J Ind Microbiol Biotechnol (2012) 39:799–803
DOI 10.1007/s10295-011-1071-z

SHORT COMMUNICATION

Hydrogen peroxide (H_2O_2) supply significantly improves xanthan gum production mediated by *Xanthomonas campestris* in vitro

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Received: 19 October 2011 / Accepted: 29 November 2011 / Published online: 20 December 2011

Abstract To improve xanthan gum productivity, a strategy of adding hydrogen peroxide (H_2O_2) was studied. The method could intensify oxygen supply through degradation of H_2O_2 to oxygen (O_2). In shake flask testing, the xanthan gum yield reached 2.8% (improved by 39.4%) when adding 12.5 mM H_2O_2 after 24 h of fermentation. In fermentor testing, it was obvious that the oxygen conditions varied with the H_2O_2 addition time. Eventually, gum yield of 4.2% (w/w) was achieved (increased by 27.3%). Compared with the method of intense mixing and increasing the air flow rate, adding H_2O_2 to improve the dissolved oxygen concentration was more effective and much better. Moreover, addition of H_2O_2 improved the quality of xanthan gum; the pyruvate content of xanthan was 4.4% (w/w), higher than that of the control (3.2%).

Keywords Xanthan gum · Hydrogen peroxide · Oxygen supply · Fermentation · *Xanthomonas campestris*

Introduction

Xanthan gum is a natural anionic polysaccharide and an important industrial biopolymer produced by the bacterium *Xanthomonas campestris*. This biopolymer is widely used in several industries, such as food, oil recovery, and cosmetics [8, 11, 18], due to its superior pseudoplasticity, thixotropy, and viscosity. Xanthan production depends on several factors, including medium composition, temperature, pH, and oxygen transfer [2, 10, 13]. In the aerobic fermentation process, oxygen is one of the decisive factors for *X. campestris* growth as well as xanthan production [6]. However, xanthan fermentation is accompanied by a dramatic increase of broth viscosity because of extracellular accumulation of the biopolymer, which leads to significant decrease of the oxygen mass transfer rate. The dissolved oxygen (DO) concentration becomes a controlling step rate for the overall process. Generally, this could be easily obtained by means of intense mixing, but the cultivated microorganisms are sensitive to shear stress, which can damage cells and lead to low biomass [7]. Though improved impellers may achieve intense mixing and decrease shear stress [15], this stress still exists and may damage cells. The work efficiency of increasing the air flow rate is not high, and excessive energy is consumed. Therefore, in order to reduce energy consumption (i.e., decrease gas flow rate and stirrer speed), we aim to obtain a more ideal result than in the control. Induced mutation of strains with low oxygen requirements is another conventional approach. However, mutation stability remains an issue. Mutant strains easily lose mutagenic activity after multigeneration genetic translation.

One of the essential conditions for optimization of oxygen transfer is reduction of the length of the oxygen diffusion route through microorganisms (i.e., reduction of the thickness of the film layer around bubbles and microbial cells). Organic compounds, such as hydrocarbons [20], fluorocarbons [5], and soybean oil [9], can be used as oxygen vectors to intensify oxygen supply. Another technology (water-in-oil fermentation technology) has been developed to enhance the performance of highly viscous fermentation processes [12]. However, the cost of the consumed oil is high, and the cost of recovery increases.

The methodology reported by Sriram et al. [19] to overcome gas–liquid transport resistance is through

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liquid-phase conversion of H_2O_2 to O_2 and H_2O. This reaction is catalyzed by catalase available from the culture itself, and gas–liquid oxygen transport does not occur. Although some studies on xanthan gum fermentation using H_2O_2 have been reported [3, 19], those reports focus on basic research of H_2O_2 used in gum production using experiments carried out in miniature bioreactor. The results achieved are unreliable for guiding industrial production. The focus of this work is the feasibility of utilizing H_2O_2 for industrial fermentation. So, our experiments were carried out in a pilot test (10 L) fermentor and paid more attention to decreasing energy consumption. The effect of dissolved oxygen level on pyruvate content, which is an important factor for gum quality, was also studied. The aim of this work is to increase gum productivity and decrease energy consumption, and the results could be useful for fermentation scale-up.

Materials and methods

Microorganism, maintenance, and growth

A wild-type strain of *X. campestris* (stored in the laboratory) was used throughout this study. Inoculum preparation was performed by transfer of the microorganism from stock solution to yeast peptone agar plates (YP agar) containing (g/L) sucrose 20, peptone 5, beef extract 3, yeast extract 1, and agar 2, pH 7.0, with subsequent incubation for 72 h at 30°C. A loopful of cells from the (YP) plates was then transferred to a 250-mL conical flask containing 50 mL sterile YP medium and incubated for 28 h at 30°C and 180 revolutions per minute (rpm) for ultimate use as inoculum.

Fermentation conditions

Experiments were carried out in 100-mL Erlenmeyer flasks with 20 mL sterile production medium containing (g/L) sucrose 50, peptone 6, K_2HPO_4 5, $MgSO_4{\cdot}7H_2O$ 0.3, $CaCO_3$ 3, and citric acid 1, pH 7.0. Before autoclaving, the components of the medium were heated until dissolved completely. The medium was inoculated with 10% (v/v) *X. campestris* culture. H_2O_2 was aseptically added to the fermentation medium after filtration with a 0.22-μm membrane. The flasks were stirred for 72 h in an orbital shaker (30°C, 180 rpm).

Six liters of production medium in a 10-L fermentor (Baoxing Bio-engineering Equipment Co., Ltd., Shanghai, China) was inoculated (10%, v/v) at 30°C. The pH value was controlled at about 7.0 by adding sodium hydroxide solution. During bioreactor cultivations, oxygen was supplied through pulse additions of H_2O_2 using the peristaltic pump of the fermentor within 30 min. Fermentation without H_2O_2 was used as control. Each datum is the mean value of three identical samples, unless otherwise stated.

Analytical methods

Xanthan gum was separated by precipitation using ethanol (three times volume of xanthan sample) and 2 g/L KCl after the cells were adsorbed by diatomite. Biopolymer production by this strain was evaluated by measuring the weight of dry product per weight of fermented broth, expressed as an average in % (w/w). Biomass determination was done by dry cell weight estimation. Cells were collected after centrifugation at 4,500 rpm for 20 min. After supernatant was discarded, biomass was washed with aseptic water and recentrifuged. This was repeated twice. Finally, cells were dried at 104°C for 24 h and weighed. Cell morphology was studied using an optical microscope (Nikon Eclipse E100). The percentage of pyruvate covalently attached to the polysaccharide was determined by a colorimetric method [4] after hydrolysis of xanthan samples in 0.1 M HCl at 100°C for 4 h. pH and DO were measured using inductors of the fermentor. The sucrose conversion rate could be calculated with Eq. (1).

$$\text{Sucrose conversion rate} = \frac{\text{Mass}_{\text{xanthan gum}}}{\text{Mass}_{\text{glucose}}} \times 100\% \qquad (1)$$

Results and discussion

Shake flask testing

The results are shown in Figs. 1 and 2. Without H_2O_2 the xanthan gum yield was around 1.9% (w/w), whereas upon addition of H_2O_2 the yield increased to 2.8% (w/w). However, adding more than 12.5 mM H_2O_2 resulted in undesirable changes and consequently decreased xanthan gum yield, probably due to toxicity at high H_2O_2 concentration (Fig. 1). On the other hand, some produced species, such as superoxide radicals and/or hydroxyl radicals, would also cause damage to cells [14]. The effect of H_2O_2 on gum production was not obvious if it was added before 24 h of culture (Fig. 2). At the initial stage of fermentation, the dissolved oxygen concentration was high and cells had fairly low demands for oxygen supply. So, excess oxygen was released from the broth. High demand for oxygen supply is shown when xanthan gum is being produced. As is well known, gum is a secondary metabolite whose production begins after cells reach the stationary growth phase. As shown in Fig. 3, after 24 h of fermentation, the cells were in the stationary growth phase in shake flask testing. Therefore, H_2O_2 should be added after cells are in the stationary growth phase.

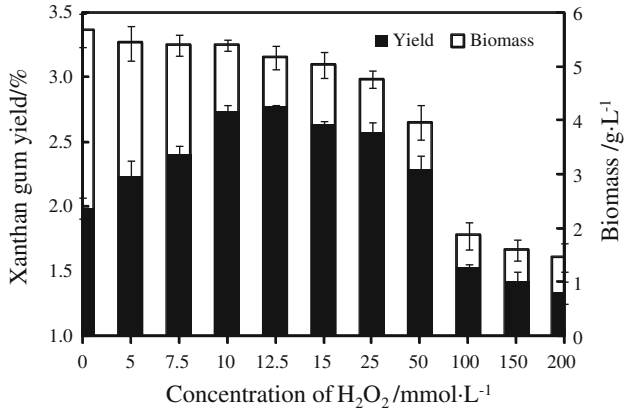

Fig. 1 Optimization of H_2O_2 concentration (0–200 mM) for xanthan gum yield. H_2O_2 was added after 24 h of culture

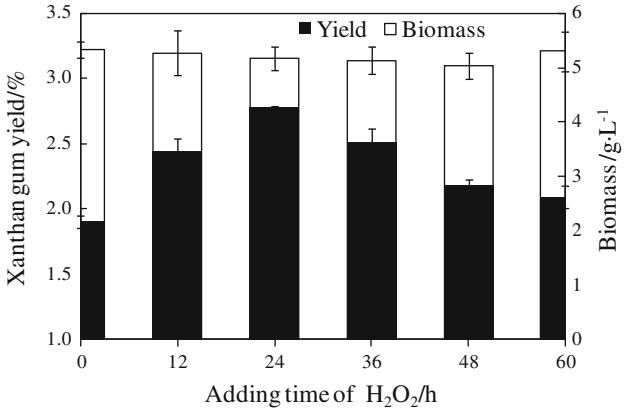

Fig. 2 Effects of H_2O_2 addition time on xanthan gum yield. H_2O_2 (12.5 mM) was added to the broth at different time points (after 0, 12, 24, 36, 48, and 60 h of culture)

In addition, gum yield was also not high when the addition time was after 24 h, though the concentration of H_2O_2 in the culture was lower than that required to kill cells (Fig. 2). This phenomenon is explained by the following fermentor experiment. Comparison of the growth curves of the control group and experimental group (Fig. 3) showed that cells did not exhibit clearly different growth rates or concentrations. Photomicrographs of free *X. campestris* are shown in Fig. 4. The secreted biopolymer (xanthan gum) was much thicker than in the control group, meaning that addition of H_2O_2 did enhance gum yield.

Fermentor production

The main process parameters are shown in Fig. 5, and experimental results of fermentor fermentation are presented in Table 1.

The cell growth curves of fermentor testing showed that cells were in stationary growth phase after 17 h of fermentation. So, H_2O_2 (12.5 mM) was added at this time according to the analysis of the results of shake flask testing. It was very obvious that high dissolved oxygen concentration could be maintained for 7 h in fermentor medium after the first addition of H_2O_2. Compared with the control group, the dissolved oxygen concentration decreased significantly, proving that H_2O_2 did provide oxygen to support cell growth and fermentation.

However, in the intermediate stage (after 28 h of fermentation), high dissolved oxygen concentration only lasted for about 1 h at the second time of 12.5 mM H_2O_2 addition. Because serious oxygen deficiency made cells quickly use the evolved oxygen, multiple pulses of H_2O_2 should be supplied when the dissolved oxygen falls to a set value for future application in production. One-time supply of H_2O_2 cannot be used because high H_2O_2 concentrations are toxic and the excess oxygen will be released.

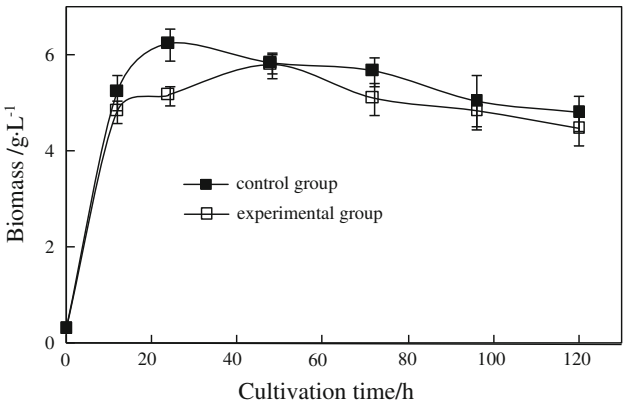

Fig. 3 Effects of H_2O_2 on *X. campestris* growth in shake flask experiments. In experimental group, H_2O_2 (12.5 mM) was added after 24 h of culture

Although enhancing the mechanical conditions could increase the oxygen level to a certain degree, this effect is limited because of extracellular accumulation of the biopolymer which decreases the oxygen mass transfer rate. After 42 h of culture, the gum yield was 4.2% (w/w) (about 34 g gum per liter of fermented broth, g/L). The results of this study are comparable to the values of 8.3 g/L and 12.16 g/L reported by other investigators [3, 19], who commenced adding H_2O_2 into gum fermentation before cells were in stationary growth phase. As they reported, this method of oxygen supply yielded lower cell yields, which was probably the reason for low gum yield.

Moreover, there was a significant difference in the pyruvate content of xanthan, which is an important factor for xanthan gum quality. The degree of pyruvilation can have a dramatic influence on gum's effectiveness in certain applications. It has been demonstrated that a strong relationship exists between microbial oxygen demand and pyruvate content [1, 16, 17]. A similar result was observed

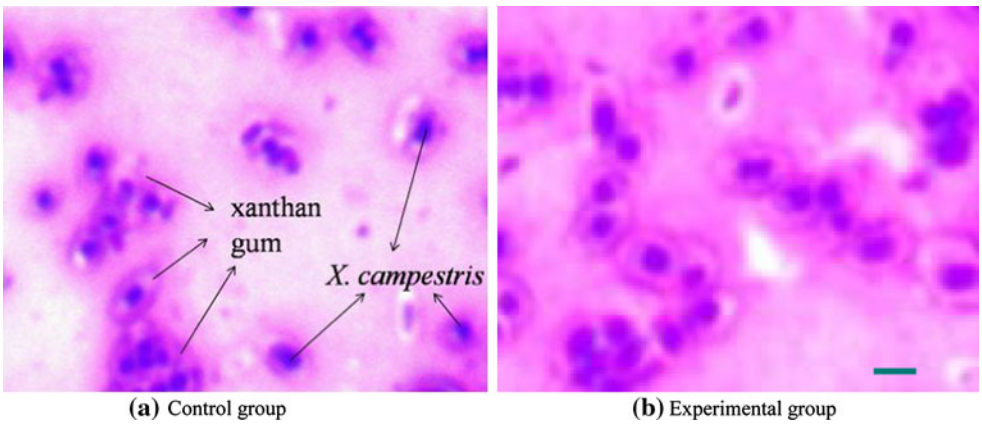

Fig. 4 Strains observed with a 100× oil-immersion lens at 72 h fermentation. In experimental group, H_2O_2 (12.5 mM) was added after 24 h of culture. The capsules of the strains were demonstrated by Anthony's capsule staining. Scale bars: 1 μm

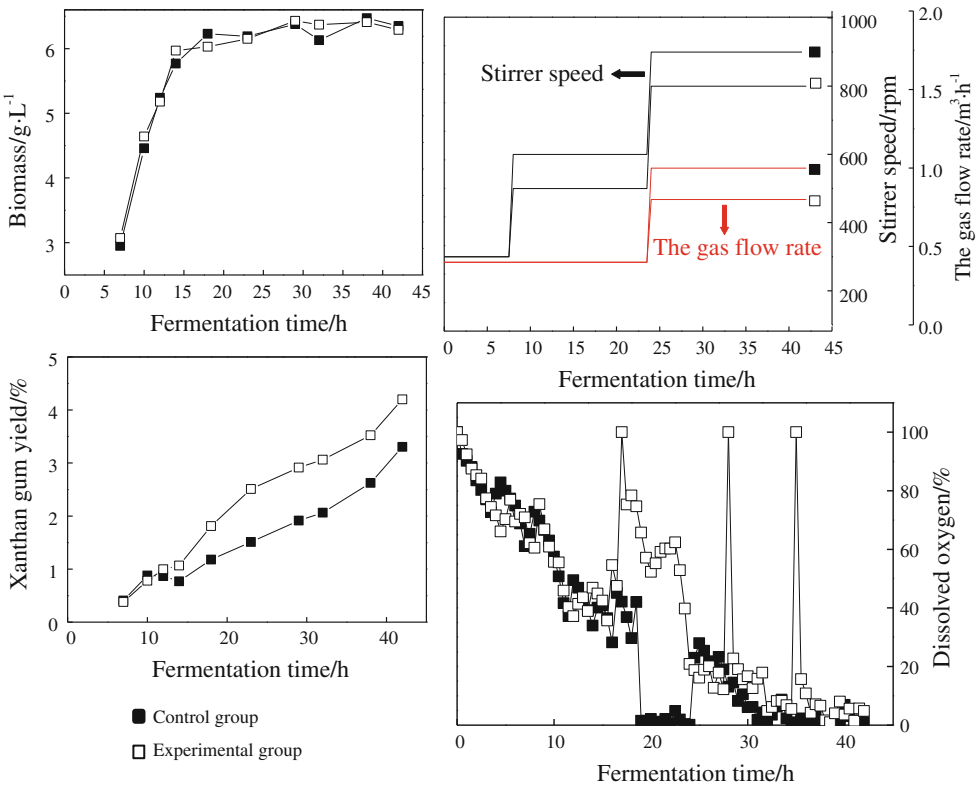

Fig. 5 Process parameters of fermentation. The fermentor experiments were carried out in a stirred tank bioreactor. In experimental group, H_2O_2 (12.5 mM) were fed after 17, 28, and 35 h of cultivation, respectively. Runs were terminated after 42 h of culture

Table 1 Results of fermentor fermentation

	Control group	Experimental group
Xanthan gum yield (%, w/w)	3.3	4.2
Broth viscosity (mPa s)	19,000	23,000
Sucrose conversion (%, w/w)	65	84
Pyruvate content (%, w/w)	3.2	4.4

H_2O_2 (12.5 mM) were fed after 17, 28, and 35 h of cultivation, respectively

Broth viscosity was measured using a rotational viscometer (NDJ-1) with spindle no. 4 at 6 rpm

here. The pyruvate content in the experiment with added H_2O_2 was higher than in the control group (Table 1). It should be realized that, in commercial processes for xanthan production, pure oxygen is added to the air input to increase the concentration of dissolved oxygen. This method is simple but expensive, and our proposed method using H_2O_2 compares favorably in terms of cost-effectiveness.

Acknowledgments The work was supported by grants from the National Natural Science Foundation (20576082) of the People's Republic of China.

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