J. Agric. Sci. Mansoura Univ., 31 (3): 1585 - 1603, 2006

STUDIES ON THE PRODUCTION OF MICROBIAL β-GALACTOSIDASE:
3- OPTIMIZATION OF β-GALACTOSIDASE PRODUCTION BY *Streptococcus salivarius* SUBSP. *thermophilus* USING WHEY.

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ABSTRACT

β-galactosidase production by *Streptococcus salivarius* subsp. *thermophilus* increased as the whey concentration raised up to 5.48% (4.0% lactose). Lactose was exhausted till 4.11% whey concentration (3.0% lactose) after 24 hours. Growth rates and lactose utilization were increased as the whey concentrations increased up to 5.48% whey (4.0% lactose). Lactose media yielded 42.93% less β-galactosidase activity than whey media. A 21.33% decrease in lactose utilization was observed while about 5.0% increase in growth rate was obtained. The decrease of pH was much smaller in the whey- than in lactose-containing medium. Maximum β-galactosidase production by bac erial species in whey medium was observed between pH 6.5 to 7.5. Whereas maximum growth rate recorded at pH 6.0 to 7.5. Cells lowered the pH from an initial pH of 7.0 to 4.5 after 24 h of growth. A progressive increase in β-galactosidase production by *Str. salivarius* subsp. *thermophilus* was observed up to 24 h of incubation, after which it become constant for another 24 h, whereas growth rate was still increased. The optimum temperature for β-galactosidase production coincided with the optimum temperature for the growth of the organism, about 40°C. Lactose utilization by the organism also increased around the optimum temperature. Higher β-galactosidase levels from *Str. salivarius* subsp. *thermophilus* were obtained when either whey (4.0% lactose) or whey (2.0% lactose) supplemented with 2.0% galactose was used. Supplementation of whey with glucose repressed β-galactosidase formation. Supplementation of whey with other sugars tended to decrease the level of β-galactosidase to variable degrees. $(NH_4)_2SO_4$ promoted the highest level of β-galactosidase biosynthesis in *Str. salivarius* subsp. *thermophilus* than other nitrogen sources. The stimulation in β-galactosidase production by organic nitrogen supplements. In general, nitrogen sources did not have great effect on the growth rate or lactose utilization. β-galactosidase production increased with increasing of $(NH_4)_2SO_4$ concentration until reaching a maximum yield for enzyme at 0.5%. Further increase $(NH_4)_2SO_4$ concentration has diverse effect on increasing enzyme production and growth rate. NaH_2PO_4 supported the highest level of β-galactosidase production by tested bacterial species. Whereas the other phosphorus sources tended to decrease the level of enzyme to variable degrees. The maximal β-galactosidase biosynthesis was reached at a 0.5% (w/v) level of NaH_2PO_4. The supplementation of whey with Mn^{++}, Mg^{++}, Ca^{++} and Na^{+} led to significant stimulation for bacterial β-galactosidase production. Monovalent salts, i.e., K^{+} or Na^{+} exerted a slight stimulatory effect indicating that the levels of these salts in whey were sufficient.

INTRODUCTION

Lactose, in milk at about 4.8%, is a rather insoluble, not sweet sugar which may have a mild laxative effect when consumed in large amounts because a large number of adults and children have a low tolerance for lactose. These lactose-sensitive individuals suffer from bloating cramps, diarrhea, and malaosorption when they consume sufficient quantities of milk or some milk products. As a result, a valuable source of food is unacceptable to them. The problem of lactose insolubility contributes to its crystallization, which is often responsible for gritty texture in concentrated dairy products. The problems of lack of sweetness, intolerance for and insolubility of lactose have limited its use in foods such as ice cream, candy, and animal feed. On the other hand, the above problems also have limited the utilization of whey because of its high lactose content. Consequently, whey constitutes a severe problem of disposal for the dairy industry. However, there is widespread interest, both from the nutritional and commercial points of view, in reducing lactose in certain dairy products. Treatment of milk and milk products with β-galactosidase, EC 3.2, 1.23 (lactase) to reduce their lactose content seems to be an appropriate method to increase their potential uses and to deal with the problems of lactose insolubility and lack of sweetness. Furthermore, this treatment could make milk, a most valuable food, available to many adults and some infants intolerant to lactose. Preparations of lactase have been obtained from several microorganisms including lactose fermenting yeasts and bacteria. *Kluyveromyces fragilis* and *Streptococcus salivarius* subsp. *thermophilus* are the best sources of the enzyme. The former has the added advantage of being approved for use in foods. The latter, which is widely used as a starter organism for yogurt manufacture, is a food-safe and promising source of β-galactosidase.

β-D-galactosidase enzyme is widely distributed in nature and can be found in plants animal organs, yeasts, fungi and bacteria (Shukla, 1975). The classical source of bacterial lactase is *Escherichia coli* (Hu *et al*., 1959). Griffiths and Muir (1979) described the properties of the intracellular β-galactosidase of a thermophilic *Bacillus*. In 1978, Ramana Rao and Dutta reviewed that 62 strains of yeasts, molds, and bacteria were screened for lactase (β-galactosidase) activity and they found that the molds exhibited lowest enzyme activity by highest cell yields, while bacteria produced lowest cell yield and maximum enzyme activity. Crude β-galactosidase was produced by lysis of cells of *Streptococcus thermophilus* grown on deproteinized whey by Greenberg and Mahoney (1982). The enzyme from this source has a neutral pH optimum, a temperature optimum in buffer at 55^{o}C and is more heat stable than the neutral-pH optimum, a temperature optimum in buffer at 55^{o}C and is more heat stable than the neutral-pH lactases from yeasts, which are available commercially. Also Ramana Rao and Dutta (1977) reported on the production of β-galactosidase from bacteria grown in deproteinized cheese whey. The activity of β-D-galactosidase was studied in 13 strains of *Lactobacillus*. Studies on the enzymes, which hydrolyze lactose in some species of Lactobacilli done by Premi *et al*. (1972)

came to the conclusion that in eight strains of *Lactobacillus* casei, no β-D-galactosidase activity was present. De Macias *et al*. (1983) reported that β-D-galactosidase has been isolated from *Lactobacillus helveticus*, strain isolated from natural starters or the manufacture of Argentine hard cheese and its properties. *Lactobacillus acidophilus* was studied by Nieisen (1987) for the isolation and characterization of lactose hydrolyzing enzyme β-galactosidase. Lactase has been also isolated from several bacterial sources.

Lactase has been solated from several microbial sources including bacteria, fungi and lactose fermenting yeasts. Thus, there are many factors which influence production of lactase by microorganisms and the selection of conditions of microbial growth maximize lactase yield during growth in medium. The effect of substrate concentration, pH, temperature and related factors were investigated by Jasewicz and Wasserman (1960). The most important factors, which affect the activity and production, are pH and temperature. In food industry conditions for use of enzymes (pH and temperature) are generally set by substrate itself.

In screening for microorganisms as a source of β-galactosidase for lactose hydrolysis, some properties of this enzyme must be considered, particularly the optimum pH for hydrolysis activity, since the pH optimum of the microbial lactases are quite varied.

The optimum pH for the species belonging to the Thermo-bacterium group was uniform, in contrast the pH for these from the Streptobacterium varied according to the species. The optimum temperature was quite uniform within each group and higher in the Streptobacterium. In 1982, Greenberg and Mahoney found that the enzyme from Streptococcus thermophilus has a neutral-pH. Another work was done by Ramana Rao and Dutta (1979) which found that the optimum conditions for maximum enzyme production were pH 7.0, 40^{o}C and 24 h.

Composition of whey (from different sources) did not affect enzyme production. Whey reconstituted to 8 – 12% total solids and adjusted to pH 4.0 afforded maximum enzyme production. Whereas inorganic nitrogen source (ammonium salts) only slightly stimulated enzyme production, organic nitrogen sources (particularly digested proteins) provided a nearly four-folds increase of enzyme production. Yeast extract and beef extract and industrial by-products like corn-steep liquor significantly had a very little stimulation effect. Addition of ammonium sulfate (0.3%) and yeast extract to the deproteinized cheese whey increased cell mass and enzyme yield. Addition of 3% lactose did not affect β-galactosidase activity (Sonawat *et al*., 1981 and Barbosa *et al*., 1984).

Proteose peptone (2.0% w/v) and com steep liquor (2.08, w/v) were highly stimulatory, increasing the enzyme units available in their absence from 660 U/liter of medium to 18,200 and 10,000 U/liter of medium, respectively, in their presence. There was an insignificant increase in the production of enzyme in the presence of added inorganic nitrogen and phosphorus sources. Various inorganic and organic nitrogen supplements were examined to determine their effect on enzyme production. Various growth factors (beef extract, yeast extract, malt extract, com steep liquor and

molasses) were tried. Also mono- and divalent salts ($MgCl_2$, $MnCl_2$, KCl and $CaCl_2$) were studied to determine their effect on enzyme production. Maximal enzyme production by *Kluyveromyces lactis* required Na^+ and Mn^{++} (Ramana Rao and Dutta, 1977 and Dickson *et al*., 1979).

Addition of tryptophane was found to stimulate growth of *Candida pseudotropicalis* (Gomez and Castillo, 1983).

The objective of this study was to investigate nutrient requirements and growth conditions, which influence biosynthesis of β-galactosidase by *Streptococcus salivarius* subsp. *thermophilus* grown in whey.

MATERIALS AND METHODS

Microorganism:

Streptococcus salivarius subsp. *thermophilus* EMCC 10509 was obtained from Microbiological Resource Center, Cairo MIRCEN (CAIM), Ain Shams University, A.R.E.

Maintenance media:

The medium was used to maintain the bacteria (at 4°C) and to prepare the inocula of bacteria had the following constitution (g/L); yeast extract (Difco), 5.0; peptone (Difco), 10.0; glucose (Difco), 5.0; NaCl, 5.0 and agar 20.0. The pH was adjusted to 7.2 – 7.4.

Media for β-galactosidase production:

The basal medium used for β-galactosidase production was a modification of that used by Mahoney *et al*. (1974). The medium consisted of whey plus supplementary nutrients consisting of: (per cent, w/v, in distilled water) K_2HPO_4, 0.3 g, yeast extract, 0.5 g and $(NH_4)_2SO_4$, 0.3 g. Insoluble protein of whey was removed by either centrifugation or filtration.

Whey:

Source:

Whey from bovine was supplied as sweet dried whey from Sigma Chem. Co., St. Louis, Mo, USA. It contained (w/w) 73% lactose, 10.8% protein and 0.75% phosphorus.

Preparation:

Deproteinized whey solutions were prepared by acidification 9pH 4.5) and heat treatment (90°C for 30 min) or by autoclaving (0.9 atmosphere for 20 min). Then, the preparation was cooled and filtered or centrifuged to remove the precipitated protein. The deproteinized whey was subjected to intermittent sterilization at 100°C for 30 minutes for three successive days.

Supplementation:

Whey solution (pH 4.5) was supplemented with (w/v): yeast extract, 0.5%; $(NH_4)_2SO_4$, 0.3% and K_2HPO_4, 0.5%. The pH of the solution was finally adjusted to 5.5 for yeast and 6.5 for bacteria with 2M H_2SO_4 and 2N KOH. The medium was sterilized at 111°C for 25 min.

Maintenance of stock cultures:

The original cultures were maintained at 4°C with monthly transfers to medium containing (w/v): 0.5% yeast extract; 0.5% peptone; 0.5% NaCl; 1.0% glucose and 2.0% agar at pH 7.2 – 7.4.

Inoculum preparation:

The maintained agar slants were used as propagation for the test organism. Slants were inoculated with the organism and after 24 hours incubation at 37°C, slants were stored in refrigerator at 4°C or used directly as working cultures for preparation of seeding material.

Consequently, the growth on the agar slant was scrapped, using 5 ml sterilized tap water then transferred to a 250 ml flask containing 30 ml sterile liquid medium. The resulting cell suspensions (after 18 h incubation at 30oC in shaking incubator) were used for inoculating the sterilized experimental (fermentation) flasks.

Cultivation:

Unless other wise stated, cultivation was made in 300 ml Erienmeye flasks each containing 45 ml of sterile medium (pH 6.0 for yeast and 7.0 for bacteria). A 5 ml of an 18-h-old inoculum was transferred to the culture medium. The flasks were incubated at 30°C (yeast) or 37°C (bacteria) with agitation (120 strokes/min) in a reciprocating shaker bath. After incubation for 48 hours, the culture broth from each flask was used for measuring pH and growth Cells were harvested by centrifugation at 10000 rpm for 15 min at 4°C.

Enzyme extraction:

The harvested cells were washed twice with 0.067 M potassium phosphate buffer (pH 6.8). Washed cells were suspended in 25 ml of 0.067 M potassium phosphate buffer, pH 6.8, containing 0.1 mM manganese chloride and 0.5 mM magnesium sulfate. 10 ml of the suspension were treated with toluene (2% -v/v- at 37°C for 15 min). Broken cells were removed from the assay mixture by centrifugation at 15000 rpm for 30 min at 4°C and the precipitated fraction was discarded. The supernatant was used as enzyme source.

β-galactosidase assay:

The assay mixture consisted of 2.8 ml of 50 mM sodium phosphate buffer (pH 6.8) and 0.1 ml of 68 mM o-nitrophenyl β-D-galactosidase (ONPG), Sigma Chem. Co., USA, then 0.1 ml of enzyme source was added. The assays were carried out at incubation temperature at 45°C. After 10 min, the reaction was stopped by addition of 3.0 ml of ice-cold 0.5 M Na_2CO_3. o-nitrophenyl liberated was estimated at 420 nm. One unit of enzyme was defined as activity, which released 1 u mol of o-nitrophenyl/ml/min, under the conditions specified above.

Analytical Methods:

Growth measurement:

Bacteria growth was measured as absorbance of cultures at 650 nm. Before reading, the suspensions always were diluted to give turbidity reading lower than 1.0 E.

Protein determination:

The protein contents of the enzyme extracts were determined by the method of Lowry *et al*. (1951), with bovine serum albumin as standard.

pH values:

These determined with pH-meter Co-710.

Lactose determination:

Lactose was estimated by the method of Nickerson *et al*. (1976).

RESULTS AND DISCUSSION

Production of β-galactosidase from *Str. salivarius* subsp. *thermophilus* grown in whey:

Utilization of whey in human and animal foods is limited in spite of its very high lactose content (-70% on a dry weight basis). Consequently, whey disposal has become a severe problem of disposal for the dairy industry (Zadow, 1986). During the last decade much emphasis has been given to the enzymatic hydrolysis of lactose in whey to reevaluate it for nutritional applications. However, there is widespread interest in reduction of lactose content of dairy products for both commercial and nutritional reasons (Toba and Adachi, 1978, Hobman, 1984 and Gekas and Lopez-Levia, 1985). β-galactosidase plays an important role in lactose hydrolysis. These experiments deal with the determination of nutrient requirements and growth conditions for maximal β-galactosidase production by *Str. salivarius* subsp. *thermophilus* grown in whey.

Effect of whey concentration on β-galactosidase production:

The effect of whey concentration on β-galactosidase production by *Str. salivarius* subsp. *thermophilus* is presented in Table (1). Whey-containing media were prepared by dissolving sufficient dried whey in H_2O to give serial concentrations of lactose, i.e., 1 - 15% lactose. The whey was deproteinized as described earlier. The results showed that β-galactosidase production increased as the whey concentration raised from low levels to 5.48%. Optimum lactose concentration in whey for production of β-galactosidase under these conditions was 4.0%. Lactose utilization by *K. fragilis* was greater than that used by *Str. salivarius* subsp. *thermophilus* (El-Sawah *et al*., 2000). Whey lactose was completely exhausted up to whey concentrations of 4.11%. However, the lactose utilization reached 3.7% after 24 h.

The growth rate of *Str. salivarius* subsp. *thermophilus* increased with increasing concentrations of whey up to 5.48% whey (4.0% lactose), while lactose utilization decreased.

The decrease in pH during the growth of *Str. salivarius* subsp. *thermophilus* was due to uptake of NH_4^+ and metabolite production.

In this connection, DE Bales and Castillo (1979) found that although the highest lactase yield of *Candida pseudotropicalis* (0.87 U/mg of whey) was obtained with 2% whey, production of enzyme, per milligram of cells and per milliliter, was maximum in 10 to 12% whey (7.3 to 8.7% lactose, in contrast with the 10 to 15% lactose reported as optimal for *K. fragilis* (Mahoney *et al*., 1974). The latter author stated a maximal enzyme yield of 15 U/ml for *K. fragilis* grown in whey, containing 15% lactose, in shake flasks. With *C. pseudotropicalis* in 12% whey (8.7% lactose), 67.5 U/ml were obtained by DE Bales and Castillo (1979).

Table 1: Effect of whey concentration on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

Whey conc. %	Final culture pH	β-galactosidase activity			Growth Rate $O.D_{650\ nm}$	% Lactose in Medium
		U/ml	U/mg whey	U/mg protein		
1.46	5.9	64.29	4.40	16.67	1.331	0.00
2.74	5.8	75.00	2.74	18.35	1.562	0.00
4.11	5.7	81.57	1.89	18.83	1.602	0.00
5.48	5.6	82.35	1.50	18.87	1.685	0.65
6.85	5.6	81.43	1.19	18.34	1.490	1.00
8.22	5.6	71.43	0.89	16.66	1.453	2.15
9.59	5.5	64.29	0.67	16.33	1.400	3.10
10.96	5.5	56.57	0.52	15.86	1.312	4.01
12.33	5.5	48.75	0.40	15.67	1.265	5.11
13.70	5.5	43.07	0.31	15.46	1.201	6.13
15.07	5.5	36.96	0.25	14.11	1.112	7.12
16.44	5.5	33.14	0.20	12.90	1.002	8.05
17.80	5.5	27.96	0.16	11.35	0.976	9.00
19.18	5.5	19.93	0.10	9.65	0.965	10.20
20.55	5.5	10.71	0.05	6.68	0.694	11.30

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**
*** Initial pH (7.0).**
*** Incubation temperature (37°C).**
*** Cultivation time (24 h).**

The biosynthesis of β-galactosidase during the growth of tested organisms was also followed in lactose containing media. Consequently, the whey was replaced by lactose for comparing each of them. The effect of lactose concentration on β-galactosidase production by *Str. salivarius* subsp. *thermophilus* is presented in Table (2). The results show that β-galactosidase activity increased slowly as lactose concentration increased (at 1-2% conc.). Thereafter, β-galactosidase activity increased sharply as the lactose concentration was raised from low levels because of a sharp increase in cell mass. Then β-galactosidase activity was approximately proportional to the growth rate. Optimal lactose concentration for β-galactosidase production from yeast and bacteria was about 11% and 5.0%, respectively. These results are in line with Wendorff *et al*. (1970) and Mahoney *et al*. (1974). They found that optimum lactose concentration for biosynthesis of lactase by yeasts, in media of lactose plus supplementary nutrients in shake flasks, was about 15%.

Whey media yielded 24.25 – 42.93% more β-galactosidase than lactose media. Thus the whey media were employed for β-galactosidase production in the subsequent experiments. The results are in agreement with the results of Wendorff *et al*. (1970) and Mahoney *et al*. (1974). In this connection, Mahoney *et al*. (1974) reported that whey media yielded 30%

more total enzyme and 80% more total enzyme activity per mg yeast than lactose media.

The decrease in pH was much smaller in the whey than in lactose-containing medium. This may be due to the greater buffer capacity of the whey. On the other hand, the decrease in pH during growth was due to uptake of NH_4^+ and metabolite production (Mahoney *et al*., 1974).

Table 2: Effect of lactose concentration on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

Whey conc. %	Final culture pH	β-galactosidase activity		Growth Rate	% Lactose in Medium
		U/ml	U/mg protein	$O.D_{650\ nm}$	
1	3.9	35.51	16.45	0.678	0.00
2	4.0	38.62	16.60	0.806	0.00
3	4.0	40.93	16.65	0.900	0.00
4	4.0	46.93	15.20	1.592	0.10
5	4.0	47.00	17.40	1.599	1.01
6	4.1	45.03	16.60	1.732	2.15
7	4.1	41.52	15.90	1.750	3.11
8	4.1	34.60	15.65	1.768	4.00
9	4.2	30.64	15.60	1.785	5.11
10	4.3	28.03	15.75	1.845	6.13
11	4.5	24.32	15.35	1.891	7.12
12	4.7	21.30	13.50	1.460	8.05
13	4.9	20.73	12.06	1.462	9.05
14	4.9	14.85	9.00	1.466	11.21
15	4.9	9.34	6.55	1.533	11.30

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**
*** Initial pH (7.0).**
*** Incubation temperature (37°C).**
*** Cultivation time (24 h).**

Effect of initial pH value in the whey medium:

In this experiment whereby effect of reaction of culture media (H^+ conc.) on the formation of β-galactosidase was studied, the same whey medium was used. Whey concentrations were adjusted taking into account the best favorite concentrations attained to in the foregone experiments. Before sterilization, the pH value of the culture medium was adjusted with dilute HCl or NaOH solution to cover a pH range from 4.0 to 9.0. Cultivation was then performed under identical conditions. The results obtained on the effects of different pH values on β-galactosidase production are recorded in Table (3). The results showed that by increasing levels of the initial pH value of the whey medium, it appeared that the best growth rate ($OD_{650\ nm}$ 1.69) was obtained at pH 7.0.

The results also indicated that the pH values of cell-free extracts ranged between 4.5 to 4.8. Greenberg and Mahoney (1982) found that the pH of the cultures of *Str. thermophilus* grown in 2% whey dropped rapidly to pH 4.5 and slowly to pH 4.2.

Maximum β-galactosidase production by *Str. salivarius* subsp. *thermophilus* was observed at pH 6.5 – 7.5. This increase in enzyme production per unit media was probably due to a greater amount of cell mass.

The results are in line with those of Wendorff *et al*. (1970), Ramana Rao and Dutta (1977), Itoh *et al*. (1982), Gomez and Castillo (1983), Lee *et al*. (1985) and Kheiralla *et al*. (1994) worked on β-galactosidase production with different yeast and bacteria species.

Table 3: Effect of different initial pH values of whey medium on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

pH of whey	Final culture pH	β-galactosidase activity			Growth Rate $O.D_{650\ nm}$	% Lactose in Medium
		U/ml	U/mg whey	U/mg protein		
5.0	4.5	41.94	0.77	10.56	0.810	74.96
5.5	4.5	49.56	0.90	12.22	1.200	76.98
6.0	4.5	67.10	1.23	16.39	1.660	82.85
6.5	4.5	79.00	1.44	18.87	1.683	83.95
7.0	4.6	82.35	1.50	18.87	1.685	83.98
7.5	4.6	78.84	1.44	17.56	1.640	82.05
8.0	4.6	64.05	1.17	10.89	1.200	81.00
8.5	4.7	41.94	0.77	9.50	1.050	78.95
9.0	4.8	16.78	0.31	8.15	0.805	76.80

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**
*** Incubation temperature (37°C).**
*** Cultivation time (24 h).**

Effect of time course:

The effect of time course on growth rate and β-galactosidase production by *Str. salivarius* subsp. *thermophilus* grown in deproteinized whey medium was studied up to a period of 96 h. For reaching to the optimum culture medium, for the production of enzyme, the best concentrations of whey and the optimum initial pH of media investigated before were taken into consideration. The results obtained are presented in Table (4). A progressive increase in β-galactosidase production by *Str. salivarius* subsp. *thermophilus* was observed up to 24 h of incubation, after which enzyme production become constant for another 24 h then decreased. The optimum period for maximum enzyme production was the same for optimum growth. The results are in line with Ramana Rao and Dutta (1977) and Greenberg and Mahoney (1982). The last authors reported high β-galactosidase production by *Str. thermophilus* by incubation period from 24 hr to 48 h.

The final pH of the cultures was acidic, namely 4.4 to 4.6, after 24 h of growth. Abo El-Dahab (1965) reported that, at the stationary phase of growth, the increase of the product metabolite material from the cell activation caused a decline in the pH value of the medium.

Increases in lactose consumption wee continued with elapsed time. The percentages of lactose utilization were 25.15% (after 6 h), 45.05% (after 12 h) and 91.30% (after 96 h) by *Str. salivarius* subsp. *thermophilus*. The results concerning the consumption of whey lactoe are in line with Mahoney *et al*. (1974) and De Bale and Castillo (1979).

Table 4: Effect of different initial pH values of whey medium on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

Time (h)	Final culture pH	β-galactosidase activity			Growth Rate $O.D_{650\ nm}$	% Lactose in Medium
		U/ml	U/mg whey	U/mg protein		
6	6.5	18.31	0.33	7.25	1.131	25.15
12	6.0	38.13	0.70	10.15	1.213	45.05
18	5.5	64.05	1.17	12.87	1.442	69.95
24	4.6	82.35	1.50	18.87	1.685	83.98
48	4.5	80.10	1.46	18.50	1.750	85.11
72	4.4	70.20	1.28	14.50	1.451	88.02
96	4.3	60.11	1.10	12.00	1.254	91.30

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**

*** Initial pH (7.0)**

*** Incubation temperature (37°C).**

Effect of incubation temperature:

The effect of incubation temperature of the growing cultures on their β-galactosidase production was studied. The optimized factors concluded from the preceding experiments were considered. Determinations of the optimum temperature for enzyme production by test organism was made by placing the inoculated flasks in shaking incubators at 25 to 60°C.

The results on the effect of incubation temperature on the β-galactosidase production are recorded in Table (5). It is seen that the temperature greatly affected the β-galactosidase activities of cells of the two strains.

The optimum temperature for β-galactosidase production by *Str. salivarius* subsp. *thermophilus* coincided with the optimum temperature for the growth of the organism, about 40°C. Therefore, incubation temperature of 40°C was used throughout the subsequent studies.

The highest levels of lactose utilization were obtained at the optimum temperature for growth. Slight change in final culture pH was observed at elevated temperature.

In this connection, similar results were reported by Wendorff *et al*. (1970), Sonawat *et al*. (1981), Gomez and Castillo (1983), Thomas *et al*. (1984), Lee *et al*. (1985), El-Sawah *et al*. (1991) and Kheiralla *et al*. (1994), working on the productivity of β-galactosidase from some yeast species. Ramana Rao and Dutta (1977) studied the cultural conditions optimum for maximum production by *Streptococcus thermophilus* grown in deproteinized

cheese whey. They reported 40°C as the optimum temperature for enzyme production.

Table 5: Effect of incubation temperature on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

Temperature (°C)	Final culture pH	β-galactosidase activity			Growth Rate O.D$_{650\ nm}$	% Lactose in Medium
		U/ml	U/mg whey	U/mg protein		
30	4.5	80.01	0.73	8.00	0.812	55.25
35	4.6	80.18	1.46	18.05	1.251	77.15
37	4.6	82.35	1.50	18.87	1.685	83.98
40	5.8	94.10	1.72	21.58	1.604	85.15
45	5.9	80.05	1.46	18.12	1.741	84.00
50	5.9	68.11	1.24	17.43	1.620	73.01
55	5.9	60.29	1.10	15.65	1.530	71.10
60	5.9	54.06	1.00	11.95	1.251	60.00

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**
*** Initial pH (7.0).**
*** Cultivation time (24 h).**

Effect of nutrients on β-galactosidase production:
Inducers:

This experiment was designed to investigate the effect of supplementation of whey with different sugars. The sugars were supplemented to whey medium at 0.5% final concentration. The best favorite conditions attained to in the foregone experiments were taken into account.

The results obtained are recorded in Table (6). It is seen that the sugars added affected greatly the yield of β-galactosidase produced in the cells of the test organism. The highest β-galactosidase level in bacteria (94.10 and 88.5 U/ml) was obtained when whey plus galactose was used as the sole carbon source. Glucose, although gave the highest growth rates, acted as a repressor for β-galactosidase production in the organism. The other sugars tended to decrease the levels of β-galactosidase to variable degrees. Therefore, the whey (without supplementation with sugars) was used in subsequent experiment.

The results recorded in Table (6) suggest the β-galactosidase of the test organism appeared to be of inducible type, in the sense that the enzyme level in the cells was not almostly of the same order whether the sugar in the medium was whey lactose, whey lactose plus galactose or another sugar. In other words, the biosynthesis of enzyme by the test organisms was highly dependent on the carbon source on which they were cultivated. The results obtained by Mahoney *et al*. (1974), Pedrique and Castillo (1982), Selim *et al*. (1983), Thornart *et al*. (1984), Selim and El-Diwany (1985), Mahmoud *et al*. (1986), Deshpande *et al*. (1989) and Kheiralla *et al*. (1994) could give support to our suggestion. They studied the induction of β-galactosidase with

different yeast species. Most of them concluded that β-galactosidase was synthesized only in the presence of certain sugars.

Table 6: Effect of supplementation of whey with some sugars on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

Supplement	Final culture pH	β-galactosidase activity			Growth Rate $O.D_{650\,nm}$	% Lactose in Medium
		U/ml	U/mg whey	U/mg protein		
Without	5.8	94.10	1.72	21.58	1.685	83.98
Galactose	4.9	88.51	1.62	20.05	1.601	90.16
Xylose	5.1	29.99	0.55	8.99	1.243	69.42
Sucrose	4.8	47.18	0.86	12.04	1.772	83.04
Fructose	5.0	30.21	0.55	7.15	1.410	65.45
Glucose	6.7	30.23	0.06	2.51	0.409	30.63
Mannose	4.9	28.30	0.52	8.95	0.615	69.30
Mannitol	4.9	45.75	0.85	13.12	1.590	80.17
Glycerol	4.8	22.65	0.41	5.36	1.551	27.26
Maltose	5.0	8.85	0.16	3.23	0.620	20.08
Arabinose	4.9	14.95	0.27	4.15	0.532	25.25

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**
*** Initial pH (7.0).**
*** Cultivation time (24 h).**
*** Incubation temperature (40°C).**

Nitrogen sources:

In this experiment where the effect of supplementation of whey with different nitrogen sources on β-galactosidase production was investigated, the $(NH_4)_2SO_4$ used in the preceding experiments was replaced by eleven of nitrogen sources, for comparing each of them with other. All of the nitrogen sources were added separately to the whey medium, in (0.3% w/v) amounts irrespective of the chemical constitution.

The results obtained on the effect of supplementation of whey with nitrogen sources on the synthesis of β-galactosidase are given in Table (7). It is seen that the kind of nitrogen source affected greatly the yield of β-galactosidase produced by *Str. salivarius* subsp. *thermophilus*. It is clear that ammonium sulfate promoted the highest β-galactosidase activities. Inorganic nitrogen sources slightly stimulated β-galactosidase production by *Str. salivarius* subsp. *thermophilus*. In this connection Sayed *et al*. (1993) found that the β-galactosidase formation by *Mucor humicolus* was favoured by using organic nitrogen sources. The effect of different nitrogen sources on the production of β-galactosidase production by various organisms was studied by several workers (Deshpande *et al*., 1989) and Khairalla *et al*., 1994).

Table 7: Effect of supplementation of whey with different nitrogen sources on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

Supplement	Final culture pH	β-galactosidase activity			Growth Rate $O.D_{650\,nm}$	% Lactose in Medium
		U/ml	U/mg whey	U/mg protein		
$(NH_4)_2SO_4$	5.8	94.10	1.72	21.58	1.685	83.98
NH_4NO_3	6.5	49.17	0.90	13.12	1.253	85.10
KNO_3	6.2	46.15	0.84	12.60	0.604	86.00
$NaNO_3$	6.9	47.16	0.86	12.95	0.908	84.20
NH_4HPO_4	5.5	53.20	0.97	14.85	1.510	83.10
$NH_4H_2PO_4$	5.5	42.12	0.77	11.12	1.681	85.00
Peptone	5.5	46.59	0.85	10.11	1.892	85.20
Urea	6.9	31.18	0.57	7.14	1.484	84.40
Casein	6.5	30.90	0.56	6.09	0.996	80.10
Malt extract	6.2	41.12	0.75	9.70	1.325	83.30
Proteose peptone	5.9	52.86	0.97	12.93	1.963	86.60

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**
*** Initial pH (7.0). * Cultivation time (24 h). * Incubation temperature (40°C).**

The effect of different concentrations of the best nitrogen sources on β-galactosidase production by the tested organism is given in Table (8). The enzyme production and growth rate of *Str. salivarius* subsp. *thermophilus* was increased with increasing $(NH_2)_2{}^{SO}{}_4$ concentration until reaching a maximum yield for enzyme (112.5 U/ml) at 5 g/l and maximum rate for growth (OD_{650nm} 1.833) at 10 g/l. Further increase of peptone or $(NH_4)_2SO_4$ concentrations resulted decline in β-galactosidase production and growth rate of the two organisms. Accordingly a whey medium with 1.0% peptone and 0.5% $(NH_4)_2SO_4$ was selected for further investigations in case of *Str. salivarius* subsp. *thermophilus*.

Table 8: Effect of different concentrations of $(NH_4)_2SO_4$ on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

$(NH_4)_2SO_4$ Conc. (%)	Final culture pH	β-galactosidase activity			Growth Rate $O.D_{650\,nm}$	% Lactose in Medium
		U/ml	U/mg whey	U/mg protein		
0.0	6.0	37.91	0.69	3.51	1.412	80.11
0.3	5.8	94.10	1.72	21.58	1.685	83.98
0.5	5.7	112.5	2.05	26.05	1.803	87.05
1.0	5.6	96.05	1.75	24.19	1.833	87.95
1.5	5.5	85.99	1.57	23.85	1.819	88.04
2.0	5.3	82.84	1.51	17.60	1.752	87.23
2.5	5.0	76.23	1.39	17.60	1.724	86.09
3.0	4.5	70.13	1.28	17.19	1.610	85.11

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**
*** Initial pH (7.0). * Cultivation time (24 h). * Incubation temperature (40°C).**

Phosphorus sources:

In this experiment attempts were made to investigate the effect of supplementation of whey with different phosphorus sources on β-galactosidase producing potential. The phosphorous sources were added to the whey medium in equimolecular phosphorous weights, i.e., g P/l. The results obtained are recorded in Table (9). It is clearly shown that all the used phosphorus sources decreased the production of β-galactosidase by the tested organism except with K_2HPO_4 where some stimulation in β-galactosidase production was observed. On the other hand, NaH_2PO_4 favoured K_2HPO_4. Thus, the results obtained show that the two experimental organism favored the utilization of K_2HPO_4 as a phosphorus source to stimulate the production of β-galactosidase. In this connection supplement of whey with phosphorus sources had no significant effect on growth rate and lactose utilization. Ramana Rao and Dutta (1977) investigated the effect of mono-, di-, and tribasic salts of phosphate on β-galactosidase production by *Str. salivarius* subsp. *thermophilus* grown in whey. The authors found that the monobasic salt (0.8%, w/v) stimulated enzyme production to the maximum extent.

Table 9: Effect of supplementation of whey with different phosphorus sources on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

Supplement	Final culture pH	β-galactosidase activity			Growth Rate $O.D_{650\ nm}$	% Lactose in Medium
		U/ml	U/mg whey	U/mg protein		
Without	5.3		1.37			
K_2HPO_4	5.7		2.05			
KH_2PO_4	5.7		1.93			
Na_2HPO_4	5.5		1.65			
NaH_2PO_4	5.5		2.10			
Na_3PO_4	5.5		1.55			

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**

*** Initial pH (7.0).**

*** Cultivation time (24 h).**

*** Incubation temperature (40°C).**

Although the phosphorous plays an important role in cell metabolism, and utilization of lactose is greatly depend on the presence of adequate supply of phosphorous in the whey medium. Further increase or decrease of K_2HPO_4 concentration than 0.3% resulted decline in β-galactosidase production. Similar observations with *Saccharomyces fragilis* and *S. thermophilus* have been made (Wendorff *et al*., 1971; Ramana Rao and Dutta, 1977 and De Bales and Castillo, 1979) upon addition of other phosphorus salts.

According to above mentioned results, it was necessary to establish the effect of different concentrations of K_2HPO_4 on growth rate, lactose consumption and β-galactosidase production by *Str. salivarius* subsp.

thermophilus. The concentrations used ranged from (0.0 to 0.9% w/v). The results obtained are presented in Table (10).

The β-galactosidase levels in the cells of *Str. salivarius* subsp. *thermophilus* were maximal when K_2HPO_4 was added at 0.5. At higher concentrations of K_2HPO_4 the β-galactosidase activity decreased.

Metal salts:

Five metal salts, namely, potassium chloride, calcium chloride, sodium chloride, magnesium chloride and manganese chloride, have been tested, concerning their effects on β-galactosidase production and growth of *Str. salivarius* subsp. *thermophilus*. Each metal salt was incorporated at a concentration of 0.1 mg/l in whey medium.

Table 10: Effect of supplementation of whey with different concentrations of NaH_2PO_4 on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

NaH_2PO_4 Conc. (%)	Final culture pH	β-galactosidase activity			Growth Rate $O.D_{650\,nm}$	% Lactose in Medium
		U/ml	U/mg whey	U/mg protein		
0.0	5.4	40.15	0.73	11.11	1.05	53.22
0.1	5.6	88.43	1.61	22.13	1.72	86.13
0.3	5.5	115.32	2.10	27.11	1.91	90.62
0.5	5.7	117.59	2.15	30.13	1.85	90.11
0.7	5.8	114.13	2.08	28.22	1.76	88.95
0.9	5.9	102.31	1.87	25.06	1.75	86.42

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**

*** Initial pH (7.0). * Cultivation time (24 h). * Incubation temperature (40°C).**

The effects of metal salts are given in Table (11). The results showed that β-galactosidase production was considerably stimulated by divalent salts comparable to stimulation by monovalent salts. The slightly stimulation exerted by monovalent salts indicates that the levels of these salts in whey were sufficient. The results are in agreement with those obtained by Ramana Rao and Dutta (1979).

Table 11: Effect of supplementation of whey with metal salts on growth rate, lactose utilization and β-galactosidase production by *Str. salivarius* subsp. *thermophilus*.

Metal salts	Final culture pH	β-galactosidase activity			Growth Rate $O.D_{650\,nm}$	% Lactose in Medium
		U/ml	U/mg whey	U/mg protein		
Control	5.7	117.59	2.15	30.13	1.85	90.11
NaCl	5.5	120.05	2.19	30.62	1.75	90.78
KCl	5.4	115.12	2.10	30.32	1.50	91.94
$CaCl_2$	5.5	125.16	2.23	30.51	1.76	90.56
$MgCl_2$	5.5	125.12	2.28	30.90	1.80	92.12
$MnCl_2$	5.4	130.25	2.38	31.21	1.84	93.30

*** The medium employed for β-galactosidase production contained different concentrations of whey in water together with "supplementary nutrients" [0.3% (w/v) K_2HPO_4; 0.5% (w/v) yeast extract, and 0.3% (wt/vl) $(NH_4)_2SO_4$].**

*** Initial pH (7.0). * Cultivation time (24 h). * Incubation temperature (40°C).**

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دراسات عن إنتاج البيتاجلاكتوسيديز الميكروبى

٣- الظروف المثلى لإنتاج البيتاجلاكتوسيديز من خميرة كليفيروميمز فرانجيليز أثناء النمو على الشرش٠

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تبلغ نسبة اللاكتوز فى اللبن ٨ر٤%، وهو سكر ضعيف الذوبان منخفض الحلاوة، ينتج من تناوله بكميات كبيرة إسهال متوسط للبالغين أو لبعض الطفال بسبب ضعف مقاومتهم له كنتيجة لغياب اللاكتيز المعوى، وعادة يعانى مثل هؤلاء الأشخاص من الإنتفاخ والمغص الحاد والإسهال والامتصاص الغير جيد حال تناولهم كميات كبيرة من اللبن أو بعض منتجاته، وعليه يحرم هؤلاء الأشخاص من أحد أقيم المصادر الغذائية، كذلك يسبب انخفاض ذوبان اللاكتوز تبلور مما يتسبب فى القوام الجريش للمنتجات اللبنية المركزة، وتحد

المقاومة المنخفضة للاكتوز وعدم ذوبانه من استخدامه في بعض الأغذية مثل الأيس كريم والكاندى والأعلاف الحيوانية، كما تحد هذه الأسباب من اتسخدام الشرش أيضاً بسبب المحتوى العالى من اللاكتوز (حوالى ٧٥% على أساس الوزن الجاف)، ومن ثم يمثل الشرش مشكلة بيئية كبيرة لمصانع الألبان تتعلق بتصريفه٠

ولذلك فهناك إهتمام كبير من لاناحيتين الغذائية والتجارية بإختزال كمية اللاكتوز فى منتجات لبنية معينة، وأحد أهم طرق تحليل اللاكتوز هو استخدام إنزيم البيتاجلاكتوسيديز (اللاكتيز) لتحليله إلى جلوكوز وجلاكتوز، وهناك مصادر ميكروبيةعديدة للبيتاجلاكتوسيديز، إلا أن الخمائر والبكتيريات التى تتوطن الألبان كبيئة طبيعية تعتبر أفضل المصادر بسبب إمتلاكها لميزة إستعمالها نفسها كغذاء مأمون٠

وعليه فقد استهدف هذا البحث دراسة إنتاج البيتاجلاكتوسيديز أثناء نمو الميكروبات على الشرش، بقصد محاولة الاستفادة من الشرش (الحلو) بمحتواه العالى من اللاكتوز (٧٦%) كبيئة لإنتاج البيتاجلاكتوسيديز، ولذلك فقد تناولت التجارب تدعيم الشرش ببعض المصادر والعناصر الغذائية إلى جانب دراسة بعض العوامل المؤثرة فى إنتاج الإنزيم وفى معدل النمو الميكروبى واستهلاك اللاكتوز٠

وجد أن أقصى إنتاج من بيتاجلاكتوسيديز البكتيريا يكون عند تركيز ٤٨ر٥% شرش (٤% لاكتوز)، وقد بلغ استهلاك لاكتوز الشرش ١٠٠% وحتى تركيز ٤٨ر٥% شرش (٤% لاكتوز) بعد ٢٤ ساعة، كما لوحظ تزايد معدلات النمو استهلاك اللاكتوز حتى تركيز ٤٨ر٥% شرش (٤% لاكتوز)٠

أدى إستبدال الشرش باللاكتوز فى بيئة البكتيريا المستخدمة للإنتاج الإنزيمى إلى حدوث ٩٣ر٤٢% نقص فى إنتاج البيتاجلاكتوسيديز، كذلك لوحظ حدوث نقص فى معدلات استهلاك اللاكتوز (٣٣ر٢١% نقص)، بينما لم يلاحظ تغيير فى النمو، أيضاً لوحظ زيادة انخفاض درجة تركيز الأس الهيدروجينى النهائية للمزارع المحتوية على اللاكتوز عن مثليتها المحتوية على الشرش٠

وجد أن لمثل تركيز أس هيدروجينى لبيئة الشرش لإعطاء أقصى كمية من البيتاجلاكتوسيديز من البكتيريا يقع بين درجتى ٥ر٦ – ٥ر٧، فى حين لوحظ أعلى معدل نمو بين درجتى ٥ر٦ – ٥ر٧، وقد انخفضت درجة تركيز الأس الهيدروجينى للمزرعة المحتوية على الشرش إلى ٥ر٤ بعد مرور ٢٤ ساعة تحضين٠

وجدت زيادة متدرجة فى إنتاج البيتاجلاكتوسيديز بمرور فترة نمو البكتيريا وحتى ٢٤ ساعة تحضين، حيث أعقب ذلك ثبات إنتاج الإنزيم، فى حين لوحظ إستمرار زيادة معدل النمو٠

لوحظ توافق درجة الحرارة المثلى لإنتاج أقصى كمية من البيتاجلاكتوسيديز مع درجة الحرارة المثلى لأعلى معدل نمو وهى درجة ٤٠ °م، وقد لوحظ زيادة استهلاك البكتيا للاكتوز حول هذه الدرجة٠

لوحظ زيادة إفراز البيتاجلاكتوسيديز فى البكتيريا وجود لاكتوز الشرش (٠ر٤% لاكتوز) أو لاكتوز للشرش (٠ر٢% لاكتوز) والجلاكتوز (٠ر٢%) معا كمصجر للكربون، وقد أدى وجود الجلوكوز إلى تثبيط إفراز الإنزيم (٢ر٩٦% تثبيط)، كما أظهر الجلوكوز أقل معدل نمو لميكروب ستربتوكوكس ساليفاريس، وقد تناقص إفراز الإنزيم فى وجود بقية السكريات، كذلك أدت إضافة السكريات إلى نقص فى استهلاك لاكتوز الشرش٠

وجد أن سلفات الأمونيوم هى أفضل مصدر نيتروجينى يمكن إضافته للشرش للحصول على أقصى إنتاج إنزيمى من البكتيريا، إلا أنه لم يكن لمصدر النيتروجين بصفة عامة تأثير كبير على زيادة النمو أو استهلاك اللاكتوز٠

كما وجد أن أفضل تركيز لسفات الأمونيوم للحصول على أقصى إنتاج من البيتاجلاكتوسيديز هو ٥ر٠%، فى حين لم يكن لتركيزات السلفات المتزايدة تأثيرعلى زيادة النمو٠

أدى تدعيم الشرش بفوسفات البوتاسيوم أحادية القاعدية إلى تنشيط تكوين البيتاجلاكتوسيديز فى البكتيريا، وقد وجد أن تركيز ٥ر٠% من فوسفات البوتاسيوم هو أفضل تركيز لأقصى إنتاج إنزيمى٠

لوحظ تنشيط إفراز البيتاجلاكتوسيديز فى وجود الأملاح المعدنية ثنائية القاعدية مقارنة بالأحادية القاعدية، بما يشير إلى احتمال وجود الأخيرة فى الشرش بالتركيز اللازم٠

أدى تدعيم بيئة الشرش بأملاح المنجنيز والماغنسيوم والكالسيوم والصوديوم إلى زيادة معنوية فى إنتاج البيتاجلاكتوسيديز، فى حين لم يؤدى التدعيم بالبوتاسيوم إلى حدوث زيادة ملحوظة بما يشير إلى وجود الأخيرة بتركيزات كافية للميكروب فى الشرش٠