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Effect of nitrogen sources on production of β-galactosidase from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 grown in whey under different culture conditions

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Abstract: The present study examined the effect of various nitrogen source such as yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey (control) on the growth of *B. animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 and production of β-galactosidase (β-gal) grown in deproteinized whey by these organisms. The organisms were grown in deprotenized whey (control) and that containing 3.5% of yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate was supplemented as a nitrogen source and β-gal activity was determined using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. The strains were able to utilize a wide range of nitrogen sources such as yeast extract, peptone, casein hydrolysate, tryptone and ammonium sulphate in deproteinized whey. In general, yeast extract and peptone were found to be more suitable for β-galactosidase production. The highest level of β-gal activity at 45.69 unit/mL was produced with peptone followed by yeast extract at 43.44 unit/mL by *B. animalis* Bb12 and 46.6 unit/mL was produced with casein hydrolysate followed by yeast extract at 46.25 unit/mL by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. This study showed that whey medium supplemented with nitrogen sources was suitable for fermentation by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and production of β-gal.

Keywords: β-galactosidase, *Bifidobacterium*, *Lactobacillus*, β-galactosidase, nitrogen sources, deprotenized whey

Introduction

β-Galactosidase (β-gal), is an important enzyme used in dairy industry for hydrolysis of lactose into glucose and galactose. β-Gal is found in abundant in biological systems and micro-organisms such as yeasts and molds and bacteria still remain the only commercially exploited sources (Agrawal, Garg and Dutta, 1989). Since, a large number of people suffer from lactose intolerance in different countries of the world (Shah, 1993), this makes the enzyme even more important. The need for low lactose milk is particularly important in food-aid programs as severe tissues dehydration, diarrhoea even death may result from feeding lactose containing milk to lactose in-tolerant children and adults suffering from protein-calories malnutrition. In addition, lactose has a low solubility which results in crystal structure at concentrations above 11% (w/v), which prevents the utilization of concentrated whey in several food processes (Bansal *et al*., 2008). Dairy industry waste whey contains lactose (5%), whey protein (0.8%), mineral and vitamins, which are essential components that have not been exploited for the cultivation of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* 11842 (Mahalakshmi *et al*., 2000). Whey contains about 6.66% of important milk nutrients (Bansal and *et al*., 2008). The disposable of whey remains a significant problem for dairy industries especially in developing countries where a relatively insignificant part of whey is used for production of whey protein concentrates and significant part of it disposed off into the water streams causing serious water pollution problems. Hence, problems associated with whey disposal, lactose crystallization and milk consumption by lactose- intolerant populations of the world have drawn the attention of several research workers. This has led to the selection of micro-organisms with view to high potentials for producing β-gal, the enzyme that hydrolyses lactose into its component monosaccharide units (Rao and Dutta, 1997). Therefore, it is important to evaluate the production of β-gal using whey by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* 11842 organisms in terms of effectiveness and enzyme production so that process could be scaled up. In this regard, not many studies have been carried out recently for economical production of β-gal. Hence, selection of micro-organisms which are safe for human use and are capable of producing high level of β-gal becomes

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vital. Thus, the present study was conducted to evaluate the effect of various nitrogen sources on the production of β-gal by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and *B. animalis* Bb12 in deprotenized whey.

Materials and Methods

Micro-organisms

The pure cultures of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). The stock cultures were stored at -80ºC in sterile MRS broth (50% w/v) and 50% glycerol.

Culture condition

The organisms were activated in two successive transfers in *lactobacilli* MRS broth (Difco, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) supplemented with 0.05% L-cysteine (Sigma Chemical Company, St. Louis, MO, USA) for growing *Bifidobacterium* and incubated at 37ºC for *B. animalis* Bb12, and 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 18 h. Deproteinized whey was prepared by heating whey at 90ºC and pH 4.5 for 10 min, filtered through Whatman no. 1 filter paper to remove the coagulated protein, adjusted to pH 7.0 and sterilized at 121ºC for 15 min. For production of β-gal, the sterile deprotenized whey was supplemented with 3.5% of each of nitrogen source individually including yeast extract, peptone, casein hydrolysate, tryptone or ammonium sulphate and inoculated with 1% of active culture of each organism. The various nitrogen sources were used in order to study their effect on β-gal production. Deproteinized whey was used as a control. All experiments were carried out for 24 h. The culture was maintained at 37ºC for *B. animalis* Bb12 and 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842.

Production of β-galactosidase

For production of β-gal, cells of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were first harvested by centrifugation (1252 x g for 20 min at 10ºC). The supernatant was discarded and cell pellets were washed twice with 5 mL of 0.03 M phosphate buffer. Lysozyme at 75 µl per millilitre of cell pellet in TE buffer (1 mM EDTA and 10 mM Tris-HCL, pH 8.0) was used to release the enzyme from the test organisms. β-Gal activity was then assayed according to the method of (Nagy *et al*., 2001). The reaction mixture consisted of 0.5 mL of enzyme source (cells treated with lysozyme) and 0.5 mL of 15 mM o-nitrophenyl β-D-galactopyranoside (ONPG) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min at 37ºC, 2 mL of 0.1 M sodium carbonate was added to stop the reaction. Absorbance was measured at 420 nm with a spectrophotometer (Model Pharmacia, Biotech LKB-Novespec II, UV/VIS spectrophotometer, Ontario, Canada). A unit of β-gal was defined as the amount of enzyme that catalysed the formation of 1 µmol of o-nitrophenyl from ONPG per min per gram under the assay condition.

Enumeration of micro-organisms

To enumerate *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, MRS agar supplemented with 1% (w/v) D-glucose was used. Peptone and water 0.15% (w/v) diluent was used to perform serial dilutions. Plates were incubated at 37ºC for *B. animalis* Bb12 and 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 for 72 h in an anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD, USA) with a gas generating kit (Oxoid Ltd., Hamshire, UK). Plates showing 25 to 250 colonies were counted and results were expressed as colonies forming units (CFU) per millilitre of sample.

Statistical analysis

All analyses were performed in triplicate and data were analysed using one-way analysis of variance (ANOVA) at 5% significance level. Analyses were performed using SAS (SAS, 1995). ANOVA data with a $p < 0.05$ were classified as statistically significant.

Results and Discussion

Effect of nitrogen source on the production of β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842

Effect of nitrogen sources on the β-galactosidase production

The influence of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey on production of β-gal by *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figures 1 and 2. In general, *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed a significant difference ($p<0.05$) in β-gal production in all nitrogen sources. *B. animalis* Bb12 produced higher ($p<0.05$) β-gal in peptone, yeast extract and casein hydrolysate compared with other nitrogen sources at 24 h (Figure 1). Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced higher ($p<0.05$) β-gal production in

yeast extract and casein hydrolysate than the other nitrogen sources (Figure 2). Statistically, *B. animalis* Bb12 showed significantly different ($p>0.05$) β-gal production at 24 h in casein hydrolysate and ammonium sulphate and others nitrogen sources. However, significant difference ($p<0.05$) was found between casein hydrolysate, tryptone and other nitrogen sources; yeast extract, peptone, casein hydrolysate and other nitrogen sources; and peptone, casein hydrolysate, tryptone and other nitrogen sources at 0 h; 6 h; and 12 h, respectively.

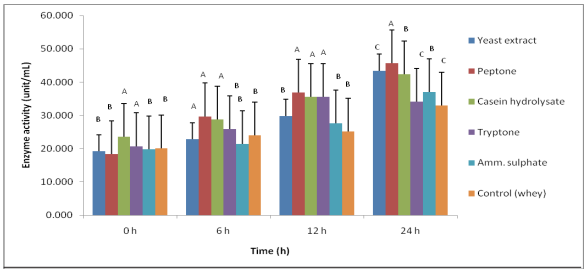

Figure 1. Effect of nitrogen source on the β-galactosidase production by *B. animalis* Bb12. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$)

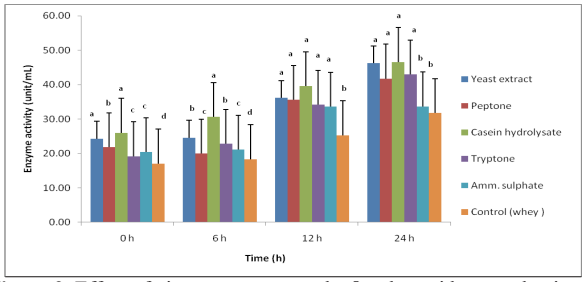

Figure 2. Effect of nitrogen source on the β-galactosidase production by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$)

Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed significantly different ($p>0.05$) β-gal production at 24 h between ammonium sulphate, whey and other nitrogen sources. However, significant difference ($p<0.05$) was found amongst yeast extract, whey and other nitrogen sources; yeast extract, peptone, casein hydrolysate, whey and rest of nitrogen sources; and whey between other nitrogen sources at 0 h; 6 h; and 12 h, respectively.

B. animalis Bb12 produced the highest amount of β-gal (45.69 unit/mL) with peptone followed by (43.44 unit/mL) with yeast extract and lowest activity (33.0 unit/mL) with whey (control) at 24 h (Figure 1). The β-gal production increased ($p<0.05$) by 126.01, 149.40, 79.35, 64.63, 86.14, and 64.49 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively, at 24 h as compared with 0 h (Figure 1). Peptone provided optimum nutrients for this organism. Hence, the organism produced the highest level of β-gal. However, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced the highest amount of β-gal (46.6 unit/mL) with casein hydrolysate followed by yeast extract (46.25 unit/mL) and lowest activity (31.8 unit/mL) with whey (Figure 2). At 24 h, the β-gal production increased ($p<0.05$) by 90.09, 91.20, 78.90, 123.74, 65.06 and 86.33 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey respectively as compared with 0 h (Figure 2).

Our results are also in line with those reported by (Mahoney *et al*., 1975), in which maximum β-gal activity was achieved at about 22 h of incubation period. Most of the available literature suggests the optimal fermentation time in the range of 20-36 h (Mahoney *et al*., 1975; Ku *et al*., 1992; Ranzi *et al*., 1987). Furthermore, the study by Bury *et al*., (2001) reveals a maximum β-gal activity after 15-17 h of growth in yeast extract medium. The β-gal activities of cultures grown with 0.2-0.8% yeast extract were approximately 2.5 times higher than for those grown without yeast extract in case of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. This may be attributed to the growth factors in addition to the nitrogen compounds present in yeast extract (Bridson and Brecker, 1970). According to Rao and Dutta (1979) and Shaikh *et al*., (1997), nitrogen sources may affect microbial biosynthesis of β-gal.

Effect of nitrogen sources on the growth

Figures 3 and 4 demonstrate the effect of nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey on the growth of *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In general, *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed a significant difference ($p<0.05$) in the viable counts in all nitrogen sources. The viable counts were higher ($p<0.05$) in yeast extract and peptone than others nitrogen source including yeast extract, peptone, tryptone and ammonium sulphate in both organisms (Figures 3 and 4). Statistically, *B. animalis* Bb12 showed no significantly difference ($p>0.05$) in the viable count at 0 h in various nitrogen sources. However, significant difference ($p<0.05$) was found between yeast extract, peptone, casein hydrolysate and other nitrogen sources; yeast extract, casein hydrolysate, whey and other nitrogen sources; and yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen sources at 6 h; 12 h; and 24 h, respectively. Similarly, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 showed no significantly difference

(p>0.05) in the viable count at 0 h and 6 h in various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey. However, a significant difference (p<0.05) was found between yeast extract, tryptone and other nitrogen sources; yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen source; at 12 h and 24 h, respectively. The final viable population of the *B. animalis* Bb12 ranged from 5.91 to 8.27 log CFU/mL and the organism showed the highest viable population of 8.27 log CFU/mL at 24 h with peptone followed by yeast extract at 8.24 log CFU/mL and lowest with ammonium sulphate at 7.26 log CFU/mL. At 24 h, the viable count increased (p<0.05) by 36.42, 39.70, 29.01, 30.80, 22.84 and 23.83 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively, compared with 0 h (Figure 3). Similarly, the final viable population of *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 ranged from 5.59 to 8.64 log CFU/mL and the organism showed the highest viable population of 8.64 log CFU/mL at 24 h with yeast extract followed by tryptone 8.59 log CFU/mL and lowest with whey 7.87 CFU/mL. The viable count increased (p<0.05) by 49.48, 44.31, 46.10, 51.23, 39.05 and 40.97 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively at 24 h as compared with 0 h (Figure 4).

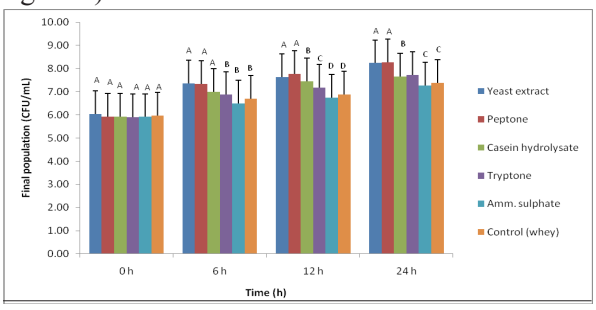

Figure 3. Effect of nitrogen source on the viable population by *B. animalis* Bb12. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference (p<0.05)

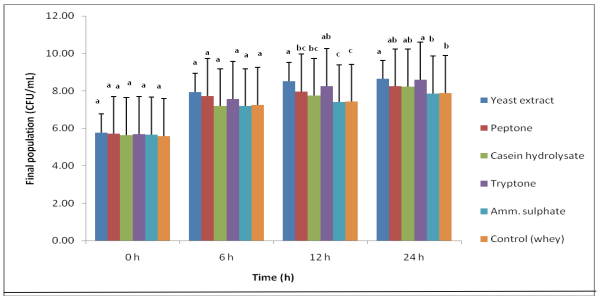

Figure 4. Effect of nitrogen source on the viable population by *L. delbrueckii* spp. *bulgaricus* ATCC 11842. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference (p<0.05)

Yeast extract provided optimum nutrients for this organism; hence, the organism produced the highest viable count (p<0.05). The bacterial grown in whey can be increased by addition of whey protein concentrate (Bury and *et al*., 1998), yeast extract (Gupta *et al*., 1995). However, effectiveness of the supplementation of these nutrients for the production of β-gal has not been studied to a great extent. Supplementation of nitrogenous sources especially yeast extract increases the amount of nutrients available to the bacteria, which could explain why there was an increase in the viable population of the organisms. This may be attributed to the growth factors in addition to the nitrogen compounds present in yeast extract (Bridson and Brecker 1970).

Effect of nitrogen sources on the pH

The effect of nitrogen source including yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey on the pH of media as affected by growing *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 is shown in Figures 5 and 6. In general, pH value of the medium containing yeast extract and peptone was lower (p>0.05) compared with other nitrogen sources for both organisms. The pH value was significantly (p<0.05) higher in tryptone among other nitrogen sources in both organisms. *B. animalis* Bb12 had significantly different (p>0.05) pH values at all fermentation times. However, a significant difference (p<0.05) was found between yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen sources; yeast extract, tryptone, ammonium sulphate and other nitrogen sources; tryptone, ammonium sulphate, whey and other nitrogen sources; and yeast extract, casein hydrolysate, ammonium sulphate and other nitrogen sources at 0 h, 6 h; 12 h; and 24 h, respectively. Likewise, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had significantly different (p>0.05) pH values at all fermentation times. The significant difference (p<0.05) was found at 0 h and 6 h in all nitrogen sources. However, significant difference was recorded between yeast extract, peptone, ammonium sulphate and rest of nitrogen sources; yeast extract, peptone, casein hydrolysate, ammonium sulphate and other nitrogen sources at 12 h and 24 h, respectively.

The decrease in pH by *B. animalis* Bb12 was lowest with yeast extract (4.53) followed by peptone (4.59) and highest with ammonium sulphate (5.47). The pH value decreased (p<0.05 by 24.37, 24.51, 22.10, 20.91, 5.53 and 14.26 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively at 24 h as compared with 0 h (Figure 5). Similarly, decrease in pH by *L.*

delbrueckii ssp. *bulgaricus* ATCC 11842 was lowest with yeast extract and casein hydrolysate (5.31) followed by peptone (5.37) and highest with tryptone (6.26). At 24 h, the pH value decreased ($p<0.05$ by 11.09, 3.10, 4.13, 4.62, and 4.23 percent in yeast extract, peptone, casein hydrolysate, tryptone, ammonium sulphate and whey, respectively as compared with 0 h (Figure 6). The drop in pH correlated with an increase in population of the two organisms.

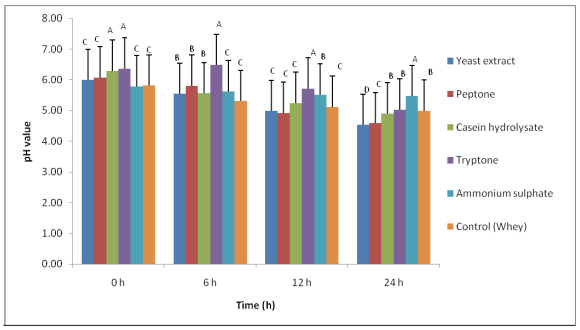

Figure 5. Effect of nitrogen source on the pH by *B. animalis* Bb12. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$)

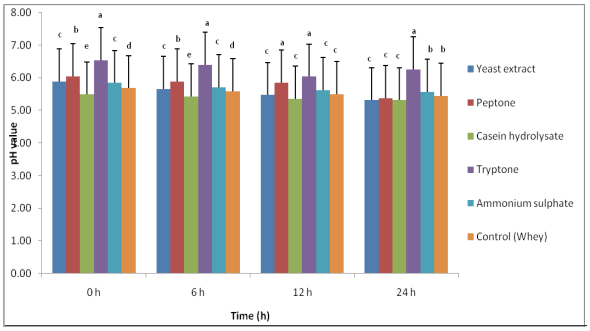

Figure 6. Effect of nitrogen source on the pH by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Medium contained 3.5% of various nitrogen sources including yeast extract, peptone, casein hydrolysate, tryptone, and ammonium sulphate in whey. Determinations were made after 24 h. Bars indicate standard deviations. Different letters, within each type of determination, indicate significant difference ($p<0.05$)

Conclusion

The addition of nitrogen sources (especially yeast extract and peptone at 3.5%) in deprotenized whey can increase the β-gal by these organisms. Whey medium supplemented with nitrogen sources could be suitable for fermentation for *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. In addition, due to high yield of β-galactosidase with *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, these organisms could be potential sources for the production of β-gal.

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