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Bioprocess optimization of Xanthan production by *Xanthomonas campestris* using semi-defined medium in batch and fed-batch culture

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ABSTRACT

Xanthan gum is an extracellular hetero polysaccharides of many industrial applications and produced by Xanthomonas campestris. This biopolymer is produced as extracellular product in submerged fermentation system. The production of xanthan was influenced by the concentration in glucose in medium. The maximal volumetric production of 17.9 g L^{-1} was obtained in 80 g L^{-1} glucose culture in shake flask level. Furthermore, cultivations were carried out in in situ sterilizable 15-L pilot scale bioreactor to improve this cultivation process. Using batch cultivation mode, the maximal xanthan production of 28.5 g L^{-1} was obtained after 60 h cultivation. Based on data of batch culture, fed-batch cultivation with increased feeding rate using either glucose or complete medium as feeding solution was developed. The results clearly showed that, fed-batch cultivation using complete medium solution (chemically defined medium supplemented with glucose), increased volumetric xanthan production up to 43.15 g L^{-1} (about 51% higher than the corresponding batch culture) but with almost the same value of specific production of about 6.35 g g^{-1}. Thus, fed-batch cultivation mode using completely defined medium could be good alternative for economic production of xanthan in industrial scale.

Keywords: Xanthan, *Xanthomonas campestris,* polysaccharides, Bioreactor cultivation, Fed-batch cultivation, Bioprocess optimization.
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INTRODUCTION

Microbial polysaccharides is important group of metabolites of wide range of industrial applications. They are widely used in food, chemicals, petroleum, and pharmaceutical industries. Beside their use as thickening agent and additives, some microbial polysaccharides showed also medicinal values as immunomodulatory, antiinflamatory, antimicrobial, and anticancer properties [1,2]. Therefore, nowadays different types of microbial polysaccharides such as alginate, levan, kefiran, pullulan, galactomannan, curdlan and glucans have been studied extensively by many authors in terms of their production methods and potential applications [3-9]. Xanthan (CAS #11138-66-2) and known as E415 is bacterial polysaccharide with chemical structure $(C_{35}H_{49}O_{29})_n$ which is composed of penta-saccharide repeated units of glucose, mannose and glucuronic acid in the molar ratio of 2:2:1. In addition, the

288

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structure of xanthan contains also acetate and pyruvate residues. The acetate and pyruvate contents are varied and are highly affected by the production process in both upstream and downstream.

Driven by its wide and extended applications in many industries, the global market of xanthan is expected to reach USD 987.7 million by 2020 [10]. Xanthan gum increase liquid viscosity even when used in very low concentration. Therefore, it becomes attractive for many food, cosmeceuticals, and pharmaceutical applications. In addition, it is also used in petroleum industries during drilling process as thickener to the drilling mud. The production of this polysaccharide is carried out mainly by submerged cultivation of the gram negative bacteria *Xanthomonas campestris*. In spite the fact that this microbe was initially isolated from plant and pathogen, however, it's widely accepted for xanthan production in industrial scale. Production is usually carried out in medium containing directly fermentable sugar such as glucose or sucrose which is usually added in high concentration. The fermentation process usually is run aerobically at 28-30 °C, with an aeration rate more than 0.3 v v^{-1} min^{-1} [11]. Beside carbon source, other nutrients such as potassium, iron, magnesium, and calcium salts are usually added to the fermentation medium. Other research proven also that addition of yeast extract in low concentration enhanced xanthan production [12]. In spite of the fact that xanthan production favors medium of high C/N ratio, high carbon source concentration can lead to growth inhibition which reduce the xanthan yield accordingly [13]. In our previous study, we optimized simple cultivation medium for xanthan production up to bioreactor level using batch cultivation medium. The present work is focused on further improvement of the xanthan production process using semi-synthetic medium in fed-batch cultivation mode in semi-industrial (16-L) stirred tank bioreactor.

MATERIALS AND METHODS

Microorganism
Xanthomonas campestris DSMZ 19000 was obtained from DSMZ culture collection (Deustche Samlung für Mikrobiolgie und Zellkulturen, Braunschweig, Germany). Once received in lyophilized form, cells were activated in modified YM broth, of the following composition (g L^{-1}): glucose, 10; K$_2$HPO$_4$, 4; yeast extract, 4; malt extract, 5.0; and MgSO$_4$.7H$_2$O, 0.5) and incubated for 4 days at 30°C. The activated cells were further plated on YM agar of the same composition and supplemented with 20 g/L agar and incubated for 48 hours. The arisen colonies were collected in glycerol culture solution (glycerol:water, 1:1 v v^{-1}) and kept frozen at -80°C until further used. To minimize results fluctuation due to intra population variation in the experiments, one frozen culture vial was taken and activated in YM broth as starting point for each experiment.

Inoculum preparation, xanthan production medium, and cultivation conditions
One deep frozen cryogenic vial (containing 1 ml glycerin culture) was used to inoculate 50 mL of modified YM broth in 250 ml Erlenmeyer flasks. The inoculated flasks were incubated on rotary shaker at 150 rpm and 30°C for 24 hours. The grown cells were used thereafter to inoculate either shake flask or stirred tank bioreactor in concentration of 5% (v v^{-1}). The Initial fermentation medium used in this study was composed of (g L^{-1}): glucose, 60.0; (NH$_4$)$_2$HPO$_4$, 0.217; MgSO$_4$.7H$_2$O, 1.0, (NH$_4$)$_2$SO$_4$, 3.0, modified from El Enshasy et al., [14]. The pH was adjusted to 7.0 before sterilization. The carbon source was autoclaved separately at 110°C for 25 min and added the fermentation medium before inoculation. In case of shake flask experiments, cultivations were carried out in rotary incubator shaker (Innova 4080, Eppendorf Co., Springfield, MA, USA). Bioreactor cultivation experiments were carried out in *in situ* SIP stainless steel 15-L stirred tank bioreactor of 10-L working volume (Biostat C, Sartorius, Melsungen, Germany). The initial agitation speed was adjusted to 200 RPM and aeration rate of 1 v/v/min. During cultivation, foam was detected using foam sensor cascaded to antifoam pump for dosing of antifoam reagent (silicon antifoam grade A, Sigma-Aldrich Inc., USA) when necessary. The pH values were monitored continuously during cultivation using pH electrode (Ingold, Mittler Toledo, Switzerland).

Sampling and Analysis
During cultivations in both shake flask and bioreactors, samples were taken at different time intervals for biomass, sugar concentration, and xanthan determination. In case of shake flasks, samples were in form of 3 flasks of 50 ml broth each or in form of 10 ml fermentation broth in case of bioreactor. Samples were centrifuged immediately (30,000 g for 30 min at 5°C) to separate cells from the broth. For samples of high viscosity, samples were first diluted in KCl (1% w/v) to improve cell separation. The supernatant were taken in stored at -20°C for further analysis. The precipitate were resuspended in 1% KCl solution and centrifuged at 10,000 g for 10 min in pre-weighed centrifuge tube, the cells were washed twice with distilled water and the tube were dried in vacuum oven at 50°C for 2 days for cell dry weight determination. The quantitative determination of xanthan in the supernatant was

289

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carried out with direct precipitation using three volumes of 95% ethanol [15]. The precipitate was resuspended in 1% KCl solution, precipitated again using 95% ethanol and dried in vacuum oven at 50°C for constant weight. Glucose in culture was determined using glucose lactate analyzer (Model 2700, YSI Inc., OH, USA).

RESULTS AND DISCUSSION

Effect of different glucose concentrations on xanthan production by *X. campestris*.
Carbon source is usually one of the critical nutrients for microbial polysaccharides production. Therefore, optimization of carbon source concentration in the production medium is critical step to improve the overall production process. In this experiment, completely chemical defined medium supplemented with glucose as carbon source have been used. Different glucose concentrations ranging between 0-100 g L^{-1} have been used for xanthan production. As shown in Figure 1, results clearly showed that glucose concentration has a great effect on xanthan production. Increasing glucose concentration up to 80 g L^{-1} proportionally increased xanthan production, where the maximal xanthan reached 17.9 g L^{-1}, afterwards xanthan production remained more or less constant. On the other hand, cell mass increased with increasing glucose concentration reaching its highest cell growth (4.84 g L^{-1}) at 50 g/L glucose, and then gradually decreased by about 2-4%. Accordingly, xanthan yield coefficient ($Y_{P/X}$) increased with glucose concentration reaching 3.896 g xanthan/g cells at 80 g L^{-1} glucose. However, the yield coefficient increased with further increase in glucose due to the decrease in cell mass at concentrations higher than 80 g/L.

Previous reports literature showed that xanthan production is highly affected by the carbon source [16]. Recently, Ben Salah et al. [17] investigated the optimization of xanthan production using response surface methodology. They found that xanthan production increased proportionally with increasing the concentration of carbon source from 40 to 80 g/L. This effect can be explained on the bases that carbon source is used for building both cell components as well as synthesis of the polysaccharide.

Figure 1. Effect of glucose concentration on the cell growth, pH of the culture medium, and xanthan production by *X. campestris*. (data wg L-1ere taken after 96 h cultivation in shake flask at 30°C and 200 RPM)

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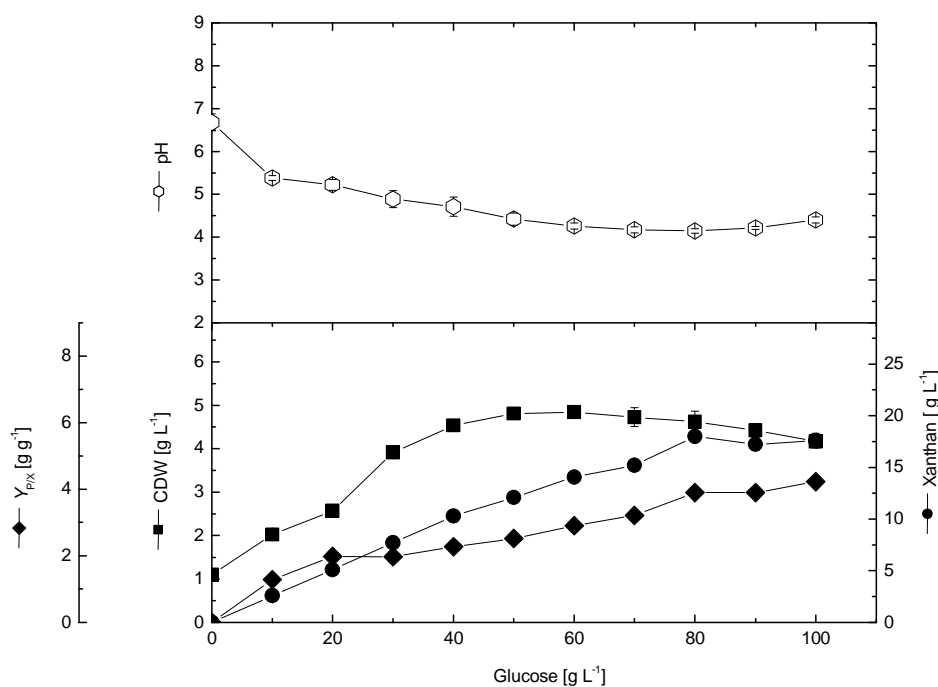
Batch cultivation in shake flask and in 15-L stirred tank bioreactor

The optimized production medium containing 80 g L^{-1}glucose was used to compare the kinetics of growth and xanthan production in both shake flask and semi-industrial scale bioreactor levels. The obtained results for shake flask (Figure 2) and bioreactor (Figure 3) levels clearly showed improvement in bioreactor cultivation in terms of growth and production kinetics. In shake flask cultivation, cells grow exponentially for the first 72 h with an average growth rate of 0.056 g L^{-1} h^{-1}, and an average glucose consumption rate of about 0.84 g L^{-1} h^{-1}. Moreover, the average cell growth yield coefficient ($Y_{X/S}$) recorded 0.0867 g cells/g glucose during that phase. At the end of the exponential phase, cell mass reached 4.05 g L^{-1}, thereafter, cell growth started to slow down mainly due to the depletion of the glucose. Concomitantly, xanthan production increased with cultivation time with an average production rate of 0.2108 g L^{-1} h^{-1} and reached a maximal of 17.9 g L^{-1} after 96 h. Furthermore, at 96 h xanthan yield coefficient ($Y_{P/X}$) reached its maximal of 4.1628 g xanthan/g cells. After which, xanthan concentration remained more or less constant.

On the other hand, results obtained for bioreactor batch cultivation (Figure 3) showed improved growth and production parameters. During the exponential growth phase, cells grew with an average growth rate of 0.0722 g L^{-1} h^{-1} and an average glucose consumption rate of about 1.238 g L^{-1} h^{-1}, which were higher than those rates recorded in shake flask cultivation by about 28.2 and 47.9%, respectively. Although the average cell growth yield coefficient ($Y_{X/S}$), 0.0804 g cells/g glucose, did not change greatly recorded, a maximal cell mass of 5.2 g L^{-1} was recorded at 72 h, which is 19.5% higher than the maximal cell mass obtained in shake flask cultivation (4.35 g L^{-1}). Accordingly, xanthan was produced by an average production rate of 0.573 g L^{-1} h^{-1}, which is about 171. 8% higher than that recorded during shake flask cultivation. Similarly, a maximal xanthan concentration of 28.5 g L^{-1} could be produced with a yield coefficient ($Y_{P/X}$) of 6.1957 g xanthan-g cells after 60 h, which is 59.2% higher than the highest xanthan concentration produced in shake flask cultivation (17.9 g L^{-1}).
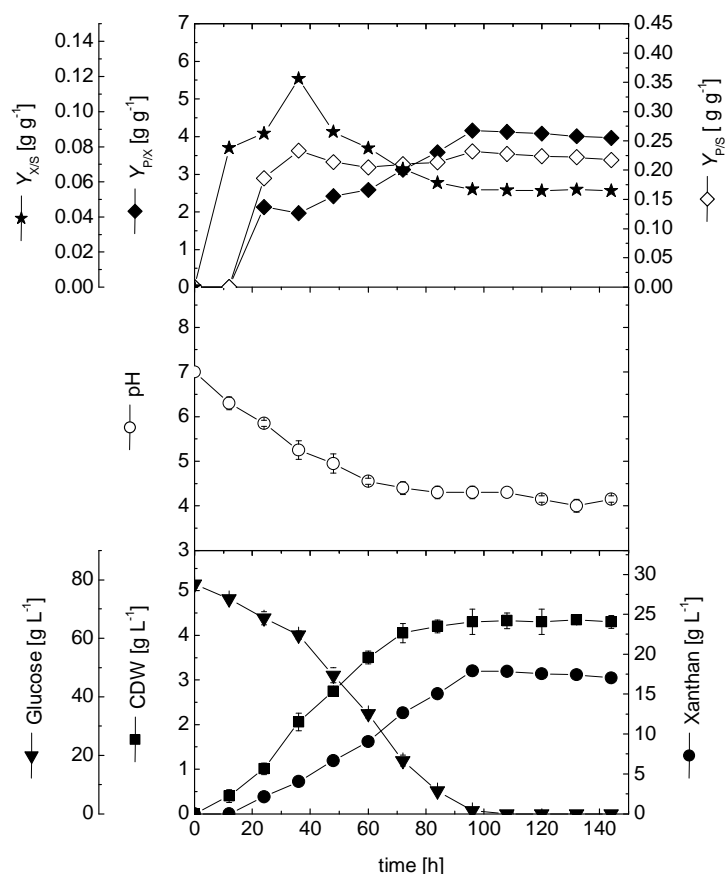

Figure 2. Kinetics of cell growth, glucose consumption, xanthan production, and pH changes during cultivation of *Xanthomonas campestris* in shake flask level

291

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The improved growth and xanthan production kinetics during bioreactor cultivation can be attributed to the better controlled bioreactor process, in terms of improved aeration and agitation conditions. This in turn allows the cells to grow at higher growth rates and produce xanthan with higher productivities. It is well known, that processes for growing and producing different primary and secondary metabolites are greatly improved when transferred to bioreactor levels [8,18]. Furthermore, Faria et al. [19] investigated the kinetics of xanthan gum production in shaker and bioreactor cultivations. They found that bioreactor cultivation increased their maximal cell mass and xanthan production by about 45.6 and 100%, respectively, from the shaker cultivation. They attributed their results to the defined operational conditions in the bioreactor, which greatly differ from those in shaker cultivation. These better operational conditions enhance better cell growth in the exponential phase due to increased substrate consumption, and hence lead to improved xanthan production titers.
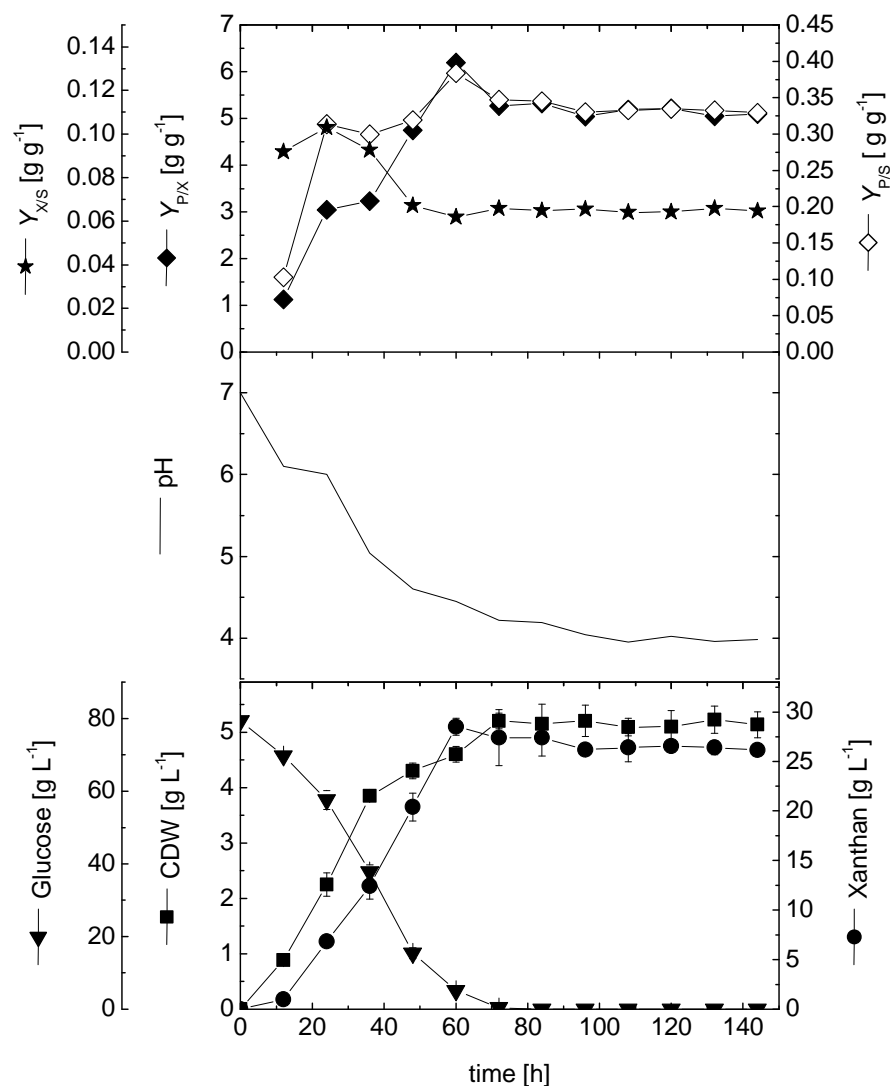

Figure 3. Kinetics of cell growth, glucose consumption, xanthan production, and pH changes during cultivation of *Xanthomonas campestris* in 15-L stirred tank bioreactor

Fed-batch cultivation in 15-L semi industrial scale bioreactor
The results of growth and xanthan production kinetics obtained from the bioreactor batch cultivation were used to design different fed-batch experiments based on changing the feeding rate of either glucose only or completer medium.

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Increased feeding rate of glucose

In this set of experiment, the fed-batch cultivation was started after 60 h and directly before complete exhaustion of glucose. Glucose feeding was started at 1.25 g L^{-1} h^{-1}, which is similar to the glucose consumption rate obtained from the batch experiment. After 108 h, the feeding rate was increased to 1.875 g L^{-1} h^{-1}. The obtained results (Figure 4) showed that cells grew exponentially for the first 60 h of cultivation with a growth rate of 0.0783 g L^{-1} h^{-1} to reach a maximal of 4.7 g L^{-1} cell mass by the end of this phase. Also, the glucose present in the medium was consumed by a rate of -1.2375 g L^{-1} h^{-1} and reached 5.15 g L^{-1}. Similarly, xanthan increased with time and was produced at a production rate of 0.405 g L^{-1} h^{-1} to reach a maximal of 24.3 g L^{-1}. Accordingly, glucose feeding was started at a feeding rate of 1.25 g L^{-1} h^{-1} for the next 48 h. During the feeding phase, growth increased slightly to 5.2 g L-1 at 72 h, and then started to slow down, which was accompanied by a decrease in glucose consumption rate. However, xanthan production continued with glucose feeding and reached 33.05 g L-1 by the end of this phase, where the production rate decreased to 0.1875 g L^{-1} h^{-1}. The glucose feeding was further increased to 1.875 g L^{-1} h^{-1} for the rest of the cultivation, where no noticeable effect was observed on cell growth, which remained more or less constant by the end of the cultivation (5.165 g L^{-1} h^{-1} at 144 h). Also, glucose started to accumulate in the medium due to the increased feeding rate and reached 22.45 g L^{-1} by the end of the cultivation. During the second feeding phase, the maximal xanthan concentration of 33.85 g L^{-1} was reached at 120 h, at which the highest yield coefficient ($Y_{P/X}$) of 6.5601 g xanthan/g cells was obtained.

Fed-batch cultivation is usually recognized as the best cultivation strategy for increasing microbial cell mass and enhances bioactive metabolite biosynthesis and thus have been successfully used for biopolymer production as in case of alginate production [20] and pleuran production [3].
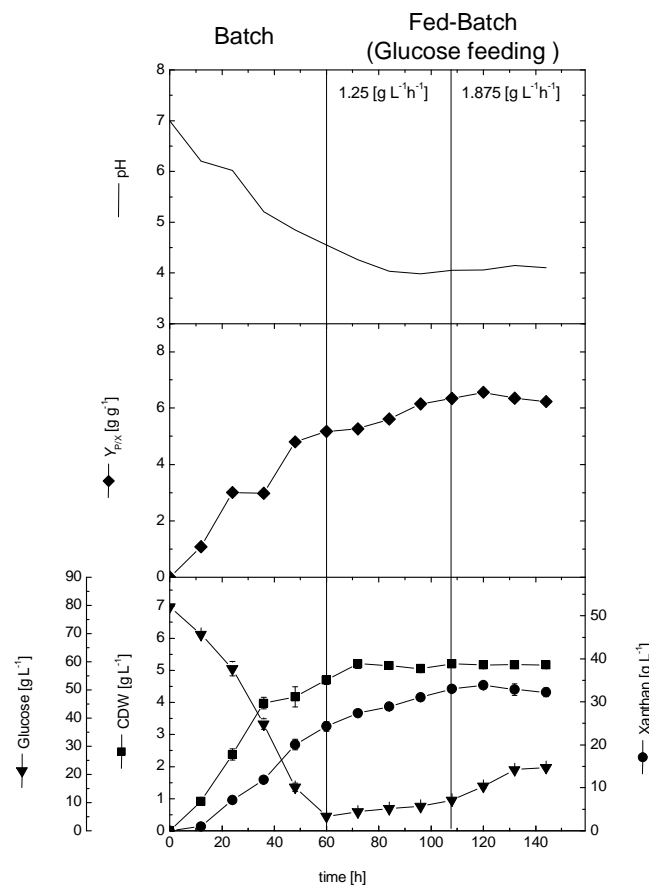

Figure 4. Kinetics of cell growth, glucose consumption, xanthan production, and pH changes during fed-batch cultivation of *Xanthomonas campestris* **in 15-L stirred tank bioreactor. Feeding started after 60 h cultivation with concentrated glucose solution with rate of 1.25 g L^{-1} h^{-1} (equal to the glucose consumption rate in batch culture), and increased up to 1.875 g L^{-1} h^{-1} after 108 h to 132h**

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Increased feeding rate of complete medium

The build-up of glucose in the cultivation medium accompanied with decrease in cell growth may be explained on the basis that glucose is not the limiting component of the cultivation medium, and those other medium components, which are depleted from the cultivation medium, are affecting the metabolic machinery of the growing cells. Therefore, the experiment was repeated applying different feeding rates of complete cultivation medium. Results presented in (Figure 5) clearly reveal that feeding complete cultivation medium is favored over glucose feeding. During the exponential growth phase, cells grew with a growth rate of 0.0737 g L^{-1} h^{-1} and reached their maximal cell mass (4.42 g L^{-1}) at 60 h. Meanwhile, glucose was consumed at a rate of 1.2042 g L^{-1} h^{-1} and reached 7.65 g L^{-1} by the end of this phase. Xanthan was produced by a rate of 0.35 g L^{-1} h^{-1} and reached a maximal of 21.0 g L^{-1} h^{-1} at 60 h. Accordingly, the first feeding phase was started for 36 h, where complete medium was fed at a rate of 1.25 g L^{-1} h^{-1}. During this phase, the glucose added was completely consumed and cells continued to grow at a growth rate of 0.0367 g L^{-1} h^{-1}. By the end of the feeding phase, a maximal cell mass of 5.74 g L and 35.35 g L^{-1} xanthan were obtained at 96 h. In addition, it can be seen that glucose did not accumulate during this phase, where its residual concentration reached 3.1 g L^{-1} at 96 h. Also, the xanthan production rate remained constant during this phase (0.3986 g L^{-1} h^{-1}). At 108 h, the second feeding phase started, where complete medium was fed at a rate of 1.875 g L^{-1} h^{-1}. Results showed that cells continued to grow, although at a slower growth rate of 0.0212 g L^{-1} h^{-1}, and reached their maximal cell mass of 6.8 g L^{-1} h^{-1} at 132 h. Consequently, xanthan production increased, where a maximal concentration of 43.15 g L^{-1} was obtained at 144 h.
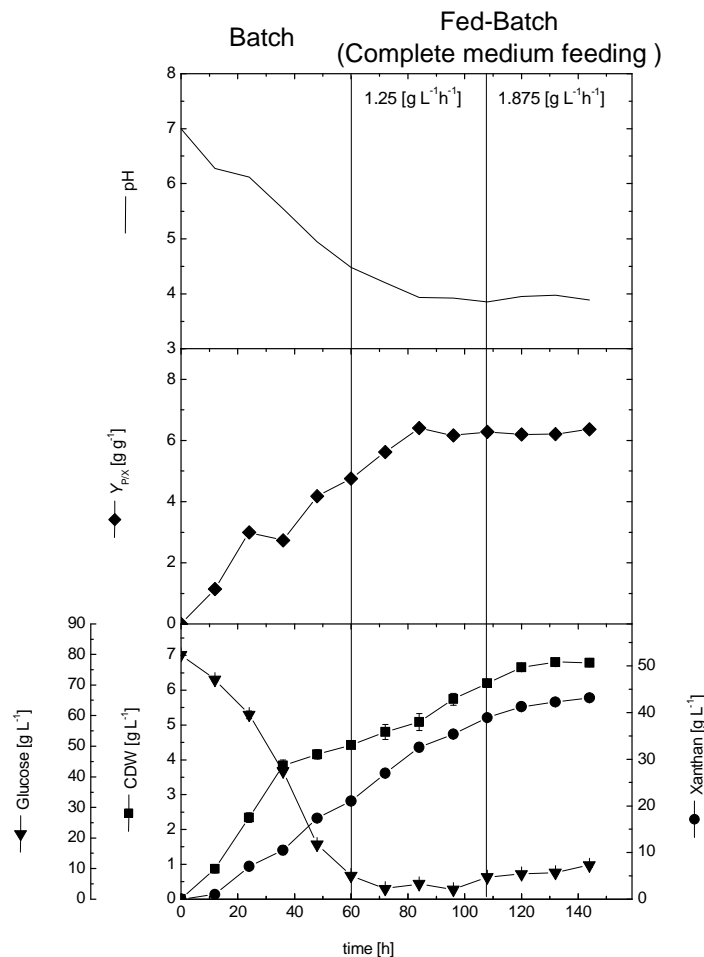

Figure 5. Kinetics of cell growth, glucose consumption, xanthan production, and pH changes during fed-batch cultivation of *Xanthomonas campestris* in 15-L stirred tank bioreactor. Feeding started after 60 h cultivation with concentrated complete medium component with rate of 1.25 g L^{-1} h^{-1} (based on the glucose concentration in the medium), and increased up to 1.875 g L^{-1} h^{-1} after 108 h to 132h

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The above obtained results can be explained on the basis that feeding complete cultivation medium provides the growing cells with the required nutrients, which have been depleted upon feeding glucose only. Besides glucose, the cultivation medium contained potassium phosphate, yeast and malt extracts, and magnesium sulphate. It has been proved that nitrogen sources, present as organic nitrogen, are required for biopolymer production, when present in smaller amounts [14, 21]. Moreover, Garcia-Ochoa et al. [22] found that phosphorus and sulphur greatly influence xanthan production. Additionally, phosphate and magnesium sulphate can act as cofactors affecting enzymes responsible for the manufacture of the pyruvate and acetate residues incorporated into xanthan structure.

CONCLUSION

Table 1 summarizes the kinetic parameters obtained for cell growth and xanthan production under different cultivation conditions. The results showed that feeding strategy using complete medium greatly enhances the productivity of the cells, where a maximal xanthan production concomitant with significant increase in xanthan volumetric production up to 43.15 g L^{-1}. Moreover, cell growth continued due to medium feeding, where the fresh medium supplies the growing cells with nutrients required for both cell growth and xanthan production. Thus, fed-batch cultivation using complete feeding solution (which composed of inorganic salts and glucose) is cost effective and scalable cultivation strategy for production of xanthan in industrial scale.

Table 1. Kinetic table for xanthan production using *X. campestris* in different cultivation scales and cultivation modes

Parameter	Batch cultivation		Fed-batch cultivation (Bioreactor level)			
	Shake flask	Bioreactor	Glucose feeding		Complete medium feeding	
			1.25 g L^{-1} h^{-1}	1.875 g L^{-1} h^{-1}	1.25 g L^{-1} h^{-1}	1.875 g L^{-1} h^{-1}
X_{max} [g L^{-1}]	4.35 (132 h)	5.2 (72 h)	5.2	5.2	5.74	6.8
dx/dt [g L^{-1} h^{-1}]	0.0563	0.0722	n.d.	n.d.	0.0367	0.0218
P_{max} [g L^{-1}]	17.9	28.5	31.05	33.85	35.35	43.15
Q_p [g L^{-1} h^{-1}]	0.2108	0.5729	0.1875	0.0239	0.3986	0.1625
$Y_{p/x}$ [g g^{-1}]	4.1628	6.1956	6.1485	6.5601	6.4012	6.3596

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