,010 Dalton and 4.58, respectively. The nucleotide sequence of the flanking region showed complete set of transcriptional regulatory sequences of ribosomal binding site (RBS), -10, and -35 regions, and transcriptional initiation site.

The complete *galA* sequence was highly homologous to those of β-galactosidases from *Bifidobacterium* species. Two conserved amino acids which act as proton donor and nucleophile base located in the catalytic site of β-galactosidase were found in the enzyme. Further investigation on comparing *galA* gene with other β-galactosidases from bacteria revealed that a β-galactosidase from *Bifidobacterium breve* B24 was the only *LacA* family member protein among bifidobacteria that is the first observation. Although a β-galactosidase of *galA* gene belongs to the *LacA* member proteins, overall amino acid sequence homology was lower than those of bifidobacteria.

The phylogenetic tree analysis showed that the *galA* gene was unique in the

LacA family protein.

After the native and recombinant β-galactosidases were successfully purified, the homogeneities of purified β-galactosidases were examined. Both the native and recombinant enzymes had similar molecular mass of 75 kDa. The result of activity staining and gel-filtration chromatography showed that the native molecular mass was 150 kDa. Comparison of the molecular mass from SDS-PAGE and gel filtration chromatography with activity staining on Native-PAGE indicated that the β-galactosidase was consisted of two 75 kDa subunits.

The optimum pHs on hydrolyzing ONPG of both native and recombinant enzymes were pH 6 and 7, respectively and optimum temperature were similar at 45 ℃. The recombinant β-galactosidase showed significant activity at pH 9.0 (42 %), whereas the native enzyme was inactive at the same pH. On the other hand, the native β-galactosidase showed enzyme activity at pH 5.0 (38 %), while the recombinant enzyme was inactivated at the same pH. Both enzymes were stable at the temperature ranging between 10 - 45 ℃ up to 5 h or more. However, both enzymes were inactivated when temperature was higher than 45 ℃. No significant activators on the enzyme activity of both enzymes were found. The K_m and V_{max} values of the native enzyme were 2.77 mM and 1.02 mM/min and those of the recombinant one were 1.82 mM and 1.4 mM/min,

respectively.

The native and recombinant β-galactosidases showed both hydrolytic and transgalactosylation activity on lactose and milk. Some parameters were examined to support maximal enzyme activity. The optimum pHs of both native and recombinant β-galactosidases were same at pH 6.0, and optimum temperatures were similar at 45 ℃. At the high concentration of lactose (1 M), about 47.5 % of lactose was hydrolyzed and 41.8 % of oligosaccharide was synthesized. Lactose in milk was completely hydrolyzed to produce 46.26 % of glucose, 46.62 % of galactose, and 7.12 % of oligosaccharide at 45 ℃ for 5 h.

The K_m values of the native and recombinant enzymes were 152.08 and 95.58 mM, respectively that are 54.9 and 52.51 times higher than ONPG. Both β-galactosidases had high affinity for ONPG than lactose.

The further studies on the over-expression of the enzyme in yeast, increasing thermal stability for higher substrate solubility and enzyme reactivity, structural identification of galacto-oligosaccharides, are required to expand the knowledges on β-galactosidase and oligosaccharide for the industrial application.

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