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Full Length Research Paper

Energy sources of yoghurt bacteria and enhancement of their galactose uptake

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The energy sources of yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) were examined with a focus on probable impact of sucrose on their galactose uptake. Yoghurt bacteria were isolated from samples of yoghurt which were purchased from different outlets and kept under refrigeration conditions throughout the period of isolation using nutrient agar (NA) and potato dextrose agar (PDA). After obtaining pure cultures of the isolates which were placed on NA and PDA slants, cultural characteristics and biochemical tests were done on them for identification purposes. Their abilities to ferment glucose, lactose fructose, maltose, sucrose and galactose as carbon source were examined. The effect of sucrose on uptake of galactose by the isolates was also examined. The four strains of *L. bulgaricus* (LBI, LB2, LB3 and LB4) and one strain of *S. thermophilus* (ST5) obtained had similar characteristics typical of lactic acid bacteria. Sugar fermentation by the isolates differed from one strain to the other. The extent of sugar fermentation by the isolates also varied depending on the types of sugar employed as carbon source. Glucose and lactose were better when used for growth by all the isolates when compared to other sugars. All the isolates had weak fermentation of galactose when compared to other sugars. All isolates however, had better fermentation of galactose in the presence of sucrose, with LB2 having the best and LB4 the least fermentation of galactose in the presence of sucrose. Appropriate amount of sucrose could thus be employed as possible enhancer for galactose uptake by galactose non-fermentive strains of yoghurt bacteria.

Key words: *Lactobacillus bulgaricus, Streptococcus thermophilus*, lactic acid bacteria, galactose, yoghurt.

INTRODUCTION

Yoghurt, which is perhaps the oldest fermented products of milk, is generally defined as coagulated milk that results from fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Zahoor et al., 2003; Ongol et al., 2007; Bari et al., 2009). Its consumption has been reported to confer several health benefits and longevity on the consumers (Davis, 1976; Rachid et al., 2002). Due to its health benefits and taste, it is known to constitute an appreciable proportion of total daily food consumption or even just as a refreshing beverage in several countries (Khan et al., 2008). It is regarded as a nutritiously balanced food containing almost all the nutrients present in milk but in a more assimilable form (Younus et al., 2002). It is actually considered to be more nutritive than milk in terms of vitamins content, digestibility and as a source of calcium and phosphorus.

These health benefits of yoghurt are attributable to the health-promoting activities of the lactic acid bacteria contained therein which are referred to as probiotics because of these inherent health beneficial characteristics (Marteau and Boutron-Ruault, 2002; Ward et al., 2002; Gueimonde et al., 2003; Tabatabaje and Mortazayi, 2008). These bacteria, referred to as yoghurt starter culture consists of two symbiotically growing bacteria, *S. thermophilus* and *L. bulgaricus* (Tamine and Marshall, 1997), which are also known as *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus*, respectively (Ginovart et al., 2002, Ongol et al., 2007).

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Yoghurt culture bacteria (*L. bulgaricus* and *S. thermophilus*) are reported to be able to preferentially metabolize and transport a few to many sugars such as lactose, glucose, sucrose and to a lesser extent galactose (O'Leary and Woychik, 1976; Tinson et al., 1982; Hutkins et al., 1985a; Hickey et al., 1985; Poolman et al., 1988). The rate of uptake of one sugar by an organism in solution sometimes depends on presence or absence of another sugar. Lactose alone, for instance was reported to be used rapidly by *Leuconostoc mesenteroides subsp. mesenteroides* but slowly in the presence of galactose (Huang et al., 1994). A combination of lactose and glucose also resulted in the largest production of exopolysaccharide (EPS) by *S. thermophilus* LY03 when compared to using separate sugars as sole energy sources (Degeest and Vuyst, 2000). The inability of many commercial strains of *L. bulgaricus* and *S. thermophilus* to utilize galactose has however, been reported to have practical undesirable implications in a number of fermented dairy products (Hutkins et al., 1985b).

This work thus aimed to examine the general characteristics of *S. thermophilus* and *L. bulgaricus* after isolation and identification, with a focus on the possibility of enhancing their galactose uptake using sucrose.

MATERIALS AND METHODS

Collection of yoghurt samples

Ten yoghurt samples were purchased from different retail outlets. The yoghurt samples were placed in an ice chest and brought to the laboratory for microbiological analysis. They were stored immediately under refrigeration conditions until further experiments.

Isolation of *S. thermophilus* and *L. bulgaricus*

Nutrient agar (NA) and potato dextrose agar (PDA) were used for the isolation of the lactic acid bacteria with pour plate method being the isolation method (Shitata and Shah, 2002). Serial dilutions of the yoghurt samples were prepared using improvised method of Harrigan and McCance (1976) and Holt et al. (1994). One milliliter of each dilution (10^{-3}, 10^{-4} and 10^{-5}) was transferred into separate sterilized Petri plates and 15 ml of the molten media was added. The Petri plates containing the mixture were gently swirled to ensure even dispersion of the inoculum. All experiments were prepared in triplicates. Petri plates were allowed to set before incubating at 37°C (Shitata and Shah, 2002). Colonies with different cultural characteristics were sub cultured into fresh NA and PDA Petri plates to obtain pure cultures. All pure cultures were stocked using NA and PDA slants.

Characterization and identification of the isolated yoghurt bacteria

Reactions of all the isolates to Gram's staining were carried out (Collins and Lyne, 1980; Awan and Rahman, 2002). Growth of pure isolates obtained was checked for morphological and cultural characteristics (Zahoor et al., 2003). The isolates were identified using their growth patterns at different temperatures and sugar fermentation (Harrigan and McCance, 1976). Biochemical tests such as catalase test, motility test, methyl red test, Voges-Proskauer test, litmus milk fermentation test, oxygen relationship test, oxidative/fermentation test, indole production test and starch hydrolysis test (Awan and Rahman, 2002) were also performed for identification purposes with reference to Bergey's Manual (Holt et al., 1994).

Effect of temperature on growth of *S. thermophilus* and *L. bulgaricus*

One milliliter culture suspension of each isolate was dispensed in conical flasks containing 25 ml of sterilized nutrient broth after cooling. Uninoculated flasks served as control. All inoculations were done in duplicates. Incubation was done at 20, 28, 40, 45 and 55°C for 72 h. Growth of the isolates was determined as optical density (OD) at 660 nm using the colorimeter (Ongol et al., 2007).

Effect of pH on growth of *S. thermophilus* and *L. bulgaricus*

Stock buffer solution (citrate phosphate buffer) was prepared using Mcilvains (1921) method which consisted of 0.1 M citric acid (21.014 g/L) and 0.2 M disodium hydrogen phosphate (28.392 g/L). Both solutions were mixed in different proportions to obtain the required pH range of 1 to 8. One milliliter of 24 h old broth culture of each isolate was then dispensed in conical flask containing equal volumes (15 ml each) of nutrient broth and the buffer solutions. All inoculations were done in duplicates. Uninoculated conical flasks served as control. Incubation was done at 37°C for 24 h. Growth of the isolates was determined as optical density (OD) using the colorimeter.

Use of carbon sources for growth by the isolates (sugar fermentation)

One gram each of glucose, lactose, galactose, fructose, sucrose and maltose was dissolved in separate conical flasks containing 10 ml of distilled water and was sterilized by passing through 0.45 µm filter (Mehmood et al., 2009). 100 µl of each sugar was then transferred into separate sterilized test tubes of 5 ml of casein broth, labeled appropriately and placed at room temperature for 24 h to check for contamination. The conical flasks were thereafter inoculated aseptically with pure colonies of the bacterial isolates. All experiments were done in duplicates. The uninoculated flasks served as control. All flasks were incubated at 37°C for 48 h (Mehmood et al., 2009). Growth of the isolates was determined as OD at 660 nm using the colorimeter (Ongol et al., 2007). The uninoculated flask was used to standardize the colorimeter to zero absorbance.

Sucrose as an enhancer for uptake of galactose by the isolates

Thirty milliliter of sterilized casein broth was measured each into six 150 ml conical flasks. Ten milliliter distilled water containing dissolved 1.0 g each of galactose and sucrose was passed through 0.45 µm filter (Mehmood et al., 2009) and added into three of the conical flasks. Equal gram of galactose only was prepared the same way and added into the remaining three conical flasks (control). All flasks were inoculated aseptically with the isolates using sterilized wire loop, plugged with cotton wool and covered with aluminum foil. Incubation was then done at 37°C for 48 h after which test for reducing sugar was performed on the cultures using Fehling's solution A and B. Growth of the isolates was later determined as OD using the colorimeter. The uninoculated flask

Table 1. Characterization of yoghurt bacteria isolates.

Test	Morphological Characteristic		Litmus milk test										Sugar fermentation						
	Gram's stain	Cell shape	Motility test	Catalase test	Coagulase test	Methyl red	Voges Proskauer	Curd Formation	Peptonization	Indole Production	Starch hydrolysis	Fermentative ability	Oxygen relationship	Lactose	Glucose	Fructose	Galactose	Sucrose	Maltose
LB1	+	Ba	-	-	-	+	-	+	+	-	+	F	An	A	A	A	a	A	A
LB2	+	Ba	-	-	-	+	-	+	+	-	+	F	An	A	A	A	a	A	A
LB3	+	Ba	-	-	-	+	-	+	+	-	+	F	An	A	A	A	a	A	A
LB4	+	Ba	-	-	-	+	-	+	+	-	-	F	An	A	A	A	a	A	A
ST5	+	Co	-	-	-	+	-	+	+	-	+	F	An	A	A	A	a	A	A

+: Positive; Ba: Bacilli; Co: Cocci; F: fermentative; An: anaerobic; A: acid production; a: weak acid production; -: negative; LB1: *L. bulgaricus* strain 1; LB2: *L. bulgaricus* strain 2; LB3: *L. bulgaricus* strain 3; LB4: *L. bulgaricus* strain 4; ST5: *S. thermophilus*

was used to standardize the colorimeter to zero absorbance.

RESULTS

Characterization of the yoghurt bacteria

Five different isolates were obtained in total viz., four strains of *L. bulgaricus* (LBI, LB2, LB3 and LB4) and one strain of *S. thermophilus* (ST5). Results of the biochemical tests and Gram's staining on the bacterial isolates are shown in Table 1. Both *S. thermophilus* and *L. bulgaricus* had similar metabolic characteristics. The rate of evolution of carbon (iv) oxide (CO_2) from different cultures varied, being slowest in cultures of LB2. LB3 had the deepest red coloration for the methyl red test, while LB2 had the lightest red coloration. All isolates grew mainly in the lower portion of the test tubes to an average of 2.8 cm up to the length of the test tubes containing Hugh and Leifson medium (1953).

Effects of temperature and pH on growth of the isolates

Effects of temperature and pH on growth of the isolates are shown in Figures 1 and 2. The optimal growth temperature for all the isolates was generally shown to be 40°C (Figure 1), even though all the isolates also had good growth at 28 and 45°C. All isolates had the poorest growth at 20°C; they also had low growth at 55°C (Figure 1). LB1 however had better growth than the rest at 55°C (Figure 1). All ranges of pH employed supported the growth of all the isolates. Optimal growth of all isolates occurred at pH 5 except isolate ST5 which had pH 6 supporting its optimal growth. However, growth of all isolates was poorest at pH 8 (Figure 2).

Utilization of carbon sources by the isolates

Extent of sugar fermentation by the isolates varied depending on the types of sugar used as carbon source. LB2 and LB4 utilized glucose best for growth followed by lactose, while LBI, LB3 and ST5 grew best in lactose, followed by glucose. However, all the isolates fermented galactose weakly with LB3 fairing slightly better than the rest in galactose fermentation. They all had poor growths in galactose when compared to their growth in other sugars (Figure 3).

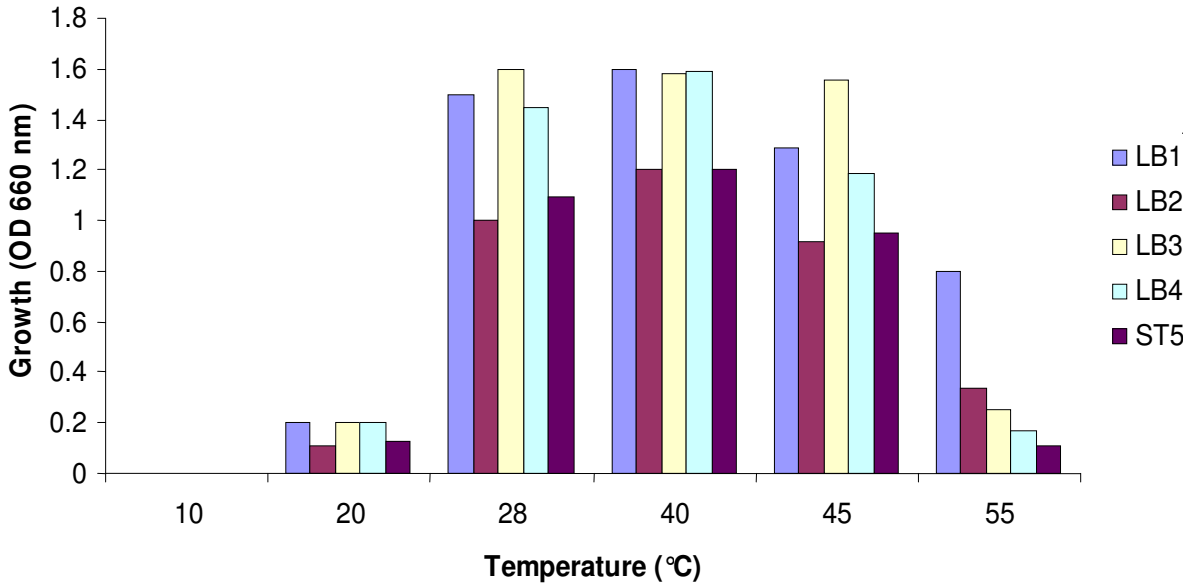

Figure 1. Effect of temperature on growth of yoghurt bacteria incubated for 72 h.

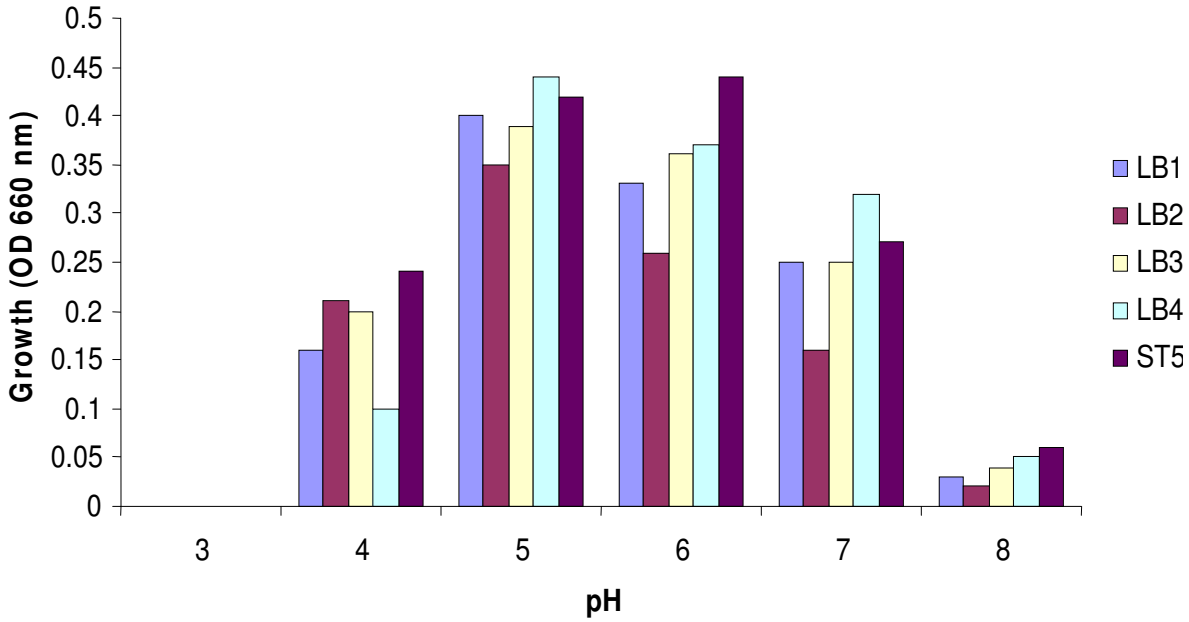

Figure 2. Effect of pH on growth of yoghurt bacteria at 37°C for 24 h.

Sucrose as an enhancer for uptake of galactose

All isolates had better utilization of galactose in the presence of sucrose. Isolate LB2 had the best and LB4 the least utilization of galactose in the presence of sucrose (Figure 3). All the cultures slowly turned a very light dirty brown colour on adding Fehling's solution A and B. The colour slowly turned light red on heating. In the confirmatory test for galactose utilization, the cultures turned brown immediately and brick red on heating.

DISCUSSION

Overall, morphological and biochemical characteristics of the isolates agreed with the works of Davis (1976), Sperber and Swan (1976), Zahoor et al. (2003) and Mehmood et al. (2009), thus confirming identity of the isolates as yoghurt bacteria. Growth of the isolates in the lower portion of test tubes containing Hugh and Leifson medium indicated their anaerobic character as reported by Taylor et al. (1974). However, their growth few

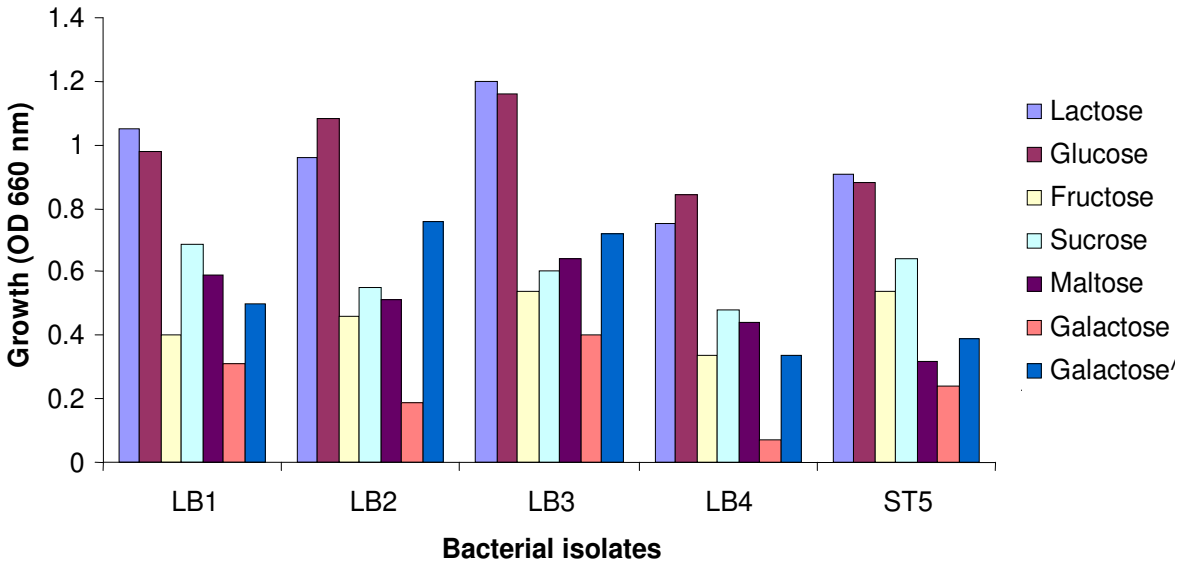

Figure 3. Sugar fermentation by yoghurt bacteria. Enhanced galactose fermentation obtained in the presence of sucrose (galactose^).

centimeters (2.8 cm) up the test tubes also showed their aero-tolerance. Drinan et al. (1975) reported that they are strongly unaffected in the presence of air.

Growth performance of the isolates at different temperatures and pH further confirms their identity. The optimum growth temperature for LBI, LB2, LB3, LB4 and ST5 which was close to 40°C agreed with the works of Davis (1976) and Shitata and Shah (2002). However growth performance of all the isolates at 28 and 45°C agreed with the results of Folkenberg et al. (2006) who concluded that changing fermentation temperature may have little effects on texture of yoghurt. Thermo-tolerance of all the isolates was shown by their growth at 55°C even though the growths were not very pronounced. However, extreme thermo tolerance of LBI over other isolates was shown by its better growth at 55°C. The pH 5 obtained for optimum growth of most of the isolates agreed with the works of Davis (1976), Younus et al. (2002) and Folkenberg et al. (2006). However, the pH 6 obtained for optimum growth of *S. thermophilus* also agreed with Wright and Klaenhammer (1983) who obtained similar results for yoghurt bacteria. Temperature and pH are among the factors affecting viability of yoghurt bacteria (Bari et al., 2009). Results obtained for sugar fermentation by the isolates agreed with those obtained by Hutkins et al. (1985b), Hickey et al. (1985), Audet et al. (1988) and Zahoor et al. (2003). It showed that all the isolates are weak galactose fermentive strains. The positive results obtained for maltose fermentation however contrasted with the reports of Davis (1976). Since maltose contains 2 molecules of glucose which is fermentable by the isolates, the results might not have been negative as reported by Davis (1976).

The utilization of glucose which is better than other sugars by LB2 and LB4 agreed with the reports of Hickey et al. (1985) who submitted that the *Lactobacilli* utilize glucose as their primary energy source. The better growth of LB1, LB3 and ST5 in lactose when compared to glucose might however not be much of a deviation from the reports of Hickey et al. (1985). This is because it has been reported that when *L. bulgaricus* and *S. thermophillus* are grown in a lactose containing medium, only the glucose moiety of the lactose is used and free galactose is released into the medium (O'Leary and Woychik, 1976; Thompson et al., 1977; Thomas and Crow, 1984).

The poor utilization of galactose by these isolates, which agreed with the work of Thomas and Crow (1984), however shows that the isolates could be regarded as poor fermenters of galactose or galactose non-fermentive ($Gal^{(-)}$) strains. In the experiments of Thomas and Crow (1984), the $Gal^{(-)}$ strains used released less galactose than the total available from lactose hydrolysis indicating partial utilization of galactose. The slow change in colour of casein broth to light dirty brown and to light red in the test for reducing sugar when sucrose was used as energizer for galactose uptake indicated the presence of galactose only in trace amount. This showed that the isolates, which are poor fermenters of galactose, were able to utilize galactose better in the presence of sucrose. This was also established in the confirmatory test where the cultures immediately turned brown and later to brick red on heating, indicating considerable presence of galactose in the medium when sucrose was absent. It showed that the addition of a suitable metabolizable energy source such as sucrose could lead to better uptake of galactose by the isolates. Suitable energizers (or enhancers) for galactose uptake was however

reported to be few or limited in number by Hutkins et al. (1985b) and Hickey et al. (1985). According to them, the range of compounds fermentable by yoghurt cultures is narrow and this limits possible energizers for galactose uptake. They reported that many of the few remaining ones (energy sources) like lactose and glucose compete with galactose, inhibiting its uptake when present together with it in a medium, thus leaving out sucrose as an appropriate possible enhancer. It has however been reported that excess amount of sucrose could affect overall quality of yoghurt. Suzuki et al. (1986) reported that presence of 4% or more of sucrose leads to decreased production of by-products of sugar fermentation such as diacetyl, acetaldehyde, pyruvic and formic acids produced by both *S. thermophilus* and *L. bulgaricus*, all of which enhanced lactic acid production and cell growth of these yoghurt organisms leading to overall, "roundness" of the characteristic yoghurt flavour. This might pose a challenge in the use of sucrose as an enhancer for galactose uptake or as a sweetener to reduce sharpness. Hutkins et al. (1985a) and Tinson et al. (1982) reported that the inability of *L. bulgaricus* and *S. thermophilus* strains to utilize galactose (in the absence of energizers) may be due to either the absence of one or more catabolic enzymes or the absence of galactose transport system through which galactose could be transported. This might be one of the reasons for the poor utilization of galactose by the isolates. Appropriate experiments will however be needed before credible assertions on this could be made. Conclusively, it could thus be said that instead of lactose, glucose could rather be tagged the primary energy source for yoghurt organisms. Galactose-fermentive ($Gal^{(+)}$) strains rather than galactose-non fermentive ($Gal^{(-)}$) strains might be employed more often as yoghurt starter cultures, as this might address to an extent, problem of early contamination of yoghurt. However, for continued use of $Gal^{(-)}$ strains of *L. bulgaricus* and *S. thermophilus* (as there are more reported cases of $Gal^{(-)}$ strains than $Gal^{(+)}$ strains), appropriate amount of sucrose could be considered as an enhancer. However, if sucrose will not be considered as an enhancer, more research may still be needed on better and more appropriate enhancer(s) for galactose uptake by yoghurt organisms. This may be so, as the inability of many commercial strains of *L. bulgaricus* and *S. thermophilus* to utilize galactose has been reported to have practical undesirable implications in a number of fermented dairy products.

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