Effect of whey permeate and yeast extract on metabolic activity of *Bifidobacterium animalis* subsp. *lactis* Bb 12 cultivated in skim milk based media

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Abstract
In fermented products containing Bifidobacteria, factors such as organic acid concentration and β-galactosidase activity are important in the development of flavor and texture of final products. Both the process conditions and medium components have significant effects on fluctuation of such factors. The effects of whey permeate powder and yeast extract concentrations, as nitrogen sources was investigated, on metabolic activity of *Bifidobacterium animalis* subsp. lactis Bb 12 in skim milk based media. Development of organic acids, growth rate and β-galactosidase activity under uncontrolled pH conditions was also monitored. Media with high concentrations of nitrogen sources showed maximum viable counts of *B. animalis*. The activity of β-galactosidase increased during the logarithmic phase and the initial stage of the stationary phase of growth and then decreased thereafter. Increasing the yeast extract concentration resulted in an increase in the specific sugar consumption rate with concomitant reduction in formation of acetic acid and formic acid. Consequently, the molar ratio of acetic acid to lactic acid was decreased. Using limited amounts of nitrogen sources resulted in more organic acid production in the tested microorganism. During the early hours of fermentation in which the amounts of nitrogen levels were limited, the specific rate of β-glactosidase activity was very high. Therefore, by evaluating the activity of this enzyme we can estimate the amount nutrient components in the medium.The production of succinic acid was observed during logarithmic and stationary phase in all fermentation media. Citric acid that naturally exists in whey and skim milk powder was consumed during the stationary phase of growth by *B. animalis* and its consumption correlated significantly with the production of succinic acid at this stage.

Keywords**:** Bifidobacterium; Nitrogen source; Metabolic activity

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INTRODUCTION

A predominant group of the colonic microflora is Bifidobacteria that can account for 25% of the total number of the colon bacteria (Macfarlane and Macfarlane, 1997). Bifidobacteria are Gram-positive, non-sporulating, non-motile, and usually catalase-negative anaerobes with various shapes (Gomes and Malcata, 1999). Bifidobacteria are heterofermentative and are thus able to produce lactic acid, ethanol, acetic acid, formic acid, small amounts of carbon dioxide and succinic acid. These bacteria provide their hosts, with several benefits like vitamin production, anticarcinogenic activity, immunostimulating effects, lowering cholesterol levels and inhibiting the growth of pathogenic bacteria by creating a shift in intestinal pH induced by the acidic metabolites during carbohydrate fermentation (Dunne and Shanahan, 2002; Buck and Gilliland, 1994). One of the strains usually used in food industries is *Bifidobacterium animalis* subsp. *lactis*, which is suitable partially due to its industrial properties such as grow on milk- based media (Janer *et al*., 2005).

Considering the successfully growing market of functional foods, the scientific and industrial divisions

working on technological and sensory aspects of the probiotic food products are of supreme importance. Besides, despite the beneficial health effects for consumers, a pleasant taste is also essential for all food products containing such supplements. For example, succinic acid has the right flavor for a dairy product but acetic acid has negative effects on the organoleptic characteristic of food. In order to get the satisfactory sensory qualities of sourness and firm coagulum in food products, the final concentration of lactate of approximately 8000 mg/kg and a pH range between 4.2 and 4.4 will be acceptable (Dudley and Steele, 2005; Østile *et al*., 2005).

Metabolic changes are very important from a technological standpoint, since the amount of organic acids plays a key role in flavor and texture development of the fermented products. Food products are complex media with different amounts of carbohydrate, organic nitrogen and other organic compounds. Besides, under natural conditions, the fermentation of food happens within an uncontrolled pH range. Each of the mentioned factors can affect metabolic activity of bifidobacteria (Marisa *et al*., 2006, Mlobeli *et al*., 1998). Metabolic activity of *Bifidobacteria* presents an interesting area of research, which concerns the effects of several factors, such as pH, temperature, encapsulation and carbohydrate source on this activity (Meulen *et al*., 2006; Marisa *et al*., 2006; Doleyres *et al*., 2004; Schmidt and Zink, 2000). However, substrate preferences of bifidobacteria especially with regard to nitrogen sources, organic acid production and mechanism of metabolic reactions are still not well understood and developed.

The aim of this study was to examine the effects of whey permeate powder and yeast extract as nitrogen sources in batch fermentation at uncontrolled pH, on the growth and metabolic activity of *Bifidobacterium animalis* subsp. *lactis* Bb 12. We also investigated the production and consumption of organic acids as well as the activity of β-galactosidase, which are important criteria in the organoleptic properties of food.

MATERIALS AND METHODS

Strain and culture Media: *Bifidobacterium animalis* subsp. *lactis* Bb 12 was purchased from Hansen factory Christian Hansen (HØrsholm, Denmark). Commercial whey (WP) and skim milk powder were supplied by a local factory (Zarin Laban Pars, Karaj, Iran). Skim milk and whey powder contained 1.3% and 1% citric acid, respectively). Skim milk and whey powder were rehydrated with distilled water were used to prepare 6 and 10% total soluble solids solutions, respectively. Culture media were prepared using these solutions and yeast extract (YE) (Sigma-Aldrich, Spain) at different levels according to Table 1. Therefore, six levels of WP and YE concentration were used. Low concentration of WP and low or high amount of YE (LWPLYE and LWPHYE), medium concentration WP and low or high amount of YE (MWPLYE and MWPHYE), high concentration of WP and low or high amount of YE (HWPLYE and HWPHYE). In all the media, lactose concentration was adjusted to 56 g/l.

Fermentation: To prepare the culture for fermentation, frozen culture of the *B*. *animalis* was added to 10 ml of Man, Rogosa and Sharpe medium (MRS) broth with 0.5 g/l cysteine hydrochloride (Difco, Detroit, USA) The culture was incubated at 37ºC under anaerobic conditions (glass jar with gas pack type C) for 10-12 h. One ml sample of this active culture was then inoculated in to batch of 150 ml of each six culture media. This culture was incubated as mentioned above and then used as a preculture.

A 2-L glass bioreactor (BIOFLO 2000, New Brunswick Scientific Co., New Brunswick, NJ, USA,

Table 1. Media composition used in the fermentation experiments.

Media	Whey rehydrated (%) (V/V)	Skim milk rehydrated (%) (V/V)	Yeast extract g/l
LWPLYE	10	80	10
LWPHYE	10	75	15
MWPLYE	30	60	10
MWPHYE	30	55	15
HWPLYE	50	40	10
HWPHYE	50	35	15

Edison) was used for all fermentations. The bioreactor vessel (autoclaved at 121ºC for 15 min), was filled with 1960 ml of six heated media in each fermentation run respectively (autoclaved at 121°C, 5 min, then cooled for 5 h at room temperature and reheated under similar condition) and was then inoculated with 40 ml of preculture. Batch fermentation with uncontrolled pH was carried out at 37 ± 1°C. Agitation speed was set at 60 rpm and fermentations were conducted under anaerobic condition with CO_2 purging for 24 h. Sampling was carried out at 2 h intervals during a 24 h period.

Viable cell enumeration: *B. animalis* viable cell counts were carried out by plating diluted samples onto MRS agar (Difco, Detroit, USA), according to the pour plate method. Plates were incubated for 48 h at 37°C in anaerobic jars.

Biomass determination: The method by Desjardins *et al*. (1990) was used for estimation of biomass concentration.

β-Galactosidase activity assay: β-Galactosidase activity was measured with the method of Hekmat and Machahon (1992).

Calculation of specific rates of enzyme synthesis (ε): Calculations were carried out using this formula:
$\varepsilon = dE.dt^{-1}.X^{-1}$
Where dE, dt, and X are: Change in enzyme activity during the time interval, time interval, and biomass (mg/ml), respectively (Astapovich and Ryabaya, 2006).

High performance liquid chromatography (HPLC): The level of organic acids' (lactic, formic, succinic, citric and acetic acids) was determined by HPLC analysis (Cecil 1100, Cambridge, UK) using a C_{18} column (Waters, USA), UV detector (Waters, USA) and gradient eluant, according to the method of Tormo and Izco (2004). Lactose concentration was also determined by HPLC, but with a Eurokat H column (Waters, USA). Acidic water (pH 2), adjusted with H_2SO_4, (Merck, Germany, Darmstadt)) was used as an eluant. Flow rate was set at 1 ml/min and operation conditions were kept constant at 65ºC.

Statistical analysis: For comparison of the mean values obtained from different treatments, the Duncan multiple range by the SPSS software (version 16) was used; the selected significance levels were $P < 0.01$ and $P < 0.05$. All experiments were carried out in triplicate.

RESULTS

Effect of different media on cell growth: Table 2 shows data obtained from the growth of *B. animalis* subsp. *Lactis* Bb 12 on different media. The cell counts were always more than 1.0×10^7 CFU/ml. A maximum population of 3.3×10^7 CFU/ml was achieved in the HWPHYE medium. A significant decline in viable count of *B. animalis* was observed during fermentation in other media. Increasing levels of YE and WP influence the growth of the cells considerably. A minimum cell count of 1.46×10^7 CFU/ml was obtained after growth in the LWPLYE medium that had minimum levels of YE and WP. Supplementation of the medium with YE resulted in an increase in the viable cell count at 10% (v/v) and 50% (v/v) WP levels. The cultures grown in all levels of WP reached maximum growth but followed by a sudden cessation after 12 h of culti-

Table 2. Chemical and microbiological characteristics of *B. animalis* subsp. *lactis* Bb12 cultured in skim milk based mediums under uncontrolled pH coditions, after 24.

Medium	Lactic acid (mM)	Acetic acid (mM)	Formic acid (mM)	Succinic acid (mM)	Citric acid (mM)	Viable count log of (CFU/ml)	Maximum β-galactosidase activity[1] (Units/ml)	Lactose consumption (g/l)	Log phase time (h)
LWPLYE	94.4^{c} (±3)	118^{ab} (±4)	13^{b} (±1)	2.8^{c} (±0.2)	10.1^{b} (±0.8)	7.16^{b} (±0.2)	351^{c} (±10)	28^{c} (±2)	12
LWPHYE	94.3^{c} (±3.1)	85.7^{c} (±4)	11^{c} (±0.7)	3^{c} (±0.8)	9.5^{b} (±1)	7.175^{b} (±0.15)	340^{c} (±8)	33^{b} (±1)	12
MWPLYE	130.7^{a} (±5)	133^{a} (±5)	15^{a} (±1)	5.75^{a} (±0.5)	13.8^{a} (±1.2)	7.18^{b} (±0.22)	405a (±11)	32^{b} (±1)	12
MWPHYE	131.8^{a} (±5.5)	101.5^{b} (±3)	13b (±0.5)	5.77^{a} (±0.9)	12.85^{a} (±0.8)	7.185^{b} (±0.18)	420^{a} (±13)	39^{a} (±3)	12
HWPLYE	112.44^{b} (±2)	123.7^{a} (±5)	15^{a} (±1.2)	5^{ab} (±1)	11.32^{a} (±0.5)	7.43^{a} (±0.31)	377^{b} (±9)	37^{a} (±3.3)	14
HWPHYE	114.41^{b} (±2.2)	91.7^{c} (±3)	11.3^{c} (±0.6)	4.4^{b} (±0.5)	11.78^{a} (±1)	7.52^{a} (±0.03)	360^{b} (±8)	39^{a} (±3.6)	14

[1]Attained by 24 h. *Means with the same letter (by columns) are not significantly different (P >0.05).

vation (in low and intermediate levels of WP) or 14 h (in high levels of WP) (Table 2). In all media growth was diauxic, similar to that in the HWPHYE medium (Fig. 1).

Effect of media on β-galactosidase synthesis rate and specific activity: As shown in Table 2, β-galactosidase activity of the strain correlated with changes in YE and WP levels. Maximum activities of the enzyme occurred in the MWPLYE and MWPHYE media, which were 420 U/ml and 405 U/ml, respectively. In all cultures, conditions similar to the HWPHYE medium, β-galactosidase activity increased during the exponential phase of growth and the beginning of the stationary phase, and decreased thereafter (Fig. 1). In the HWPLYE and HWPLYE media, the time period for β-galactosidase activity were 2 longer than other media. At three levels of WP, increasing theYE concentration from 10 g/l to 15 g/l was accompanied with increase in enzyme activity. As the Figure 2 shows, the MWPLYE and MWPHYE media had the maximum specific rate of enzyme synthesis and the HWPHYE medium resulted in the minimum ε during this time period.

Variation of product formation and selected kinetic parameters: Results of acid production or consumption, substrate utilization and some kinetic parameters related to growth and product formation are illustrated in Tables 2 and 3 and Figure 3. Considering the specific sugar consumption rate, lactose was consumed faster in the MWPLYE and MWPHYE media (Table 3), although total lactose consumption in the HWPLYE and HWPHYE media was higher than the other media (Table 2). According to initial and residual lactose concentrations, the carbohydrate source was not a limiting factor in all cultivations. The final concentration of organic acids produced after 24 h of fermentation with uncontrolled pH, varied with respect to the type of culture medium used (Table 2). Similar to what was observed in the HWPHYE medium, lactic and acetic acids were produced throughout the exponential and stationary phases of growth in other mediums. Total amounts of lactic and acetic acids formed during the stationary phase were less than that formed during the exponential phase (Fig. 3). At three levels of WP, the increase in YE concentration resulted in the elevation of total lactic acid production. Maximum quantities of lactic acid were produced by fermentation in MWPLYE and MWPHYE media, and minimum lactic acid formation occurred in the LWPLYE and LWPHYE medium (Table 2). Maximum levels of of acetic acid were formed by fermentation in the MWPLYE medium (Table 2). The molar ratio of acetic acid to lactic acid (A/L) varied between 1.25-0.78 and was different according to the medium composition. At three levels of WP, an increase in the YE concentration from 10 g/l to 15 g/l caused a reduction in the molar ratio. In all cultivations, production of formic acid started from late stage of the growth phase and continued well into the stationary (Fig. 3). MWPLYE medium resulted in the formation of the highest quantities of formic acid (Table 2). Faster rates of specific sugar consumption, correlated with larger amounts of lactic acid and smaller amounts of acetic and formic acids formation (Table 2 and 3).

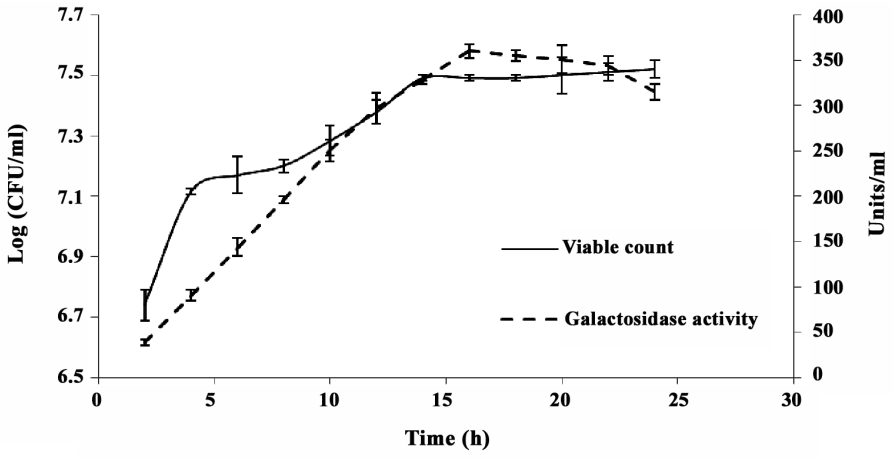

Figure 1. Growth and β-galactosidase activity of *B. animalis* subsp. *lactis* Bb 12 growth in the HWPHYE medium.

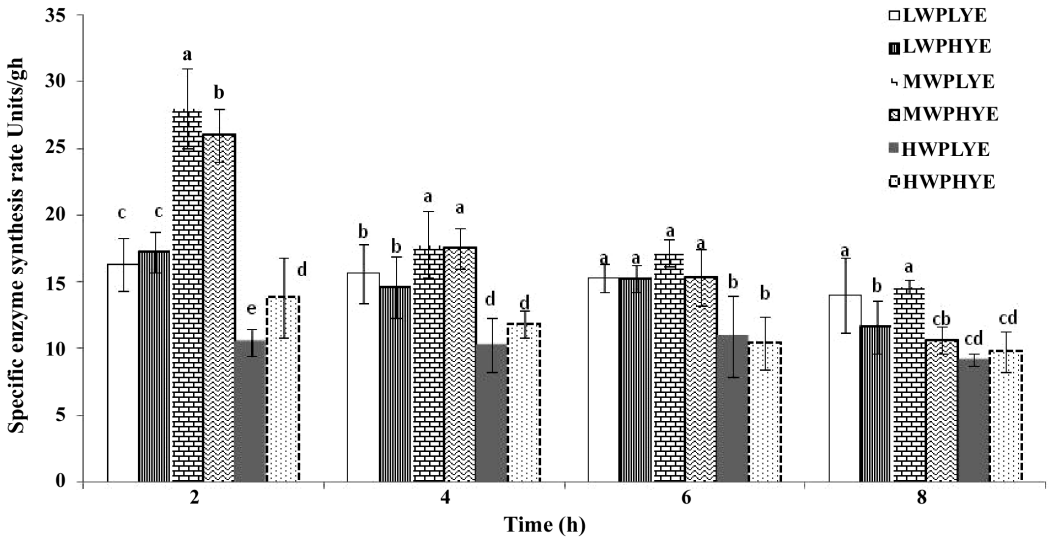

Figure 2. The specific β- galactosidase synthesis rate of cells cultivated in skim milk based media with different concentrations of WP and YE. Means are significantly different (P<0.01).

In all the media, succinic acid production coincided with the logarithmic and stationary phases of growth. Succinic acid levels produced during the growth phase was smaller than those in the stationary phase (Fig. 3). Maximum aforementioned acid concentrations belonged to the MWPLYE and MWPHYE media. At the 10% (v/v) WP level, succinic acid production did not cease after 24 h of fermentation. At other WP levels, the said acid production stopped at the late stage of the stationary phase (data not shown). Reduction in citric acid levels occured during stationary phase for HWPHYE cultivation (Fig. 3). A similar reduction pattern was observed in other mediums. Regression calculation showed sever correlating between succinic acid production and citric acid reduction during stationary phase (R^2 = 0.9). Therefore, each medium leading to larger levels of of succinic acid production, also had greater levels of citric acid consumption. Consequently, maximum citric acid reduction was observed in MWPLYE medium.

Product yield coefficient [$Y_{p/x}$ g acid (lactic, acetic and formic acid) g/biomass] varied with culture fermentation too (Table 3). Maximum coefficient values were determined at the 30% (v/v) WP concentration level. At 50% (v/v) WP concentration level, increasing the YE concentration from 10 to 15 g/l accompanied with the product yield coefficient reduction.

DISCUSSION

Variations in cell population can correspond with

Table 3. Specific lactose consumption rate and $Y_{P/X}$ of *B. animalis* subsp. *Lactis* Bb 12 grown in skim milk based media with different concentration of WP and YE.

Media	$Y_{p/x}$	Specific sugar consumption rate (g.lactose/g.bi omass/h)
LWPLYE	9.1^{c} (±1)	0.86^{b} (±0.01)
LWPHYE	8.3^{cd} (±0.9)	0.93^{b} (±0.02)
MWPLYE	10.7^{b} (±0.5)	0.99^{a} (±0.05)
MWPHYE	13.8^{a} (±0.8)	1.44^{a} (±0.1)
HWPLYE	8.5^{c} (±1.1)	0.93^{b} (±0.06)
HWPHYE	7.1^{d} (±0.4)	0.94^{b} (± 0.04)

* Means with the same letter (by columns) are not significantly different (*P* >0.05).

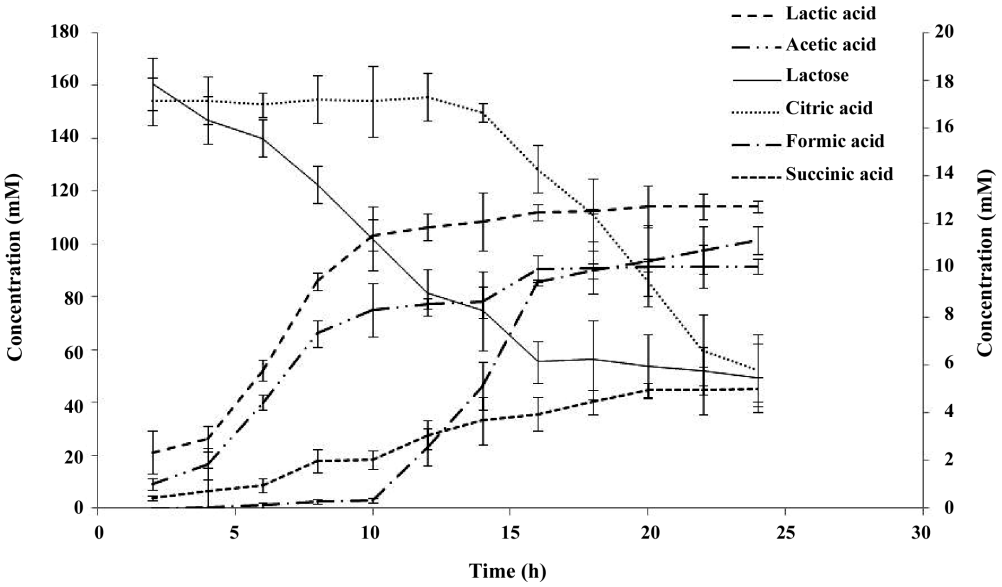

Figure 3. Organic acid production of *B. animalis* subsp. *lactis* Bb 12 in the HWPHYE medium during a 24 h time period. Right axis indicates succinic acid, formic acid and citric acid concentration and left axis indicates lactic acid, acetic acid and lactose concentration.

changes in organic acids production or substrate limitation. Deleterious factors such as high concentrations of hydrogen ions that are supplied by the acidic metabolic products function as auto-inhibitors of cell growth (Wang-june and Seong-kwan, 1995). Therefore, accumulation of this factor in the culture medium is the main reason for cessation of growth and reduction in the viable cell population. Some researchers have reported that initial lactate and acetate concentrations higher than 10 g/l rapidly decreasing the growth rate of *B. longurn* YT 4021**,** or causing it to cease, occur in media containing both lactate and acetate at concentrations higher than 18 g/l (Desjardins *et al*., 1990; Taniguchi *et al*., 1987). According to the results of this study, growth cessation occured at different concentrations of organic acids present in the culture medium. For example in the MWPLYE media, which led to maximum acids production, growth cessation occurred at 245.6 mM lactic, acetic and formic acid concentrations and this inhibitor concentration for HWPLYE and LWPHYE mediums was 210 and 149.7 mM respectively. This variation can interoperate with different redox potentials with respect to the varying media. Whey powder and milk are rich in sulfur-containing amino acids and liberate these amino acids during heat treatment, which can decrease medium's redox potential and neutralize the acidic inhibitory effect (Dave and Shah, 1998). As a result, in each media total acid concentration that causes cessation growth related to maximum possible redox potential of media.

On the other hand, in this study carbon source was not a limitation factor (Fig. 3). Therefore, related to cells population, more acid production in MWPLYE and MWPHYE mediums than other media could be explained by usable nitrogen amounts. Whey and yeast extract have nitrogen compounds that can be used by bifidobacteria (Hsu *et al*., 2005; Ibrahim and Bezkorovainty, 1994). Milk does not have sufficient amounts of this type of nitrogen source (Gomez and Malcata, 1999). In cases of carbon sufficiency and nitrogen source limitation, $Y_{P/X}$ will increase (Amrane and Prigent, 1998a; Amrane and Prigent, 1998b). As Table 3. shows, at the 30% (v/v) WP level, $Y_{P/X}$ increases when compared to other levels. It can be concluded that the bacteria may show more catabolic than anabolic activity at this level or it could be that bacteria directly use up the available energy for maintenance rather than growth (Beal and Corrieu, 1995). Therefore, at 50% (v/v) WP level, 10 or 15 g/l of YE may be sufficient sources of usable nitrogen and thus the microorganism can have normal anabolism and catabolic behavior, and at 10% (v/v) WP, with 10 or 15 g/l YE usable nitrogen amount is not sufficient for either good growth or acid production.

As observed in all experiments mediums *B. animalis* had diauxic growth and these results were in agreement with behaviour of this microorganism pre-

viously recorded for cultivation on milk based media (ABU-Tarboush *et al*., 1998).

Some researchers have reported that yeast extract contains small peptides and vitamins that improve growth and β-galactosidase activity at the 1.5-10% concentration level (Hsu *et al*., 2005; Vasiljevic and Jelen, 2002). The results of this investigation were in accordance with these findings, except at the 30% (v/v) WP level where there was no correlation observed between growth and enzyme activity and hence with specific rate of enzyme synthesis. Bifidobacteria can degrade carbohydrates to acetic and lactic acids as major metabolites through the fructose-6-phosphate shunt, and theoretically acetic to lactic acid (A/L) molar ratio formation is 3:2 (Wolin *et al*., 1998). At high intracellular sugar concentrations (medium with no carbohydrate-limitation), such as media in this study, bifidobacteria preferentially convert pyruvate to lactic acid trough the conventional catabolic route (Madiedo *et al*., 2005). In lactic acid bacteria, lactate dehydrogenase that have major role in conversion of pyruvate to lactate has inhibitory effects on acetic and formic acid production (Neves *et al*., 2005). In this research, increasing in specific sugar consumption rate accompanied with lager amount of lactic acid production and lesser quantity acetic and formic acid formation, and the A/L molar ratio decreased and in agreement with previously recorded. (Meulen *et al*., 2006). This investigation has shown that several correlations existed between citric acid consumption and succinic acid production during the stationary phase of growth. In Kegg site [http://www.genom.jp/kegg/kegg2.html] for *B*. *animalis* subsp. *lactis* Ad011 two mechanism has been suggested for production of succinic acid from citric acid. Therefore, it can be assumed that in the case of *B*. *animalis* subsp. *Lactis* Bb 12, succinic acid could be produced from similar pathways. Production of succinic acid can be a way to equilibrate the redox balance (Meulen *et al*., 2006). Thus, in the media that the tested microorganism produced larger amount of lactic, acetic and formic acid, greater quantity of succinic acid formation and citric acid reduction was observed. Variation in the initial concentration of citric acid was related to the WP and skim milk percentage in the culture medium. When whey permeates percentage increased in culture media, media have larger amounts of citric acid.

A major problem with food that contain Bifidobacteria are the organoleptic properties. This study showed that nitrogen compounds have strong affects on the metabolic activity of bifidobacteria. In the case of insufficient nitrogen amounts, metabolic activity shifts from anabolism to catabolism and this shift is concomitant with variations in β-galactosidase activity and organic acid production, especially with regard to the A/L molar ratio that plays an important role in the organoleptic acceptance of the final fermented food. Results of this research showed that the specific β-galactosidase synthetic rate during the early hours of fermentation can be used as an indicator for this shift.

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