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Full Length Research Paper

Enhanced xanthan production process in shake flasks and pilot scale bioreactors using industrial semi-defined medium

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Xanthan gum, a heteropolysaccharide produced by *Xanthomonas campasteris*, is a widely used biopolymer in food industries. The production process is highly influenced by the type and concentration of the different carbon and nitrogen source as well as other medium components. The aim of this work was to develop an economic industrial medium for industrial production. In shake flask level, the maximal production of xanthan of 19.9 gL^{-1} was achieved by excluding ammonium nitrate from the industrial medium and making new medium formulation composed of (sucrose, soybean meal, ammonium phosphate and magnesium sulphate). Further optimization in the production process was achieved by transferring the process to 16-L bioreactor and cultivation under controlled pH condition. The maximal volumetric and specific xanthan production [$Y_{P/X}$] obtained were 28 gL^{-1} and 11.06 g g^{-1}, respectively. Thus, the semi-defined medium formulation developed in this work could be better and alternatively used for large scale production process for xanthan production when compared to other published media in respect to yield and cost.

Key words: Xanthan gum, *Xanthomonas campestris*, batch culture, semi-defined medium, biopolymer.

INTRODUCTION

Xanthan gum is an extracellular heteropolysaccharide produced by many types of bacteria belonging to *Xanthomonas* spp. such as *Xanthomonas campestris, Xanthomonas phaseoli* and *Xanthomonas malvacearum* (Leela and Sharma, 2000). Of all these strains, the plant pathogen, *X. campestris*, is the most widely used for the industrial production of xanthan based on its high yield and the high quality product suitable for many applications. Xanthan is one of very few microbial polysaccharides that have received GRAS (Generally Regarded as Safe) listing for food use according to FDA (Food and Drug Administration). Xanthan polymer has a primary structure consisting of repeated pentasaccharide units.

Each unit is formed by two glucose units, two mannose units and one glucoronic acid unit (Grula et al., 1989; Garcia-Ochoa et. al., 2000a). Among microbial polysaccharides, xanthan plays a dominant role due to the relative easiness to produce it and as a result of its outstanding properties (Candia et al., 1999).

Due to its unique structure, xanthan displays special pseudoplastic properties, high viscosity and solubility, enhanced stability over a wide range of pH values and temperatures, as well as compatibility with many salts, food ingredients and other polysaccharides used as thickening agents (Kalogiannis et al., 2003). These properties give rise to numerous applications of xanthan in various industries ranging from food industries to enhanced oil recovery in the petroleum fields. In food industry, xanthan contributes to consumer acceptability of foodstuff by improving various sensory properties such as flavor

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release, texture and appearance (Candia et al., 1999). Xanthan is also used in pharmaceutical formulations, cosmetics and agricultural products such as herbicides and fertilizers (Garcia-Ochoa et al., 2000a). In the petroleum industry, xanthan is considered the most widely used biopolymer and used not only as a mobility control agent in enhanced oil recovery but also to control the rheological properties of the oil well drilling mud and to impart high viscosity at low concentrations to fracturing fluids (Grula et al., 1989; Sutherland, 2002). Nowadays, the global xanthan market is estimated to worth $400 million and is growing at a rate of more than 5% per year. Analysts also estimate this growth to continue through 2012. The main contributing factor to this growth is the market expansion in Asia, Eastern Europe and the U.S. Asia, particularly China, is growing at more than 10% per year, while Europe is growing at about 4.5% per year. The industrial production of xanthan gum is carried out mainly in submerged culture and regulated by many cultivation conditions such as temperature, pH, aeration and agitation (Shu and Yang, 1990; Garcia-Ochoa et al., 2000b; Borges et al., 2008; Borges et al., 2009). On the other hand, the medium used in the industrial scale is composed of different types of carbon sources such as glucose, sucrose and molasses (De Vuyst and Vermeire, 1994; Kalogiannis et al., 2003), different types of organic nitrogen sources such as yeast extract, soybean meal, corn steep liquors (Molina et al., 1993; Lo et al., 1997), inorganic nitrogen source, inorganic phosphate source and many other macro and micronutrients (Umashankar et al., 1996). The purpose of the present work is to report the development of economically semi-defined industrial media for xanthan production. This was followed by evaluation of the suitability of the designed medium for large scale production using pilot scale 16-L *in situ* stirred tank bioreactor operated under uncontrolled and controlled pH conditions.

MATERIALS AND METHODS

Microorganism

The strain used throughout this work was *X. campestris* DSMZ-19000. This strain was obtained in lyophilized form from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany). To minimize the productivity loss by subsequent cultivations and preservation of *X. campestris* as reported by Galindo et al. (1994), cells were preserved in glycerin culture (50% v/v) at -80°C. Each experiment was started by revival of one glycerol vial in vegetative culture. This was also necessary to minimize the results fluctuation based on intra-population variation of this strain.

Cell propagation

For the first cell propagation, 1 cryogenic vial from stock culture (containing 1 ml) was taken and used to inoculate 250 ml Erlenmeyer flask containing 50 ml modified YM broth (glucose, 10 gL^{-1}; K_2HPO_4, 4 gL^{-1}; yeast extract, 4.0 gL^{-1}; $MgSO_4.7H_2O$, 0.5 g L^{-1} and malt extract, 5.0 gL^{-1}). The inoculated flasks were incubated on rotary shaker (Innova 4080, New Brunswick Scientific Co., NJ, USA) at 150 rpm and 30°C for 24 h. Cells were used thereafter to inoculate either 250 ml Erlenmeyer flasks of 50 ml working volume or stirred tank bioreactor with inoculum concentration of 5% (v v^{-1}).

Xanthan production medium and cultivation conditions

Five different types of broth medium were used in this study for primary evaluation for medium optimization process. All these media were reported before for their high support for xanthan production. The compositions of these media in (gL^{-1}) were as follows: Medium 1: Glucose, 55.0; defatted soybean flour, 4.6; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.5; $FeCl_3.6H_2O$, 0.00333; $MnSO_4.H_2O$, 0.00022; $ZnSO_4.7H_2O$, 0.00117; $CuSO_4.5H_2O$, 0.00062 and H_3BO_3, 0.000067 (Honma et al., 1996). Medium 2: Sucrose, 42.0; NH_4NO_3, 1.125; $(NH_4)_2HPO_4$, 0.217; $MgSO_4.7H_2O$, 0.25 and defatted soybean meal, 15.0 (Letisse et al., 2001). Medium 3: Glucose, 27.5; yeast extract, 3.0; KH_2PO_4, 2.0 and $MgSO_4.7H_2O$, 0.1 (Shu and Yang, 1990). Medium 4: Sugar cane molasses, 175.0; yeast extract, 5.0; peptone, 10.0; NaCl, 10.0 and K_2HPO_4, 4.0 (Kalogiannis et al., 2003). Medium 5: Glucose, 33.0; citric acid, 2.0; yeast extract, 0.75; peptone, 0.34; $MgSO_4.7H_2O$, 0.49; NH_4SO_4, 3.33; H_3BO_4, 0.0072; $FeCl_3.6H_2O$; 0.0042; KH_2PO_4, 0.0042; $CaCO_3$, 0.029, and ZnO, 0.006 (Esgalhado et al., 1995).

For all media used, the pH was adjusted to 7.0 before sterilization. The carbon source was autoclaved separately and added to the fermentation medium before inoculation. In case of shake flasks, the inoculated flasks were incubated on rotary shaker at 200 rpm and 30°C. The bioreactor used in this study was carried out using 16-L pilot scale stirred tank bioreactor (BioEngineering, Wald, Switzerland) with working volume of 8 L. The stirrer was equipped with two 6-bladded rushton turbine impellers ($d_{i\ (impeller\ diameter)}$ = 85 mm; $d_{t\ (tank\ diameter)}$ = 214 mm, $d_i\ d_t^{-1}$ = 0.397). The agitation speed was 200 rpm throughout the cultivation. Aeration was performed using filtered sterile air and supplied continuously to the bioreactor with rate of 1 v min^{-1}. Foam was suppressed by the addition of silicon antifoam grade A (Sigma-Aldrich Inc., USA). During the cultivation process, pH value and dissolved oxygen concentration were determined using pH and DO polarographic electrodes, respectively (Ingold, Mittler-Toledo, Switzerland). In case of pH controlled culture, the medium pH was adjusted to 5.5 by cascading the pH controller with acid/base feeding peristaltic pumps connected with 2.5 M HCl and NH_4OH, respectively.

Analysis

Samples in form of 3 flasks of 50 ml broth for each (in case of shake flask experiments) or 30 ml (in case of bioreactor cultivations) were taken at different time intervals during cultivations. Samples were chilled on ice and centrifuged immediately (33,000 g for 30 min at 4°C) to separate cells from the broth. In samples of high xanthan concentration, samples were diluted in KCl (1% w/v) to decrease sample viscosity and thereby improve cell separation from broth. The supernatant was removed and stored at -20°C for further analysis. Cell biomass was washed in KCl solution 1%, centrifuged at 9,000 g for 10 min at 4°C in pre-weighed falcon tubes, the cells were washed two times with distilled water and the tubes were dried to constant weight at 60°C in vacuum oven. The difference in tube weight was used to calculate the cell dry weight concentration by relating this value to the initial sample volume. Determination of xanthan concentration in the supernatant broth was carried out by polysaccharide precipitation with three volumes of 95% ethanol according to the method of Cadmus and Knutson (1983). The precipitate was dissolved in 1% KCl solution, re-precipitated again using ethanol and dried to constant weight at

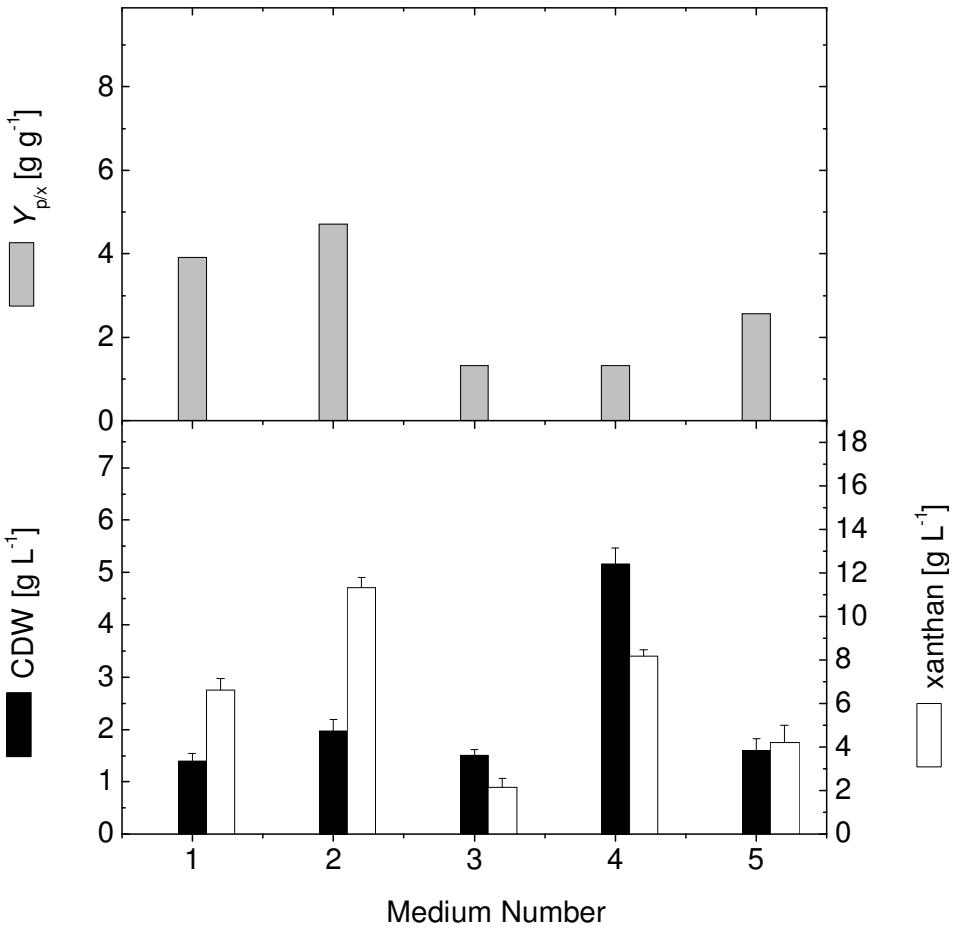

Figure 1. Cell growth and xanthan production in different types of media.

60°C in vacuum oven.

RESULTS AND DISCUSSION

Xanthan production using different production media

Different production media were used in this experiment to evaluate their capacity to support cell growth and xanthan production. These media have different composition and were used by previous authors for xanthan production as shown in materials and methods. As shown in Figure 1, all media supported cell growth and xanthan production in different extents. The maximal cell mass of about 5.2 gL^{-1} was obtained in medium 4 followed by medium 2 which yielded about 2 gL^{-1}. Other cell growth in other medium (which include glucose as sole carbon source) was ranged between 1.6 - 1.5 gL^{-1}. This indicates direct relation between the type of carbon source used and cell mass. As shown, sucrose in either pure form or in the form of cane molasses supported better growth over glucose medium. On the other hand, the maximal xanthan production of about 9.29 gL^{-1} was obtained in medium containing sucrose in pure form (medium 2) followed by medium containing sugar cane molasses which gave 6.8 gL^{-1}. The production of xanthan in other media was ranged between 2.0 and 5.44 gL^{-1}. For better understanding of the cell efficiency for xanthan production, the yield coefficient [$Y_{P/X}$], which represents the gram of polysaccharide produced by gram cell biomass, was calculated. As shown in Figure 1, the maximal cell productivity of about 5.27 gg^{-1} was obtained in medium 2 which include pure sucrose as sole carbon source. The type of medium and the role of carbon source on xanthan production were discussed by many authors. Glucose and sucrose (either in pure or commercial low market value molasses) were always the preferred carbon sources for xanthan production (Kalogiannis et al., 2003; Kurbanoglu and Kurbanoglu, 2007). This is because of the ease of assimilation of these sugars and their direct integration in xanthan biosynthesis pathway (Letisse et al., 2002).

Therefore, medium number 2 which composed of sucrose, ammonium nitrate, ammonium phosphate, magnesium sulphate and defatted soybean powder was used for further medium optimization in the following experiments.

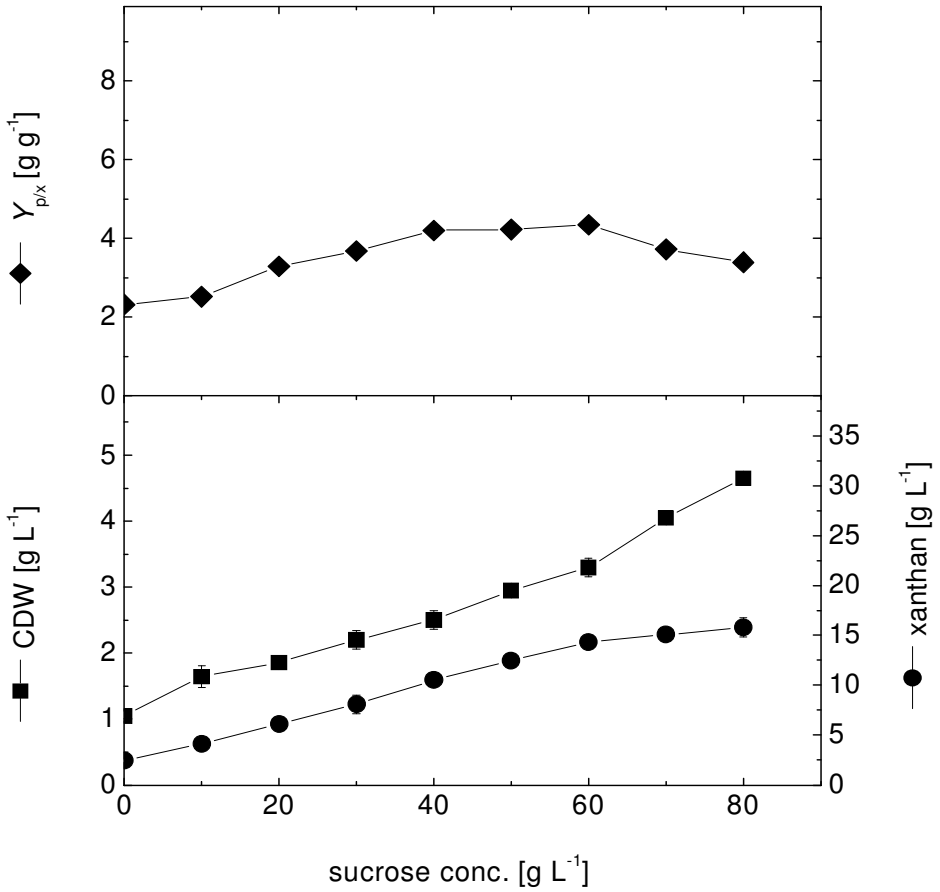

Figure 2. Effect of different sucrose concentrations on the cell growth and xanthan production by *X. campestris* in semi-defined medium.

Effect of different sucrose concentrations on xanthan production

Type and concentration of carbon source as well as C/N ratio are the three main key parameters for xanthan production as reported by many authors (Fuahashi et al., 1987; Umashankar et al., 1996; Leela and Sharma, 2000). In the present experiment, the effect of different sucrose concentrations ranged from 0 to 80 gL^{-1} on cell growth and xanthan production was investigated. Cells were cultivated in media of different sucrose concentrations for 120 h in shake flask cultures. As shown in Figure 2, there is direction relation between cell growth and sucrose concentration in medium for all concentrations applied. The maximal cell mass of 4.65 gL^{-1} was obtained in 80 gL^{-1} sucrose culture. However, the higher levels of biopolymer were not correlated with the level of sucrose in culture. Xanthan production was increased with the increase of sucrose concentration in medium up to 60 gL^{-1} which yielded 9.5 gL^{-1}. Further increase in sucrose concentration did not show significant effect on xanthan production. On the other hand, the cell productivity was increased gradually by the increase of sucrose concentration from 0 to 40 gL^{-1} reaching about 4 gg^{-1}. Further increase in sucrose concentration did not show significant influence on cell specific production. This indicates that the increase of volumetric xanthan production in cultures beyond 40 gL^{-1} was as a result of more cell growth. When the sucrose in culture exceeded 60 gL^{-1} (the maximal for xanthan production), the specific xanthan production decreased gradually as a function of further cell growth without significant increase in biopolymer production. Based on these data together, 60 gL^{-1} was selected for the subsequent experiments. The effect of sucrose concentration on xanthan production was studied by De Vuyst and Vermeire (1994) and they reported that the production of biopolymer was increased by increasing the sucrose concentration from 20 to 50 gL^{-1}. Further increase in sucrose concentration resulted in slight decrease in xanthan volumetric production.

Effect of ammonium nitrate concentrations on the cell growth and xanthan production

The nitrogen source of the fermentation medium under

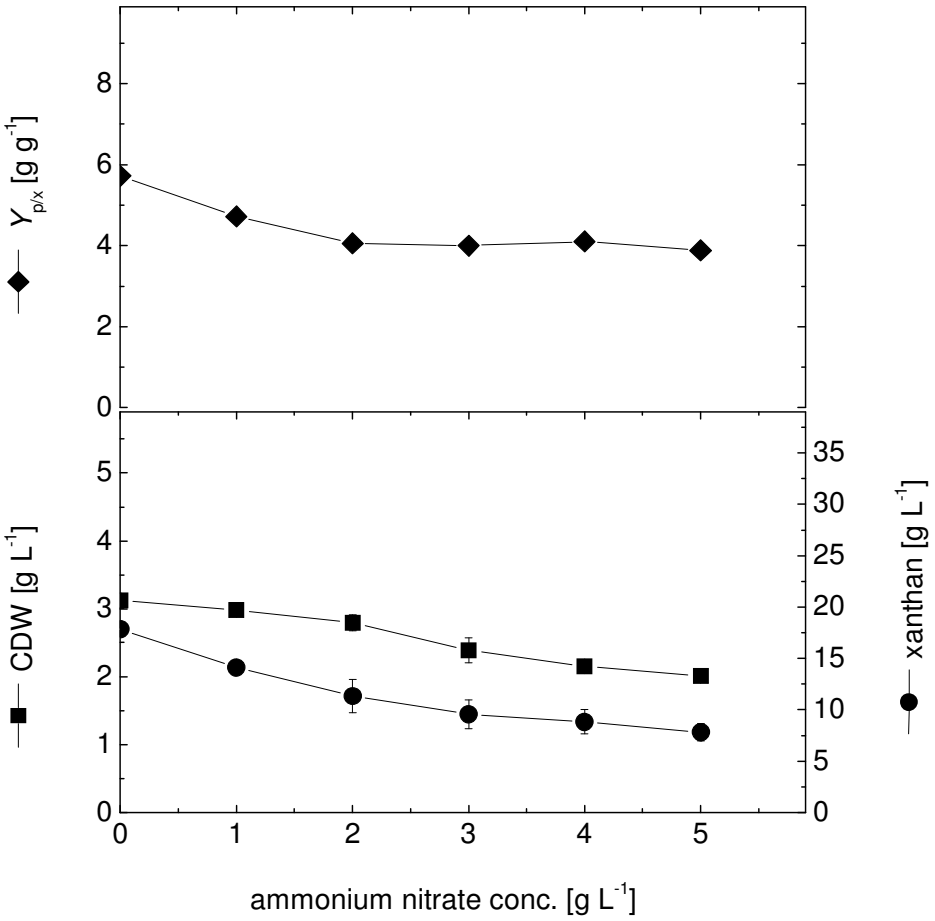

Figure 3. Effect of different ammonium nitrates concentrations on the cell growth and xanthan production by *X. campestris* in shake flask cultures.

study was in the form of organic source (soybean meal) and inorganic sources (ammonium nitrate and ammonium phosphate). The primary results showed that soybean meal did not show inhibitory effect on xanthan production if used in higher concentration than those applied in medium (15 gL^{-1}) but showed significant effect on biopolymer production when applied in lower concentrations (data not shown). Based on the molar ratio of nitrogen in culture, ammonium nitrate is the main source of inorganic nitrogen. Thus, effect of different concentrations of this salt on xanthan production was studied in more detail. As shown in Figure 3, both cell mass and xanthan production were decreased by the increase of ammonium nitrate in culture from 0 up to 5 gL^{-1}. Medium without ammonium nitrate yielded high volumetric and specific xanthan production of 17.85 gL^{-1} and 5.86 gg^{-1}, respectively. This high xanthan production observed in ammonium nitrate free culture may be attributed to the fact that low nitrogen concentration in culture (which is added in form of soybean meal) encourages the biopolymer production. This is based on the fact that xanthan production is highly induced at high C/N ratio (Lo et al., 1997).

Effect of different magnesium sulphate concentrations on cell growth and xanthan production

The magnesium concentration and increasing such concentration were found to influence both the cell growth and xanthan production (Figure 4). As shown, the amount of cell growth was increase by increasing the magnesium sulphate concentration in culture from 0 to 1.0 gL^{-1}. Further increase in magnesium concentration beyond this value did not show significant effect on cell growth. On the other hand, the highest volumetric and specific polysaccharide production of 19.5 gL^{-1} and 5.3 gg^{-1}, respectively, were achieved in medium with 1 gL^{-1} magnesium sulphate. In general, magnesium is a key cofactor for some enzymes and is present in cell walls and membranes as well. Magnesium also plays crucial role in activating the sugar uptake system. Thus, magnesium sulphate is one of the main fermentation medium ingredients to support cell growth and different metabolite production. For biopolymer production by microorganism, magnesium sulphate is always present in the cultivation medium in the range of between 0.1 and 2.0 gL^{-1}. The

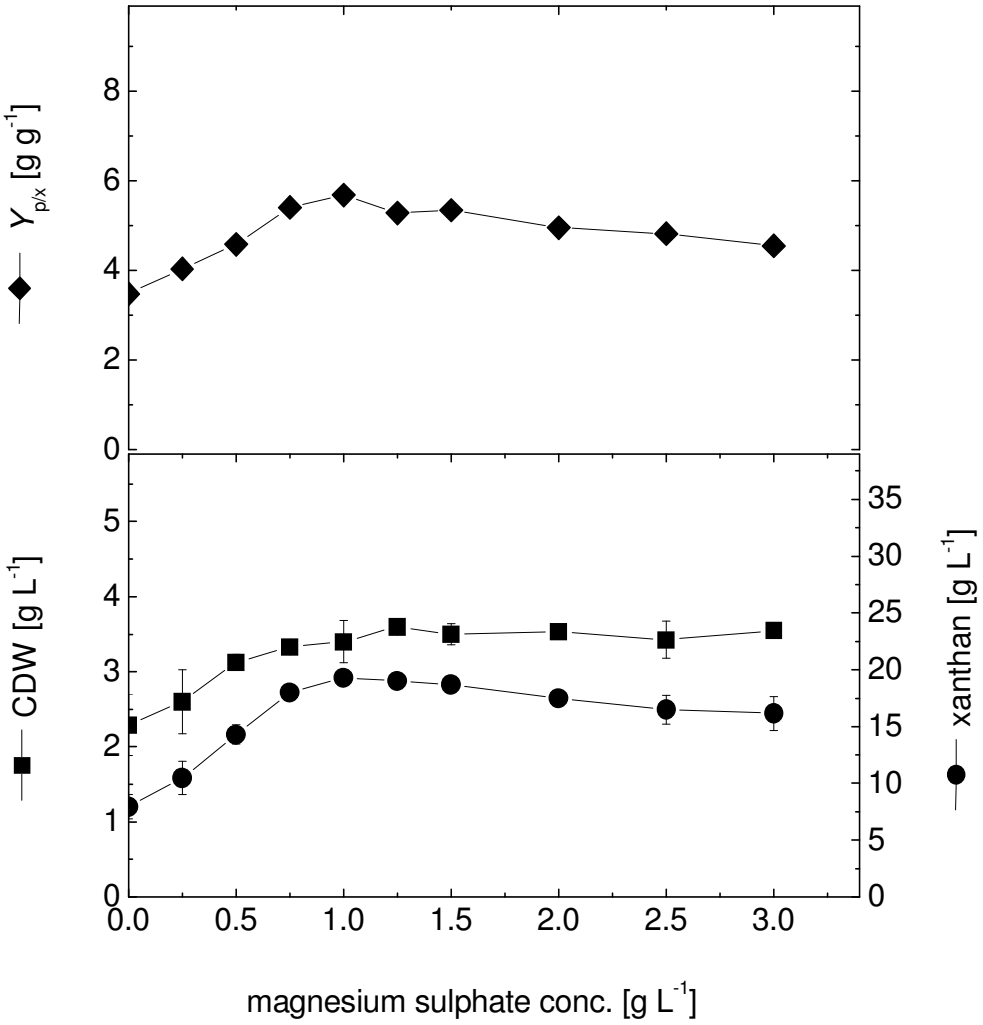

Figure 4. Effect of different magnesium sulphate concentrations on the cell growth and xanthan production by *X. campestris* in shake flask level.

previous study for xanthan production by *X. campestris* showed that magnesium did not influence cell growth significantly, but it supported xanthan production when added to the cultivation medium in concentrations more than 0.2 gL^{-1}. Another study for fungal polysaccharide production by *Ganoderma lucidans* revealed that the maximal production was achieved when magnesium sulphate was added to the medium in concentration of 1 gL^{-1} (Hsieh et al., 2006). However, the negative effect of low magnesium concentration on polysaccharide production is mediated by the reduction of phosphomannose isomerase enzyme activity which is the rate limiting step for xanthan production (Roseiro et al., 1993). However, the full function of magnesium with regard to polysaccharides production is still unclear.

In general, the optimized medium for high xanthan production had the following composition (g L^{-1}): Sucrose, 60.0; $(NH_4)_2HPO_4$, 0.217; $MgSO_4.7H_2O$, 1.0 and defatted soybean meal, 15.0.

Cell growth and xanthan production in optimized and unoptimized media in shake flask

For direct comparison between the growth kinetics and xanthan production before and after medium optimization, batch cultivation for both medium was conducted for 120 h in a shake flask. During cultivation time, samples were taken at 12 h intervals and analyzed. As shown in Figure 5, cells grew exponentially during the first 72 h and reached about 3.2 and 2.1 gL^{-1} for optimized and unoptimized media, respectively. After that time, the growth rate decreased significantly in both cultures. The production of xanthan started in both culture after 24 h and increased gradually as well up to 96 h and kept more or less constant for the rest of cultivation time. The maximal biopolymer production in optimized culture was 19.9 gL^{-1}. This value was about 70% higher than those obtained in shake flask in non-optimized medium. On the other hand, to have better

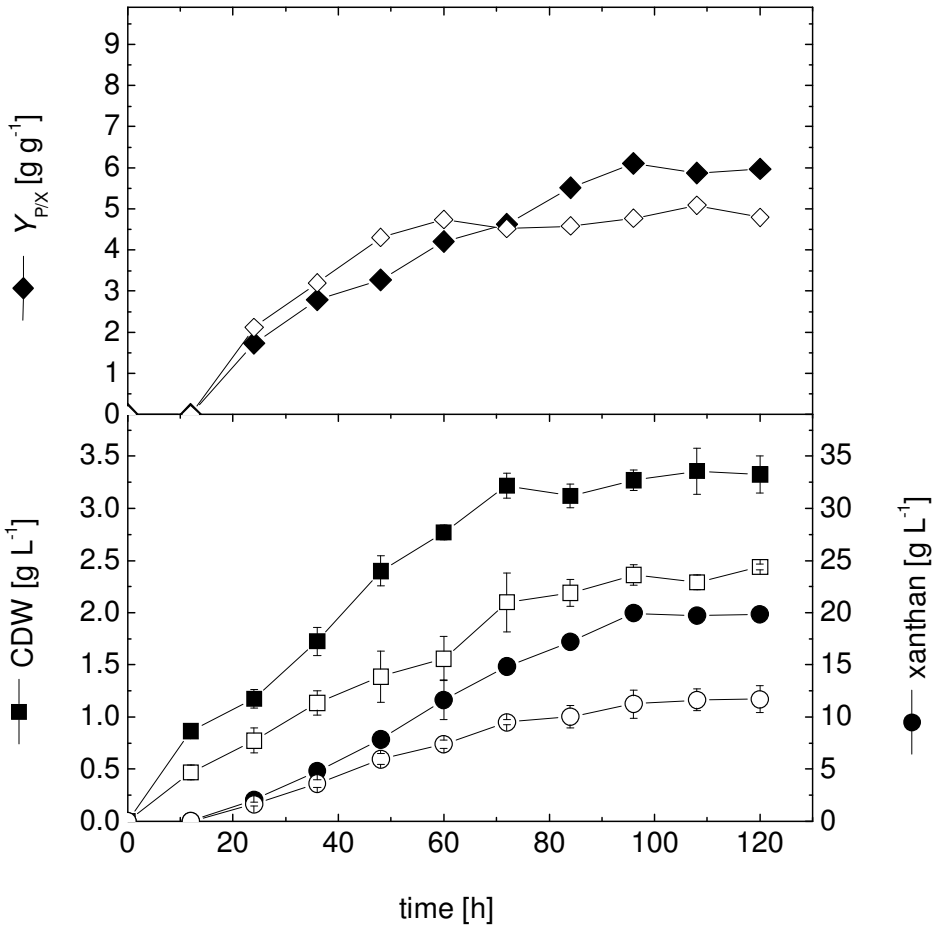

Figure 5. Cell growth and xanthan production in shake flask cultures. Closed and opened symbols represent optimized and unoptimized medium, respectively.

Table 1. Kinetics of cell growth and xanthan production in different media, production scales and conditions.

Parameters	Shake flask		Bioreactor	
	Non-optimized medium	Optimized medium	pH uncontrolled	pH controlled
Growth parameters				
X_{max} [g L^{-1}]	2.40	3.36	4.45	2.77
dx/dt [g L^{-1} h^{-1}]	0.029	0.045	0.067	0.044
μ [h^{-1}]	0.028	0.028	0.030	0.033
Production parameters				
P_{max} [g L^{-1}]	11.7	19.9	28.45	27.85
Q_P [g L^{-1} h^{-1}]	0.14	0.24	0.44	0.49
$Y_{P/X}$ [g g^{-1}]	4.9	6.38	6.79	11.06

understanding of the new medium formulation on the cell productivity, comparison between the yield coefficients [$Y_{P/X}$] in both cultures were calculated. The results in Figure 5 revealed that this value was increased from 4.9 to 6.38 gg^{-1}. This clearly indicates that the increase in volumetric xanthan production after medium optimization was not only attributed to the increase in cell growth but also to the increase of cell productivity as well. However, Table 1 summarizes all kinetic data calculated to compare between both cultures.

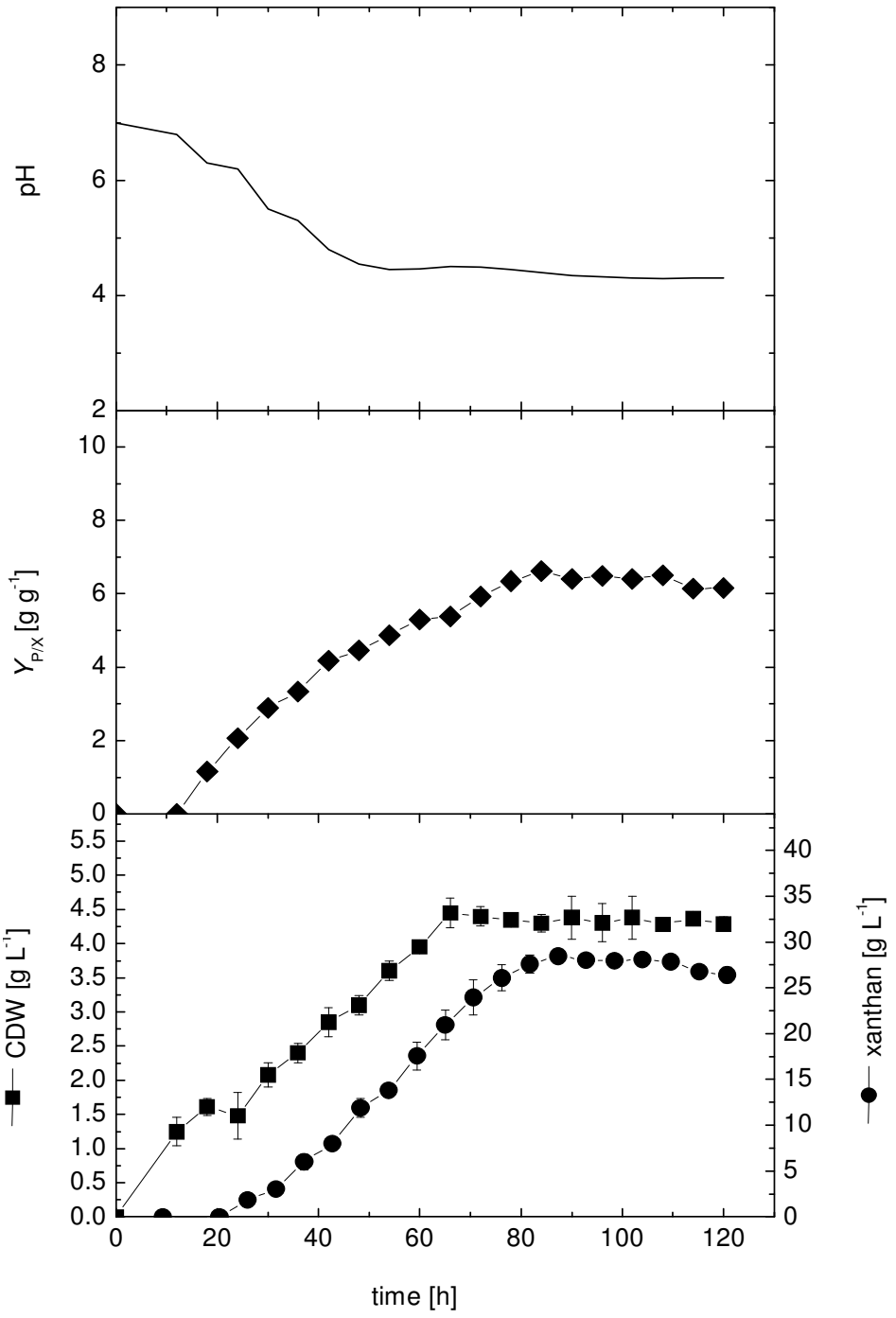

Figure 6. Kinetics of cell growth and xanthan production using optimized semi-defined medium in 16-L bioreactor under uncontrolled pH condition.

Cell growth and xanthan production in 16-L bioreactor using optimized semi-defined medium in uncontrolled and controlled pH cultures

The cell growth and xanthan production kinetics were studied during batch cultivation of *X. campestris* in a pilot scale bioreactor using the same medium composition (optimized previously) and inoculum size used in shake flask experiments. Two sets of experiments were performed using stirred tank bioreactor with a 9-L working volume under controlled and uncontrolled pH conditions.

Figure 6 shows the time profile of cell growth, volumetric and specific xanthan production during a typical batch cultivation of *X. campestris* in optimized medium without

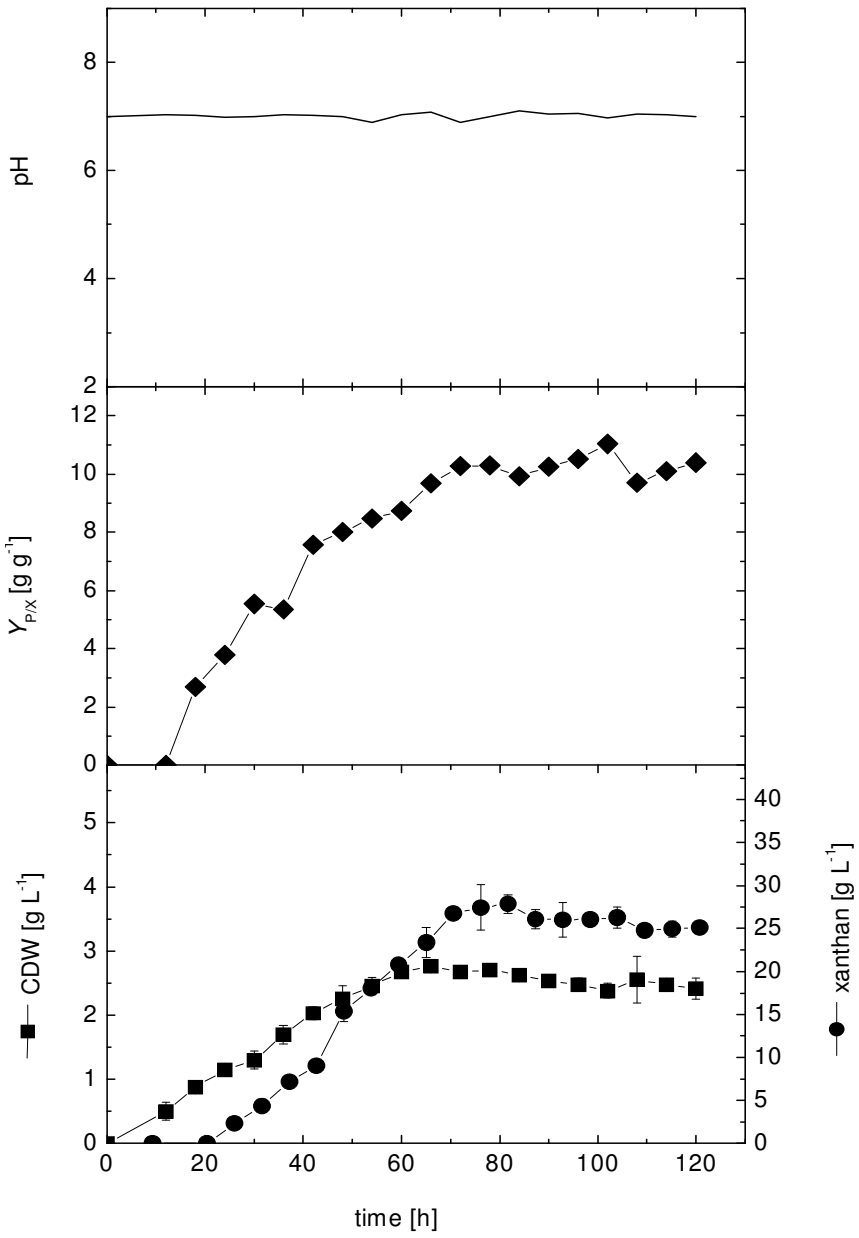

Figure 7. Kinetics of cell growth and xanthan production in optimized semi-defined medium in 16-L bioreactor under controlled pH condition.

pH control. As shown, cells grew exponentially during the first 64 h with growth rate of 0.067 gL^{-1} h^{-1} reaching 4.45 gL^{-1}. After this phase, cell concentration remained more or less constant for the rest of the cultivation time. In the meantime, xanthan started to be produced in culture after lag phase of 24 h and increased with constant rate of 0.44 gL^{-1} h^{-1}. The maximal volu-metric polysaccharide production of 28.45 gL^{-1} was obtained after 84 h cultivation. However, during the growth phase, the yield coefficient of xanthan production was increased concomitantly. The maximal value of xanthan specific production of 6.79 gg^{-1} was obtained at 84 h (the time at which the maximal volumetric production was obtained). On following the pH profile of culture, it is clearly shown that the pH decreased gradually by time, reached 4.55 after 48 h and remained more or less constant for the rest of the cultivation time. This drop in pH value was due to the presence of acid groups in xanthan structure. Some other authors suggested that pH control is not necessary for the process but others recommended the control of pH 7.0 throughout the cultivation process (Garcia-Ochoa et al., 2000a). Thus, the effect of medium pH control at 7.0 was studied in this work (Figure 7). As shown, cells grew exponentially during the first 60 h with rate of 0.044

gL^{-1} h^{-1} (about 35% less than growth rate in uncontrolled pH culture). How-ever, there was no significant difference in volumetric xanthan production rate and total volumetric production between controlled and uncontrolled pH cultures (Table 1). Even in lower cell mass in pH controlled culture, the final biopolymer concentration was not affected, this directly indicated the high cell productivity in this culture which was higher by about 63% when compared to the other culture.

However, some studies showed that pH control did enhance cell growth but had no effect on xanthan production. Under controlled pH, xanthan production stopped once the cells entered stationary phase, whereas the xanthan production continued during the stationary phase of growth when the pH was not controlled in culture. However, this phenomenon was not obvious in our experiment and the xanthan gum production stopped in both cultures about 10 h after the cells has entered stationary phase. Moreover, the control of pH increased the cell productivity in the semi-defined medium under study.

Conclusion

Table 1 summarizes the kinetics data obtained for cell cultivation in optimized and unoptimized semi-defined medium in shake flask and bioreactors. The results clearly demonstrate that, in shake flask level, the new medium supported better volumetric and specific xanthan production by about 70 and 30%, respectively. When the process was scaled up to 16-L bioreactor under controlled pH condition, the final volumetric and specific xanthan production was about 27.85 gL^{-1} and 11.06 gg^{-1}, respectively. This was almost 138 and 125% higher than those values obtained from shake flask using unoptimized medium.

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