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ORIGINAL PAPER

Xanthan production by *Xanthomonas campestris* using whey permeate medium

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Abstract Xanthan gum is a polysaccharide that is widely used as stabilizer and thickener with many industrial applications in food industry. Our aim was to estimate the ability of *Xanthomonas campestris* ATCC 13951 for the production of xanthan gum by using whey as a growth medium, a by-product of dairy industry. *X. campestris* ATCC 13951 has been studied in batch cultures using a complex medium for the determination of the optimal concentration of glucose, galactose and lactose. In addition, whey was used under various treatment procedures (de-proteinated, partially hydrolyzed by β-lactamase and partially hydrolyzed and de-proteinated) as culture medium, to study the production of xanthan in a 2 l bioreactor with constant stirring and aeration. A production of 28 g/l was obtained when partially hydrolysed β-lactamase was used, which proved to be one of the highest xanthan gum production reported so far. At the same time, an effort has been made for the control and selection of the most appropriate procedure for the preservation of the strain and its use as inoculant in batch cultures, without loss of its viability and its capability of xanthan gum production. The pre-treatment of whey (whey permeate medium hydrolyzed, WPH) was very important for the production of xanthan by the strain *X. campestris* ATCC 13951 during batch culture conditions in a 2 l bioreactor. Preservation methods such as lyophilization, cryopreservation at various glycerol solution and temperatures have been examined. The results indicated that the best preservation method for the producing strain *X. campestris* ATCC 13951 was the lyophilization. Taking into account that whey permeate is a low cost by-product of the dairy industry, the production of xanthan achieved under the studied conditions was considered very promising for industrial application.

Keywords *X. campestris* · Whey permeate · Xanthan · Preservation method

Introduction

Xanthomonas campestris, a Gram-negative bacterium, produces xanthan, a water-soluble extracellular heteropolysaccharide, with a great industrial importance. Due to its reological properties such as high viscosity and pseudoelasticity, xanthan is used in oil drilling, in building products to optimize material properties and in textile and food industries as thickener, emulsifier and stabilizer (Garcia-Ochoa et al. 2000; Plank 2004). One of the major disadvantages for the industrial use of *X. campestris* strains, is the loss of xanthan production after a long term preservation of the strain, probably due to a gene mutation at the metabolism pathway of xanthan gum production (Martinez-Salazar et al. 1993).

Whey is the major by-product of the dairy industry. It is characterized by its elevated BOD values, mainly resulting from its high content of lactose (Hatzinikolaou et al. 2005). Milk proteins, the second major constituent of whey, at a concentration of 1 % (w/v), are almost universally recovered via ultrafiltration by the dairy companies and used widely in infant nutrition industry (Outinen et al. 2010). The resulting whey permeate though, still represents a major disposal problem for the dairy industries. A typical dairy agro-industry generates 500 m^3 of wastes per day on

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average (Demirel et al. 2005). A number of applications for whey permeate have been developed, in an effort to overcome the problem of the disposal. Its concentration and subsequent use as an additive for animal feedstock seems to outstand between the various applications (Huang and Yang 1998; Koutinas et al. 2009). Another alternative is the use of whey as the basic medium for various fermentations as the production of ethanol, methane, yeast protein, lactate, propionate, acetate and xanthan gum (Koutinas et al. 2009).

In this study, the effect of whey pre-treatment on xanthan production by *X. campestris* ATCC 13951 was examined in batch cultures. The pre-treated medium which led to the highest xanthan production was used in a laboratory bioreactor. Additionally, three preservation methods of *X. campestris* ATCC 13951 strain (lyophilization, cryopreservation at different glycerol concentration solutions and storage at low temperatures) have been examined.

Materials and methods

Microorganisms, culture media and growth conditions

The *X. campestris* ATCC 13951 was kindly provided by the laboratory of Professor D. A. Kyriakides, Department of Chemistry, Aristotle University of Thessalonica, Greece. The above microorganism was grown in different media for selected study purposes (Table 1): (a) Yeast Extract Malt Agar (YMA) described by Rodríguez et al. (1997) (in g/l: glucose 20, yeast extract 3, peptone 5, malt extract 3, Oxoid No. 2 agar 20) and (b) Yeast Extract Malt-Lactose Agar (YM-LA) containing lactose instead of glucose, as its sole carbon source. Media (a) and (b) were used mainly for maintenance of the strain whilst when they were modified as (c) Yeast Extract Malt-Lactose Broth containing 43 g/l of lactose (instead of 20 g/l), coded as YM-LB and (d) Yeast Extract Malt-Glucose and Galactose Broth coded as YM-GGB (containing 21.5 g/l glucose and 21.5 g/l galactose instead of lactose) were used for the estimation of the carbon source type and its concentration on the xanthan gum production.

Whey and de-proteinated Whey, which were kindly provided by the Greek dairy industry MEVGAL SA; Thessalonica, Greece were used after specific treatment for the removal of any suspended insoluble materials (Hatzinikolaou et al. 2005), in the ultrafiltration unit for lactose hydrolysis. The treaded whey had a final lactose concentration of 43 g/l, only trace amounts of glucose and galactose (less than 0.02 g/l) and a protein content of less than 0.1 g/l (de-proteinated whey permeate). The first whey based medium was (e) called Whey Permeate Medium with total lactose concentration of 43 g/l, coded as WP, and derived after the pre-treatment as described above. The second whey based medium was (f) called Whey Permeate Medium Hydrolyzed according to Hatzinikolaou et al. (2005) with final concentration of lactose 7.4 g/l, glucose 17.8 g/l and galactose 17.8 g/l, coded as WPH. The third whey based medium was (g) called Whey Permeate Medium Hydrolyzed and De-proteinated according to Hatzinikolaou et al. (2005) with final concentration of lactose, glucose and galactose the same as in medium WPH and coded as WPD (Table 1). All whey permeate media were supplemented with 5 g of KH_2PO_4 and 1 g of yeast extract per litre.

The *X. campestris* ATCC 13951 was grown in a bioreactor with a culture volume of 2 l. The culture conditions were 30 °C, 600 rpm and pH 6.75 in YM-glucose and

Table 1 Complex and whey based media used throughout the experimental work of this study for the cultivation of *X. campestris* ATCC 13951

Medium (abbreviation)	Carbon source (g/l)	Experimental studies for
Yeast Extract Malt Agar (YMA)*	Glucose (20.0)	Maintenance of xanthan gum production ability
Yeast Extract Malt-Lactose Agar (YM-LA)*	Lactose (20.0)	Maintenance of xanthan gum production ability
Yeast Extract Malt-Lactose Broth (YM-LB)*	Lactose (43.0)	The effect of complex and whey based media on the production of xanthan gum
Yeast Extract Malt-Glucose and Galactose Broth (YM-GGB)*	Glucose (21.5) and Galactose (21.5)	(a) The effect of complex and whey based media on the production of xanthan gum (b) xanthan gum production in bioreactor
Whey Permeate Medium (WP)**	Lactose (43.0)	The effect of complex and whey based media on the production of xanthan gum
Whey Permeate Medium Hydrolyzed (WPH)**	Lactose (7.4), Glucose (17.8) and Galactose (17.8)	(a) The effect of complex and whey based media on the production of xanthan gum (b) xanthan gum production in bioreactor
Whey Permeate Medium Hydrolyzed and De-proteinated (WPD)**	Lactose (7.4), Glucose (17.8) and Galactose (17.8)	The effect of complex and whey based media on the production of xanthan gum

* Complex media

** Whey based media

galactose broth (YM-GG) and whey permeate hydrolyzed medium (WPH).

In order to estimate the xanthan gum production in complex and Whey based medium, *X. campestris* ATCC 13951 was grown in a bioreactor (Biostat B, B BRAUN, Germany) with a culture volume of 2 l and at 30 °C temperature, 500 rev/min stirring with aeration and the pH was controlled at 6.75 using 1 M of NaOH and 1 M of HCl.

Batch cultures of *X. campestris* ATCC 13951 which were used as inocula for all experiments were carried out in 300 ml conical flasks containing 50 ml of the appropriate growth medium at 30 °C and pH 6.75 and incubated in an orbital shaker at 250 rev/min for 48–96 h (Table 1). The inoculum quantity was 10^9 cells per litre in all cases and was estimated by both cell counts using a Neubauer improved counting chamber and spectrophotometrically as described in Analytical methods section.

Microorganism preservation and maintenance of the xanthan gum production ability

Three preservation procedures have been examined for 22 months period: (a) storage at low temperatures (4 °C)—microorganisms were kept as stab cultures on YMA and YM-LA plates for 1 month, (b) frozen storage—microorganisms were kept in a glycerol solution as protective agent at −70 °C. The concentration of glycerol solutions that have been examined were in g/l: 200, 300, 400 and 500, (c) lyophilization—microorganisms were freeze-dried after cultivation in YM-LA medium using skim milk 2 % (w/v) as protective agent. In all cases, every 30 days, microorganisms were transferred on YMA and YM-LA through a 22 months period. The formation of xanthan colonies (yellow coloured), after 48 h of incubation on YMA and YM-LA plates at 30 °C and the production of xanthan gum in batch cultures using YM-LB, was examined.

Analytical methods

For the estimation of growth in liquid cultures, cells were collected by centrifugation (21,250 g, 20 min, 30 °C) and washed in Ringer's solution three times and resuspended in the corresponding culture medium and then the absorbance at 600 nm was measured using a spectrophotometer (Hitachi model U-1100). Additionally, cell numbers were estimated using a Neubauer improved counting chamber. Also viable counts were measured into appropriate agar plates (Table 1) following serial dilutions up to 10^{10}.

The viscosity of the culture broth was assayed in a Brookfield DV-E Viscometer according to the method of Shu and Yang (1990).

Xanthan gum was estimated in culture supernatants by dry weight determination, as described by Ekateriniadou et al. (1994) modified as follows; Samples of 10 ml were first diluted with distilled water by a factor of 2–5 and then were centrifuged at 21,250 g, for 20 min at 4 °C to precipitate the suspended cells. Xanthan gum was obtained from the supernatant by adding 88 ml of ethanol (100 % v/v) and 4 ml of KCl (10 % w/v). The polysaccharide solution was stored for 30 min in −20 °C for precipitation. Xanthan was then collected using pre-weighed filters, dried at 40 °C for 48 h and weighed on an analytical balance.

Lactose, galactose and glucose were determined in culture supernatants by high-performance liquid chromatography (Hewlett Packard, lit with a refractive index detector) with a Kromasil-100 C_{18} column 250 × 4 mm (MZ ANALYSENTECHNIK). Analysis was carried out isocratically with CH_3CN:H_2O (80:20 %), at 40 °C, and a flow rate of 1 ml/min.

All points on graphs are the mean of four independent experiments.

Results

Microorganism preservation and maintenance of the Xanthan Gum production ability

It is well documented that master strains often lose their ability of xanthan gum production after many repetitions of inoculation in agar plates due to a gene mutation at the metabolism pathway of xanthan gum production. Therefore the study of different preservation methods of the *X. campestris* ATCC 13951 strains seemed essential.

The results of the first preservation method (storage at temperatures 4 °C) indicated that the strain loses the ability of xanthan gum production after seven consecutive inoculation trials in agar plates, independently the media used in a time course of 14 weeks.

Concerning the second preservation method (frozen storage in a glycerol solution), the results indicated that, the glycerol concentration among the concentrations studied that gave the highest xanthan gum production, was 300 g/l at −70 °C. In all glycerol solutions that have been kept at −70 °C, no significant lose of the xanthan gum production ability was observed. After a period of 22 months, a transformation of the colonies of *X. campestris* ATCC 13951 and the loss of xanthan gum production, has been noticed (Fig. 1).

The best method for the preservation of *X. campestris* ATCC 13951 was by lyophilization (Fig. 2). The microorganism was able to produce xanthan gum after been lyophilized for 22 months. As it can be seen there was a drop of 50 % of the xanthan production when the strain was kept lyophilized for two and a half months. The xanthan gum production ability of the inoculum remained stable from the second until the 22nd month of the study.

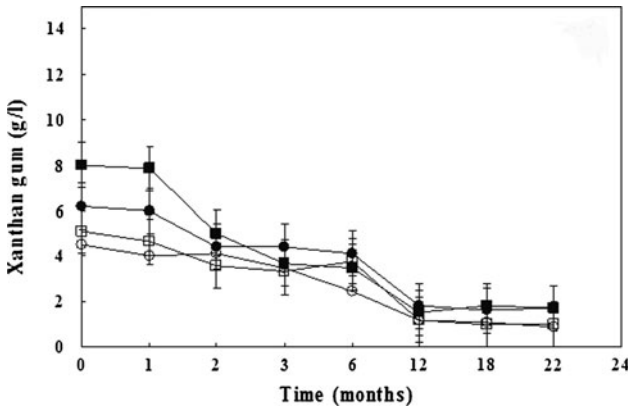

Fig. 1 Effect of glycerol concentration on xanthan gum production (g/l) during preservation of the inoculum at −70 °C. 200 g/l (*filled square*), 300 g/l (*filled circle*), 400 g/l (*open square*) and 500 g/l (*open circle*). Experiments were carried out in YM-LB for 96 h at 30 °C and pH 6.75

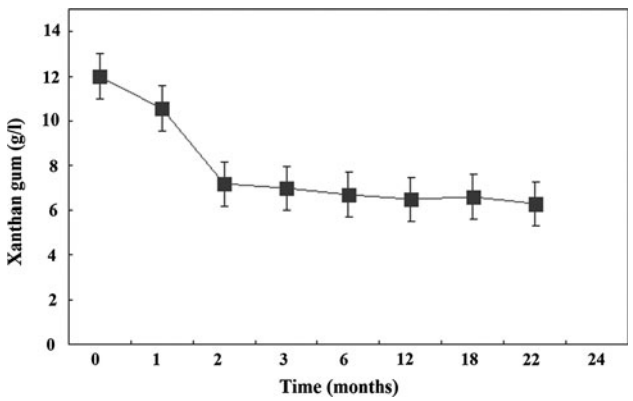

Fig. 2 Effect of preservation period on xanthan gum production (g/l) from lyophilized strain. Experiments were carried out with *X. campestris* ATCC 13951 in YM-LB for 96 h at 30 °C and pH 6.75

Concluding, among the three preservation methods tested during a period of 22 months, formation of xanthan gum—forming colonies (yellow coloured), were monitored. Thereafter, white—non xanthan gum forming—colonies started to appear in agar plates independently the medium used. Similar results were obtained from the production of xanthan gum in batch cultures using YM-L medium.

The effect of complex and whey based media on the production of xanthan gum using *X. campestris* ATCC 13951.

Results from batch cultures of *X. campestris* ATCC 13951 at stationary phase using different concentrations of lactose or equimolar concentrations of glucose—galactose in the media showed that in both cases the best concentration of carbon source for xanthan production was the same and equal to 43 g/l (Fig. 3).

When the microorganism was grown in complex (YM-GGB and YM-LB) and Whey based media (WP, WPH and

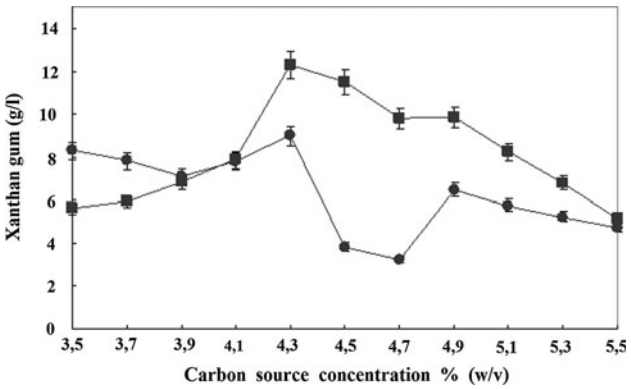

Fig. 3 Xanthan gum production in batch cultures of *X. campestris* ATCC 13951 at different concentrations of carbon sources: Experiments were carried out in YM-LB (*filled circle*), YM-GGB (*filled square*) for 96 h at 30 °C and pH 6.75

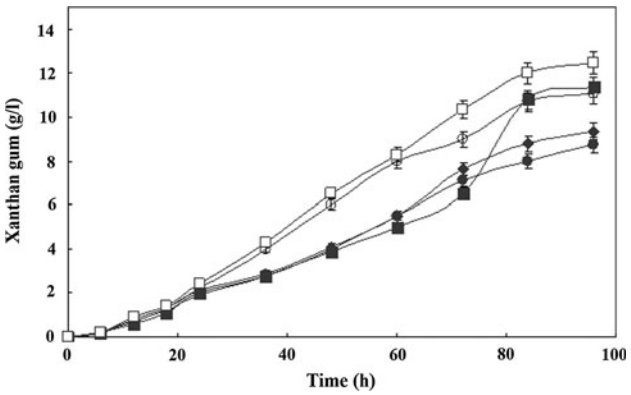

Fig. 4 Xanthan gum production in batch cultures of *X. campestris* ATCC 13951 in complex and Whey based media at 30 °C and pH 6.75. YM-GGB (*open square*), YM-LB (*open circle*), WP (*filled circle*), WPH (*filled square*) and WPD (*open diamond*)

WPD), with total carbon source concentration at 43 g/l, after 96 h of growth (stationary phase), the xanthan gum production was between 8.72 and 12.46 (g/l) with the highest value obtained in YM-GGB (Fig. 4). The xanthan gum production using WPH was 8 % less, compared with the xanthan gum produced when YM-GGB was used.

Xanthan gum production in a bioreactor using treated whey as a growth media

The *X. campestris* ATCC 13951 was grown in a bioreactor with a culture volume of 2 l using YM-glucose and galactose broth (YM-GGB) and whey permeate hydrolyzed medium (WPH). The maximum specific growth rate of *X. campestris* ATCC 13951 in YM-GGB medium was $\mu_{max} = 0.09\ h^{-1}$ (Fig. 5). Production of xanthan started after 12 h of inoculation with a continuous increase until

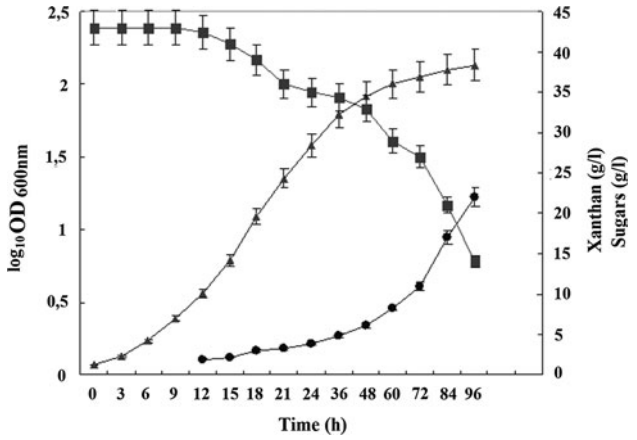

Fig. 5 Growth of *X. campestris* ATCC 13951 in YM-GGB in a 2 l bioreactor at 30 °C, stirred at 500 rpm with aeration and pH controlled at 6.75. Biomass (*filled triangle*), xanthan gum (*filled circle*) and sugar concentration (*filled square*)

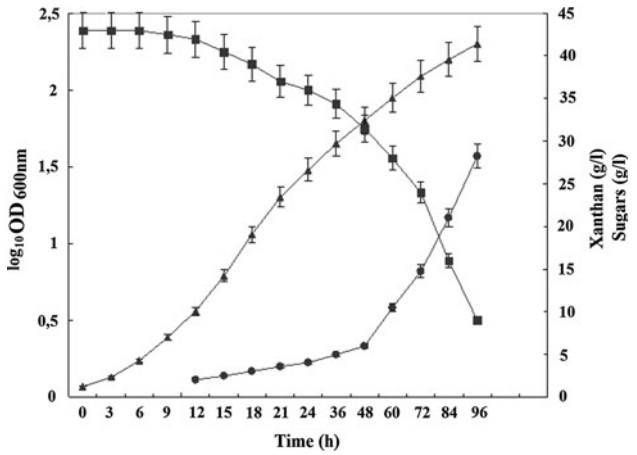

Fig. 6 Growth of *X. campestris* ATCC 13951 in WPH in a 2 l bioreactor at 30 °C, stirred at 500 rpm with aeration and pH controlled at 6.75. Biomass (*filled triangle*), xanthan gum (*filled circle*) and sugar concentration (*filled square*)

96 h. The final xanthan concentration was 22 g/l. In the case of the WPH medium, the maximum specific growth rate of *X. campestris* ATCC 13951 was $\mu_{max} = 0.08\ h^{-1}$ (Fig. 6). The production rate seemed to be very high after the 60 h of growth and until the 96th h of study. Thereby it was not possible to keep the culture growing due to the high viscosity of the culture (272 cP).

According to the equation P = C/t (P, xanthan productivity, C, xanthan concentration and t, time of fermentation) the overall xanthan productivity (P) was estimated at 0.29 g/(l.h). The xanthan efficiency ($Y_{x/g-g}$) using the equation $Y = C/S_R$ ($Y_{x/c}$ g of xanthan produced per g of carbon source consumed, C, xanthan concentration and S_R, carbon source concentration) was 0.65.

Discussion

In this work, a selection of the most appropriate procedure for the preservation of the strain *X. campestris* ATCC 13951 and its use as inoculant in batch cultures, without loss of the viability and capability of xanthan gum production was investigated. In parallel, the effect of carbon source on xanthan gum production was estimated.

Three preservation techniques were investigated and the best method was the lyophilization. The strain had retained its ability to produce quite high xanthan gum productivity almost 2 years after its lyophilization. The other two techniques (storage at temperatures of 4 °C and preservation in glycerol solution at −70 °C) illustrated that, after a short period of time, the strain lost the ability of xanthan gum production, probably due to a gene mutation at the metabolism pathway of xanthan gum production (Martinez-Salazar et al. 1993).

The effect of the quality and the quantity of the carbon source on the xanthan gum production was also studied. The best xanthan gum production in complex medium was obtained when equimolar solution of glucose and galactose was added. Although, media containing glucose ant sucrose are most frequently used for xanthan gum production, we tried to use whey permeate, a low cot by-product of the dairy industry, where lactose is the carbon source (Garcia-Ochoa et al. 2000). Concerning the quantity of carbon source, the best xanthan gum production was obtained when equimolar solution of glucose and galactose at a concentration of 43 g/l was used. This concentration was the most favourable for xanthan production in batch cultures in other studies as well (De Vuyst and Vermeire 1994). Among the different treatments of whey media that have been studied, only the growth of *X. campestris* ATCC 13951 in whey permeate medium hydrolyzed (WPH) was able to exhibit almost the same amount of xanthan gum as the growth in YM-GGB medium. This result indicated that, it is not necessary to de-proteinate the whey permeate when used as medium, but just to hydrolyze its lactose.

In addition xanthan gum production by *X. campestris* ATCC 13951 in a laboratory bioreactor using YM-GGB and WPH was studied. The xanthan production was higher when the strain grew in WPH (28 g/l) compared with that obtained in YM-GGB (22 g/l). In similar studies on complex media, the xanthan gum production varied from 22 g/l (Faria et al. 2009) to 32 g/l (Amanullah et al. 1998). This is the first report on xanthan gum production using whey based media. Its impact in the industry can be significant because the xanthan gum production achieved in the present study is among the best obtained even after comparison with the production achieved when complex media are used. Concerning the overall xanthan productivity (P) that was estimated in this study [0.29 g/(l.h)], it was

among the values obtained from similar studies like Faria et al. (2009) [0.16 g/(l.h)] and Amanullah et al. (1998) [0.47 g/(l.h)]. Corresponding results were also obtained for measurements of the efficiency (Amanullah et al. 1998; Faria et al. 2009). Further work could improve the productivity and efficiency in whey based media, by altering culture conditions such as aeration and agitation speed (Amanullah et al. 1998; Chaitali et al. 2003).

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References

Amanullah A, Tuttiett B, Nienow AW (1998) Agitator speed and dissolved oxygen effects in xanthan fermentations. Biotechnol Bioeng 57:198–210

Chaitali M, Kapadi M, Suraishkumar GK, Gudi RD (2003) Productivity improvement in xanthan gum fermentation using multiple substrate optimization. Biotechnol Prog 19:1190–1198

De Vuyst L, Vermeire A (1994) Use of industrial medium components for xanthan gum production by *Xanthomonas campestris* NRRL B-1459. Appl Microbiol Biotechnol 42:187–191

Demirel B, Yenigun O, Onay TT (2005) Anaerobic treatment of dairy wastewaters: a review. Process Biochem 40:2583–2595

Ekateriniadou LV, Papoutsopoulou SV, Kyriakidis DA (1994) High production of xanthan gum by a strain of *Xanthomonas campestris* conjugated with *Lactococcus lactis*. Biotechnol Lett 16:517–522

Faria S, Vieira PA, Resende MM, França FP, Cardoso VL (2009) A comparison between shaker and bioreactor performance based on the kinetic parameters of xanthan gum production. Appl Biochem Biotechnol 156:475–488

Garcia-Ochoa F, Santos VE, Casas JA, Gomez E (2000) Xanthan gum: production, recovery, and properties. Biotechnol Adv 18: 549–579

Hatzinikolaou DG, Katsifas E, Mamma D, Karagouni AD, Christakopoulos P, Kekos D (2005) Modelling of the simultaneous hydrolysis–ultrafiltration of whey permeate by a thermostable *β*-galactosidase from *Aspergillus niger*. Biochem Eng J 24: 161–172

Huang Y, Yang ST (1998) Acetate production from whey lactose using co-immobilized cells of homolactic and homoacetic bacteria in a fibrous-bed bioreactor. Biotechnol Bioeng 20:498–507

Koutinas AA, Papapostolou H, Dimitrellou D, Kopsahelis N, Katechaki E, Bekatorou A, Bosnea LA (2009) Whey valorisation: a complete and novel technology development for dairy industry starter culture production. Bioresour Technol 100:3734–3739

Martinez-Salazar JM, Palacios AN, Sanchez R, Caro AD, Soberdn-Chavez G (1993) Genetic stability and xanthan gum production in *Xanthomonas campestris* pv, *campestris* NRRL B1459. Mol Microbiol 8:1053–1061

Outinen M, Rantamäki P, Heino A (2010) Effect of milk pretreatment on the whey composition and whey powder functionality. J Food Sci 75:E1–E10

Plank J (2004) Applications of biopolymers and other biotechnological products in building materials. Appl Microbiol Biotechnol 66:1–9

Rodríguez H, Aguilar L, LaO M (1997) Variations in xanthan production by antibiotic-resistant mutants of *Xanthomonas campestris*. Appl Microbiol Biotechnol 48:626–629

Shu CH, Yang ST (1990) Effects of temperature on cell growth and xanthan production in batch cultures of *Xanthomonas campestris*. Biotechnol Bioeng 35:454–458