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Growth of *Bifidobacterium bifidum* in whey-based media

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Bifidobacteria play an important role in human health including the enhancement of resistance against infection in infants. To develop an inexpensive whey-based medium for *Bifidobacterium bifidum*, potential growth promoters — yeast extract, casein, bovine casein digest, tryptone, peptone and glucosamine — singly or in combinations, were evaluated for their bifidus growth-promoting activity. The effect of environmental conditions on growth in cheese whey was also evaluated. A whey-based medium for *B. bifidum* was formulated. Cheese whey supplemented with *N*-acetylglucosamine (1 mg/ml) and yeast extract (10 mg/ml) in the presence of sodium thioglycolate (0.1%) at pH 6.8 promoted the growth of *B. bifidum* at 37°C. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 177–179.

Keywords: *B. bifidum*; cheese whey; growth promoters; medium development

Introduction

Bifidobacteria are major components of the intestinal flora of human beings [16]. Bifidobacteria break down carcinogenic nitrosamines [24], suppress liver tumorigenesis in mice [17], help in the absorption of nutritionally important minerals [10], exert detoxification influences in chronic liver diseases [14] and synthesize B-complex vitamins [21]. In adults with antibiotic-induced diarrhoea, feeding a bifidobacterial inoculum corrects the problem [7]. Bifidobacteria normalise the large intestinal flora of astronauts [13]. Media generally used for administering bifidobacteria to patients are yoghurt and milk [6,7].

Several synthetic and semisynthetic media containing lactose (3–7%), protein, amino acids, B-complex vitamins and bovine casein digests have been developed for the growth of bifidobacteria [1,8,22,26]. In addition, an attempt was made to grow bifidobacteria in milk supplemented with several nutrients [6,15]. Commercial media such as MMRS and TPY are expensive.

Dairy industry effluents like whey, which contain lactose (5%), whey proteins (0.8%), minerals and vitamins as essential components have not been exploited for the cultivation of *Bifidobacterium bifidum*. The present study was undertaken to develop an inexpensive whey-based medium for *B. bifidum*. Growth promoters previously reported in the literature were incorporated in the whey and evaluated for their bifidus growth-promoting activity. Effects of environmental conditions on the bifidobacterial growth in cheese whey were also evaluated. Based on the results obtained, a whey-based medium for *B. bifidum* was formulated.

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Materials and methods

Microorganism

B. bifidum var *tissier* (ATCC 11863) was obtained from the National Council for Food Bacteria (NCFB, UK) in the freeze-dried stage. Cultures were rehydrated and maintained as suggested by NCFB in the modified MRS medium (Himedia, India) containing 0.05% L-cysteine–HCl (Himedia) and incubated at 37°C. The identity of the bacterium was confirmed by biochemical tests and microscopic examinations [5].

Inoculum preparation

B. bifidum was routinely grown in MMRS broth+0.05% cysteine–HCl at 37°C for 24 h. This served as inoculum for subsequent purposes.

Cheese whey

Cheese whey was procured from the Ravileela Dairy (Hyderabad, India). It contained 4.65% lactose and 0.8% total proteins.

Cheese preparation at the dairy

To cow's milk, 1% starter culture was added and incubated at 37°C for 2 h. Rennet (0.01%) was added to the acidified curds and incubated at 37°C for 45 min. The soft rubbery curd is cut with a knife to expel the whey which was drained out and collected.

Sterilization of cheese whey

Cheese whey was sterilized by autoclaving it at 121°C for 15 min. Growth factors that were added to the whey at the time of autoclaving are yeast extract, bovine casein, bovine casein digest, tryptone, peptone and *N*-acetyl-D-glucosamine. Growth factors were added to the whey at 10 mg/ml [23], except for glucosamine which was added at 1 mg/ml [9]. Fifty-milliliter media were dispensed to sterilized screw-capped bottles.

Effect of anaerobic conditions

Anaerobic conditions were created by adding 0.05% cysteine–HCl [18], 0.2% ascorbic acid [6] and 0.1% sodium thioglycolate [26]. The solutions were sterilized by autoclaving them at 121°C for 15 min.

Effect of pH and temperature

Cheese whey was adjusted to pH 6.6, 6.8, 7.0, 7.2 and 7.4 and whey in 50-ml bottles inoculated with *B. bifidum* was incubated at temperatures of 35, 37, 40 or 43°C.

Bacterial growth

Microbial assay tubes were placed in a N_2-CO_2 incubator at 37°C for 18 h. The pH at this point was not very low and the rate of microbial growth was entirely limited by the amount of growth promoter present in the medium. The final pH was never below 4.8 under these conditions. Unsupplemented whey served as a control. Bacteria were enumerated in MMRS agar at 37°C for 72 h under the conditions described above [25].

All experiments were repeated twice and the results presented are the means of two values.

Results and discussion

Effect of growth promoters

Table 1 illustrates the extent of bifidobacterial growth in cheese whey augmented with potential growth promoters. None of the nutrients evaluated inhibited growth of *B. bifidum* in cheese whey. Bovine whole casein did not stimulate growth of *B. bifidum*. Bovine casein digest, tryptone, peptone and their combinations moderately stimulated growth. Glucosamine, singly or in combination with other nutrients, stimulated growth to the highest extent. Supplementing cheese whey with yeast extract and glucosamine gave the highest cell count (2×10^7 cfu/ml) and successfully replaced MMRS broth.

Aminosugars stimulated the growth of *B. bifidum* var *pennsylvanicus* both in pure and in mixed culture systems containing *Escherichia coli* [9]. Different aminosugar isomers varied in the magnitude of their stimulation, but glucosamine exerted the highest activity. Similar results were reported by Lambert and Zilliken [12]. O'Brien *et al.* [20] provided evidence for an almost quantitative incorporation of 1-C^{14}-labeled α, β-methyl-*N*-acetyl-D-glucosaminide in the cell wall of the *B. bifidum* under the conditions of growth. *B. bifidum* is not capable of supplying sufficient amounts of glucosamine from its anabolic processes to synthesise its cell wall; it has to rely upon external sources. The higher growth-promoting activity of the β-glycosides might be explained by a hypothetical phosphorylase, which could convert the glycoside directly into glucosarium 1-phosphate. Glucosamine would have to be present in the crude extracts of *B. bifidum* and subsequently converted to the 1-phosphate.

Bezkorovainy *et al.* [3] compared bifidus growth responses in a semisynthetic medium and observed that glycopeptide purified from casein digest supported better growth than casein digest or whole casein. Casein digest may serve as the nitrogen and the growth factor source. It has also been reported that the bifidus growth-promoting activity of glycoproteins isolated from human milk, bovine milk, bovine casein and casein digest was directly proportional to their glucosamine content [3] (Gyorgy *et al.*, 1973). It has been proposed that whole casein has little, if any, microbial growth-promoting activity, yet when digested, a highly active fraction can be isolated therefrom. This indicates that the microbial growth-promoting fraction is not available to the microorganisms unless the casein is digested by proteolytic enzymes, as happens in the gastrointestinal tract. A sustained release of microbial growth promoters may take place during casein digestion, and as long as casein is present in the gastrointestinal tract, microbial growth promoters will continue to be released and maintain the presence of bifidobacteria in the intestinal flora [1].

Table 1 Effect of growth promoters on the growth of *B. bifidum* in cheese whey

Sample number	Growth promoters	cfu/ml
1	unsupplemented cheese whey	$2.00\times10^5\pm0.1$
2	cheese whey+casein	$3.50\times10^6\pm0.3$
3	cheese whey+peptone	$5.20\times10^6\pm0.5$
4	cheese whey+tryptone	$5.30\times10^6\pm0.4$
5	cheese whey+yeast extract	$5.40\times10^6\pm0.1$
6	cheese whey+bovine casein digest	$5.50\times10^6\pm0.3$
7	cheese whey+peptone+casein	$5.50\times10^6\pm0.3$
8	cheese whey+casein+bovine casein digest	$5.60\times10^6\pm0.3$
9	cheese whey+tryptone+casein	$6.00\times10^6\pm0.5$
10	cheese whey+yeast extract+casein	$6.00\times10^6\pm0.1$
11	cheese whey+peptone+tryptone	$7.00\times10^6\pm0.2$
12	cheese whey+peptone+bovine casein digest	$7.30\times10^6\pm0.2$
13	cheese whey+tryptone+bovine casein digest	$7.50\times10^6\pm0.3$
14	cheese whey+yeast extract+peptone	$8.70\times10^6\pm0.4$
15	cheese whey+yeast extract+tryptone	$8.90\times10^6\pm0.1$
16	cheese whey+yeast extract+bovine casein digest	$9.20\times10^6\pm0.1$
17	cheese whey+glucosamine	$1.70\times10^7\pm0.1$
18	cheese whey+casein+glucosamine	$1.73\times10^7\pm0.2$
19	cheese whey+peptone+glucosamine	$1.76\times10^7\pm0.3$
20	cheese whey+tryptone+glucosamine	$1.76\times10^7\pm0.1$
21	cheese whey+bovine casein digest+glucosamine	$1.96\times10^7\pm0.1$
22	cheese whey+yeast extract+glucosamine	$1.99\times10^7\pm0.1$
23	MMRS broth+0.05%cysteine–HCl	$2.13\times10^7\pm0.4$

Effect of anaerobic environment

The effect of reducing agents on bifidus growth-promoting activity was tested in cheese whey supplemented with yeast extract and glucosamine. Three reducing agents tested were 1% sodium thioglycolate, 0.05% cysteine–HCl and 0.2% ascorbic acid and the corresponding bifidus growth-promoting activities in colony-forming units per milliliter were $2.4\times10^7\pm0.4$, $2.4\times10^7\pm0.5$ and $2.3\times10^7\pm0.2$, respectively. No significant difference was observed in the activity of the three reducing agents. Supplementation of whey with sodium thioglycolate created a better environment for *B. bifidum* when compared to the other two reducing agents. Bifidobacteria are strict anaerobes [5]. Norris *et al.* [19] reported that growth of these organisms in a solid medium required small amounts of atmospheric carbon dioxide and tolerated up to 3% of atmospheric oxygen, but when grown in liquid medium, the organisms tolerated atmospheric oxygen and did not require the addition of carbon dioxide. Mayer [14] made similar observations. Brown and Townsley [4] also reported that after original faecal isolation, no special anaerobic condition was required for growth.

Effect of pH

Cheese whey was adjusted to pH 6.6, 6.8, 7.0, 7.2 or 7.4 and the corresponding growth responses were $2.3\times10^7\pm0.1$, $2.4\times10^7\pm0.2$, $2.3\times10^7\pm0.4$, $2.3\times10^7\pm0.5$ and $2.0\times10^7\pm0.1$ cfu/ml, respectively. No improvement in growth was observed by varying pH from 6.8. Cell counts were similar for pH 6.8, 7.0 and 7.4. As the pH was increased to 7.6, the cell count decreased.

Effect of temperature

At 32, 35, 37, 40, 43 or 45°C, the corresponding growth responses were $2.4\times10^7\pm0.1$, $2.4\times10^7\pm0.1$, $2.4\times10^7\pm0.3$, $2.4\times10^7\pm0.1$, $2.3\times10^7\pm0.5$ and $2.3\times10^7\pm0.1$ cfu/ml, respectively. *B. bifidum* growth was maximum at 35°C and 37°C. Temperatures above 37°C decreased the cell count. Our results are in agreement with those of Misra and Kuila [15] who reported that 37°C is the optimal growth temperature for strains of *B. bifidum*.

Growth of *B. bifidum* in unsupplemented cheese whey, supplemented cheese whey and MMRS broth yielded $2\times10^5\pm0.1$, $2.4\times10^7\pm0.2$ and $2.1\times10^7\pm0.4$ cfu/ml, respectively. No significant difference in the growth-promoting activities of supplemented cheese whey and MMRS broth was observed. Growth-promoting activities of supplemented cheese whey and MMRS broths were significantly different from that of unsupplemented cheese whey. Whey supplemented with *N*-acetyl-D-glucosamine (1 mg/ml) and yeast extract (10 mg/ml) in the presence of sodium thioglycolate (0.1%) at pH 6.8 and 37°C serves as an inexpensive medium for the growth of *B. bifidum*.

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