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Alginate biopolymer production by *Azotobacter chroococcum* from whey degradation

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ABSTRACT: The potential of three *Azotobacter chroococcum* strains for whey degradation and alginate production were investigated. After dilution, samples were spread plated on isolation agar and Manitol agar and incubated at 30 °C for 24 h. Microorganisms were screened for their ability to whey degradation and alginate production based on colony morphology, negative and capsule staining, ability to decrease the apparent turbidity of the fermentation broths in batch and semi continuous culture by spectrophotometer assay at 400 nanometer and tensiometer assay. Of the three strains tested for whey degradation, only *Azotobacter chroococcum* 1723 produced significant apparent growth on whey broth and could decrease about 70 % of turbidity in fermentation broth during 6 days in batch culture. Colonies of this strain was characteristically yellow, large, moucoid and slimy on whey agar than Manitol agar after 24 h at 30 °C. Transmission electron microscopy assay and Carbazole reagent were used to recognize the alginate biopolymer. After optimizing environmental factors such as pH, salt concentration and temperature, this strain was able to produce exopolysaccharide greater than 5 mg/mL. Optimum results were obtained when using whey broth as a fermentation medium without extra salt, temperature at 35 °C and pH 7. Increasing inorganic and organic nitrogen sources (yeast extract and NH_4NO_3) reduced whey degradation at least 30%. Transmission electron microscopy assay showed a net-structured polysaccharide capsule around the cells. Semi-continuous culture results demonstrated that, alginate production as well as whey degradation was decreased (1 mg/mL and 30 %).

Key words: *Azotobacter chroococcum, whey degradation, alginate production and exopolysaccharide*

INTRODUCTION

Extracellular polysaccharide (EPS) is required for wild-type virulence of *Pseudomonas solanaceaum* and other microorganisms (Willis, *et al.*, 2001). Many of them produced by microorganisms have been studied and are currently used in a wide range of industries due to their functions such as gel formation, emulsifying film formation, and antitumor activity (Low, *et al.*, 1998). Alginates are linear copolymers of β-D-mannuronic acid (M) and it's C-5-epimer, α-L-guluronic acid (G) and it is a commercially important polysaccharide which has many applications in biotechnology and food industry (Grasdalen, *et al.*, 1983). Alginate immobilized cell systems are used as biocatalysts in several industrial processes such as ethanol production by yeast cell, production of monoclonal antibodies from hybridoma cells (Crescenzi, 1995), stimulating immune cells to secrete cytokines, such as Tumor Necrosis Factor- α (TNF-α), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) (Otterlei, *et al.*, 1991), used in water treatment processes since they help in increasing the aggregate sizes in the flocculation processes (Dekwer and Hempel, 1999), used mainly in food industry, for example, in ice-creams, frozen custards, as well as cream, cake mixtures and in beer manufacture to enhance the foam and fruit drinks to assist the suspension of fruit pulp, which makes the product more appealing to the consumer (Neidleman, 1991). All alginates used for commercial purposes are currently being produced the harvesting of brown seaweeds. However, considering the quality of bacterial alginate and the environmental impact associated with seaweed harvesting and processing, it is more probable that bacterial alginate may become commercial product (Dekwer and Hempel, 1999). Several bacteria specially *Pseudomonas* spp. and *Azotobacter* spp. can synthesize alginate. The species *Azotobacter vinelandii* seems to be the best candidate for the industrial production of alginate molecules characterized by a chemical composition, molecular mass and molecular mass distribution suited to a well defined application, especially required in the biotechnological, biomedical

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and pharmaceutical fields (Clementi, 1997). All alginates produced by bacteria were, in all cases, reported to be rich in mannuronic acid (Dekwer and Hempel, 1999). *Azotobacter* spp is a gram negative soil bacterium which under adverse environmental conditions undergoes a differentiation process leading to the formation of desiccation- resistant cysts (Dekwer and Hempel, 1999). The mature cysts are surrounded by two capsule-like layers containing a high proportion of the exopolysaccharide alginate. This exopolysaccharide is essential for the encystment process, since nonmucoid strains fail to encyst (Campos, *et al*., 1996). In *Azotobacter vinelandii*, alginate protects nitrogenase from oxygen and increases nitrogen fixation (Sabra, *et al*., 2000). In the case of the dairy industry, one such 'waste' which is produced in enormous quantities is the whey resulting from cheese and casein manufacture. Whey is the serum part of milk remaining after separation of the curds during casein or cheese making (Zadow, 1986). It is rich in nutrients and contains at least half the solids found in whole milk (Horton, 1993). The other perspective, whey has a very strong polluting capacity, with a biological oxygen demand (BOD) of 40,000 to 45,000 mg/L (Hacking, 1988; Kemp and Quickenden, 1989). On a world wide scale, only 50 % (v/v) of total whey produced is utilized, and the remainder is disposed of disposed whey is either discharged into the sewage or ocean outfall or is sprayed on pastures (Zadow, 1987;1992). In recent years, value added products form whey have attracted much interest. One such area is the production of microbial biopolymer (Schwartz and Bodie, 1985; Fu and Tseng, 1990; Flatt, *et al*., 1992; Konicek, *et al*., 1993). In this study, the potential of native *Azotobacter chroococcum* strains for degradation whey when grown on whey medium to produce exopolymer like alginate were investigated.

MATERIALS AND METHODS

This research was carried out in Department of Microbiology in the Microbiology Laboratory, Islamic Azad University, North Tehran Branch, Iran during 2006 to 2007. All data reported in this study are from triplicate measurement.

Bacterial strains, culture conditions, and estimation of bacterial concentration

Three strains of *Azotobacter chroococcum* were studied for alginate production (Table 1). The strains were originally obtained from Microbiology Laboratory, Faculty of Science, Islamic Azad University, North Tehran Branch, Iran. Samples subcultured on Manitol agar (Oxoid) and Whey agar [0.1 % (w/v) yeast extract, 0.1 % (w/v) yeast extract (Oxoid), 0.05 % (w/v) K_2HPO_4, 0.01 % (w/v) NH_4NO_3, 100 % (w/v) whey broth, 1.5% Bacto agar (Merk), pH was adjusted to 7 using 5 M NaOH] and incubated at 30 °C for 24-48 h. After dilution, samples were spread plated on Isolation agar (IA), [2 % (w/v) lactose (Oxoid), 0.5 % (w/v) peptone (BBL), 0.25 % (w/v) yeast extract (Oxoid), 1.5 % Bacto agar (Merk), pH was adjusted to 7 using 5 M NaOH] and incubated at 30 °C for 24 h to screening biopolymer producer bacteria. The broth used for screening of whey utilizing commercial biopolymer producer contained: 0.1 % (w/v) yeast extract, 0.05 % (w/v) malt extract (Oxoid), 0.05 % (w/v) K_2HPO_4, 0.01 % (w/v) NH_4Cl, 0.01 % (w/v) $MgSO_4$. This medium was prepared with different pH (4-9), added inorganic and organic nitrogen sources (yeast extract and NH_4NO_3, 1 %), and kept at different temperature (25, 30, 35, 37 ºC) to optimize alginate production. Gram and capsules stains were used for staining bacteria.

Proximate analysis of whey

The whey used in this study was characterized for its composition. The lactose, proteins and fats were determined using the Milko Scan method (Micro-balance 4503 MP6). Mineral composition, Ca, K and Na were determined by atomic absorption (Varian Spectra A A. 200). The nitrogen was determined using the Kjeldahl method (Kirk and Sawyer, 1991).

Exopolysaccharide production

To test the ability of bacterial cultures to produce biopolymers in whey, colonies that were mucoid, ropy and capsule positive were cultured in whey broth as fermentation broth. This medium was prepared in 250 mL conical flasks containing 50 mL of broth. Shake flask incubations were done at 30 ºC and 120 rpm. Samples were taken every 24 h to measure optical density (OD_{400}) by spectrophotometer assay (CARY 50 scan UV/Vis spectrum spectrophotometer). The ability of bacteria to produce exopolysaccharide was determined by increase of broth apparent viscosity or presence of gel or pellicle in fermentation broth (Schwartz and Bodie, 1985).

Table 1: Source of bacterial strains used in the study

Identification	Strain	Source
Azotobacter chroococcum	DSM No. 281	Soil
Azotobacter chroococcum	DSM No. 398	Soil
Azotobacter chroococcum	DSM No. 1723	Soil

Biopolymer recovery and purification

Recovery and purification of microbial exopolysaccharide were adapted with some slight modifications according to Vendrusculo, *et al.*, (1994). The fermentation broths were diluted 5 to 10 fold with redistilled water to reduce the viscosity. Cells were removed by centrifugation by Sorval Model RC5B at 39000 g for 30 min and supernatant was recovered by decanting. Freeze dried samples were ground into fine powder and stored in a desicator. The dilute supernatant was concentrated to its initial volume by ultra-filtration, using the YM30 membranes. The concentrates were treated with 5 M Trichloro acetic acid (30 mL/100 mL sample) to remove the proteins, and then neutralized with 5 M NaOH. The protein precipitates were further concentrated by ultra-filtration. The retentate was washed two times to remove lactose or other low molecular mass carbohydrates. In permeate and lactose rich broths, acid treatment were omitted. There volumes of chilled absolute ethanol were added to precipitate the polysaccharide. To enhance precipitation, these samples were stored at 40 °C for 24 h. The precipitates were recovered by centrifugation at 12,000 g at 40 °C for 30 min and dissolved in distilled water. This solution was subjected to ultra-filtration and washed twice redistilled water. The retained material was dissolved in distilled water for 24 h. This solution was precipitated as described above and lyophilized by Snijders scientific 2040 VDH lyophilizer.

Drying of polysaccharide samples

The alcohol precipitated samples were transferred to sample jars and frozen at 20 ºC for 24 h. The frozen samples were freeze dried at -500c and 100 kPa vacuum. Freeze drying was done until constant weights were achieved (30 to 48 h).

Surface tension measurement

Surface tension was measured using a Du Nouy tensiometer. Measurements were made on supernatant samples after centrifugation.

Alginate Recognition assays

Transmission electron microscopy assay

Transmission electron microscopy was used to investigate the alginate concentration gradient around the cells was adapted with some slight modifications according to Dekwer and Hempel, (1999). Samples were picked up with carbon-coated collodion grids. The grids were plotted with filter paper, and alginate was positively contrasted by incubation on freshly prepared 1% aqueous ruthenium red solution for 1-2 min at room temperature. The grids were washed three times with distilled water by paper blotting. Finally the cells were negatively stained with 1% uranyl acetate for 10 s, blotted, and air dried. Electron microscopy was done with LEOi at 80 kV with a magnification between 16,000 and 25,000.

Reaction of alginate sample with and without borate (by use Carbazole reagent)

Reaction of alginate sample with Carbazole reagent was adapted with some slight modifications according to Dekwer and Hempel, (1999). After Carbazole reagent was added to sample, mixed and heated at 55 ºC for 30 min, color was stable for 2 h at room temperature. Standard curves of mannuronic acid as well as guluronic acid were made for the two reaction conditions (with and without borate).

Semi continuous culture of Azotobacter spp to whey degradation

A semi continuous culture was performed using *Azotobacter chroococcum* growing in whey broth medium to test the ability of whey degradation. The culture was then aerated and agitation speed was set at 200 rpm. Samples incubated at 30 °C for 24 h and optical density was measured after every replacement times at 400 nm (CARY 50 scan UV/Vis spectrum spectrophotometer).

Statistical analysis

Significant differences between bacterial treatments were tested by analysis of variance using Minitab. Data were normalized, and Tukey tests were performed. The results of different incubated time experiments were subjected to Student's *t* test to identify significant differences between bacterial treatments. Probability (*P*) values of <0.05 were considered significant.

RESULTS

All strains were studied exhibited significant growth in whey agar than Manitol agar media. The results also show that two strains of *Azotobacter chroococum* (1723 and 398) in whey agar media, were produced colonies that were mucoid, ropy and capsule positive with yellow pigment in 24 h at 30 °C. There was insignificant growth observed up to 48 h in Manitol agar.

These strains were regarded as efficient producer and were subjected to further study. They were able to use whey as fermentation substrate for biopolymer production. After optimizing environmental factors such as pH, salt concentration and temperature, *Azotobacter chroococum* 1723 was able to produce exopolysaccharide greater than 5 mg/mL. This corresponded to broths apparent viscosities of at least 25 cP at 0.6/s. In *Azotobacter chroococum* 1723, biopolymer production was minimum yield in Manitol broth, while maximum production was obtained from whey broth. Composition of the whey broth medium used in this study presented on Table 2. Optimum results were obtained when using whey broth as a fermentation medium without extra salt, temperature at 35 °C and pH 7 (P<0.05). This strain was able to produce biopolymer under uncontrolled pH condition. Maximum results were obtained in fermentations where the pH was maintained at 7. When fermentations were done in whey broth under aerated conditions, there was no improvement in polymer production and whey degradation. Increasing yeast extract and NH_4NO_3 as organic and inorganic nitrogen sources to this level reduced to whey degradation at least 30 % (Fig 1). Although *Azotobacter chroococum* 1723 could produce biopolymer at 25 °C incubation, maximum yields were obtained from fermentation done at 35 °C. Semi-continuous culture results demonstrated that, alginate production as well as whey degradation was decreased (1 mg/mL and 30%). Transmission electron microscopy assay showed that, bacterial cells have a net-structured polysaccharide capsule around the cells. It formed a compact dense layer of capsular polysaccharide which totally covered the membrane. The color was produced by reaction of alginate samples with and without borate was stable for 2 h.

Table 2: Composition of the whey broth medium

Whey Constituents	Amount (% w/v)	SD
Lactose	4.91	0.3
Proteins	0.93	0.05
N_2	0.13	0.04
Fats	0.71	0.01
Ca	4.95 ppm	0.1
K	0.93 ppm	0.04
Na	0.078 ppm	0.07
Total solids	6.25	0.05

Each value is a mean ± (standard deviation=SD) of triplicate assays.
*Indicates a significant (P < 0.05) difference

DISCUSSION AND CONCLUSION

Whey is a by product of the dairy industry that has presented many disposal problems (Horton, 1993). It is available abundantly in a potential substrate for the production of commercially valuable products (Zadow, 1992). It composed mainly of lactose (4.9 %), protein (0.9 %), and fat (0.7 %) that may be used by microorganisms as carbon and nitrogen source for metabolism to support growth and biopolymer production (Table 2). Lactose is the most abundant constituent of whey and it forms at least 78 % (w/w) of the whey's total solids. This indicates that for an organism to efficiently use whey as substrate, it must be able to metabolize the lactose as its carbon source. Such organisms should produce sufficient lactose hydrolyzing enzymes (β-galactosidase) to hydrolyze the lactose to its monomers. The media used for the production of commercial microbial polysaccharides usually contains glucose or sucrose as carbon source (Belder, 1993; Kang, *et al*., 1993).

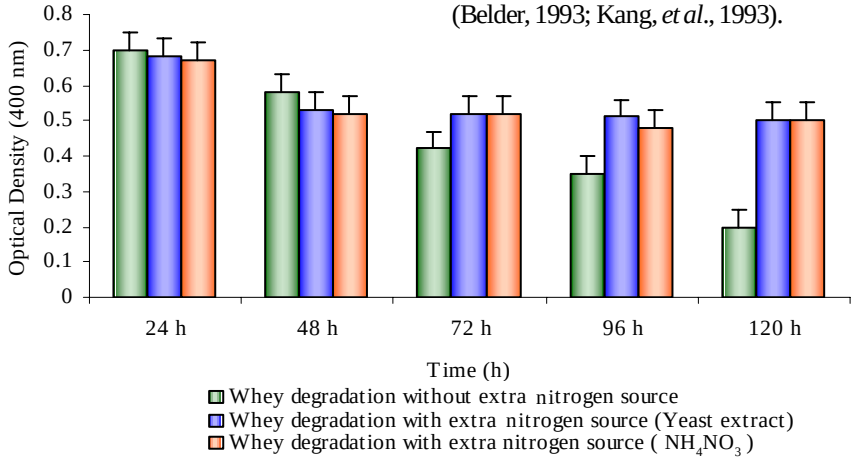

Fig. 1: The effect of different nitrogenous rich compounds (yeast extract and NH_4NO_3) on whey degradation

Incorporation of nitrogenous compounds into the medium did not increase whey degradation by *Azotobacter chroococcum* 1723(P<0.05)This increases the production costs of microbial biopolymer (Hansen, 1993). The growth of the bacteria in whey broths and efficient whey degradation, suggested that the organisms were using other carbon sources present in the whey too. Probably whey proteins are used too because whey total solids are composed of 12 % (w/w) proteins as shown at Table 2. Different nitrogen concentrations in fermentation broths were achieved by addition of different levels of yeast extract and NH_4NO_3 (organic and inorganic nitrogen sources) to a basal medium. Racine, *et al*. (1991) showed that biopolymer production was enhanced when nitrogen supply was limited and the carbon source was in excess. The results presented in this study Showed that biopolymer production decreased as the nitrogen concentration increased. Embuscado, *et al*. (1994) observed that inorganic sources gave relatively low to moderate yield of cellulose. The only difference is that in their investigation ammonium nitrogen sources gave better yields, whereas in this study, organic and inorganic sources didn't give better results. This may suggest that in different organic nitrogen sources *Azotobacter chroococum* 1723 produces biopolymers that differ in rheological quality. Similar observations were made by Kennedy, *et al*. (1982). Emtiazi, *et al*. (2004) showed that, addition of vitamin, different nitrogen sources (Ammonium salts, yeast extract and peptone) did not effect exopolymer production in *Azotobacter* spp. The effect of pH on biopolymer production has been explained before. Embuscado, *et al*. (1994) explained that pH affects both bacterial growth and biopolymer production. When bacterial cells are exposed to pH beyond their optimum range, maintenance energy is used for pH control. This reduces the energy available for biopolymer production, thus the bacterial ability to produce the biopolymer is reduced. The media pH also affects the permeability of the bacterial cell membrane thus affecting the biochemical activities of the cell required for biopolymer production. Emtiazi, *et al*. (2004) showed that, *Azotobacter* AC2 produced maximum (7.5 mg/mL) alginate in media with sucrose as the only carbon source but in this study *Azotobacter chroococum* 1723 was able to produce exopolysaccharide greater than 5 mg/mL. NMR studies by Moe, *et al*., (1995) suggested a possible binding site for calcium ions in a single alginate chain. Only alginates having G blocks (alga and *Azotobacter* alginates) can bind calcium to form rigid gels. Salt concentration of less than 3 mM is sufficient to slow down the kinetics of the dissolution process and hence limits the solubility. Calcium concentration of more than 4.95 ppm in whey broth, so alginate can attach to bacterial cell wall and a few alginate concentrations can be found in supernatant. In semi-continuous culture minimum yields of alginate production and whey degradation were obtained. Dekwer and Hempel (1999) demonstrated that, increasing the dissolved oxygen concentrations in the chemostat condition, the sugar was mainly wasted in respiration. So both growth and alginate formation were clearly affected by dissolved oxygen tensions of the culture medium. They showed that, at the lowest O_2 value as well as the highest value, the biomass and alginate formation was minimum yield. Ashraf, *et al.*, (2006) also showed that EPS-producing bacterial isolates was affected by environmental stress. *Azotobacter chroococum* strains were good N_2-fixation strains that investigated in current study. Under conditions of oxygen stress (nitrogenase inactivation) the bacterium produced compact alginate capsules to stabilize the intracellular oxygen concentration at minimal value suitable for nitrogenase activity (Dekwer and Hempel, 1999). These results have clearly shown that whey can be used as a fermentation substrate for the production of biopolymers. *Azotobacter* spp produced biopolymers (alginate and PHB) of commercial importance. Therefore the production of these biopolymers by whey fermentation can ensure that not only are value added products obtained, but the costly problem of the disposal of whey is also addressed.

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