Pakistan J. Sci. Ind. Res., Vol. 28, No. 2, April 1985

SCALE-UP STUDIES OF EXTRACELLULAR POLYSACCHARIDE PRODUCTION BY *XANTHOMONAS CUCURBITAE* IN STIRRED *FERMENTERS**

Shahjahan Baig, M.A. Qadeer and S.R.A. Shamsi,

PCSIR Laboratories, Lahore-16,

(Received January 31, 1984)

Xanthomonas cucurbitae (PCSIR-52) isolated from infected plant leaves, was grown in stirred fermenters of 10, 50, and 100 capacity using sucrose-salt medium by batch process. The fermentation patterns of microbial polysaccharides were greatly improved by the addition of cottonseed meal as "proflo extract", a source of amino acids and other growth factors. The supply of oxygen also plays an important role in such non-Newtonian fermentation process. Partial replacement of air by oxygen or addition of hydrogen peroxide as a source of oxygen concentrate, enhanced the rate of gum fermentation.

INTRODUCTION

Xanthan gum, an excellular anionic heteropolysaccharide, is commercially produced by fermenting carbohydrates, using *Xanthomonas campestris* NRRL B-1459 [1-6, 10, 12-16]. The annual production of xanthan gum is about 10,000 tons and due to its unique flow properties, has found many industrial applications in oil well drilling and recovery, drilling mud, paints, detergents, adhesives rheological control and in food industry [13]. Xanthan gum is composed of D-glucose, D-mannose and D-glucosonic acid in ratio of 2:2:1, [7] and varying amount of pyruvic acid and acetic acid [8]. The present work is a continuation of our studies reported earlier using locally isolated culture of *X, cucurbitae* (PCSIR-52) in shake flasks and 10 litre fermenter [14]. The conditions for xanthan gum fermentation were further optimised in laboratory to pilot plant scale fermenters by batch system.

MATERIALS AND METHODS

Organism Xanthomoas cucurbitae (PCSIR-52), isolated from infected leaves of cucurbita plant, as described earlier [14], was used in the present work. The culture was maintained on YM (Yeast Malt Extract) agar slants containing (g/l): yeast extract, 3.0; malt extract, 3.0; peptone, 5.0; glucose, 10.0; $CaCO_3$, 1.0 and agar, 20.0. All media, unless otherwise stated, were sterilized at 121° for 15 min. The cultures were incubated at 30 ± 1°.

Inoculum preparation. The Fresh cells from 24 hr. old YM agar slants were inoculated into 50 ml YM brother contained in 250 ml conical flasks and placed on a rotary shaker, rotating at 150 rpm. The shake flask cultures were incubated at 30 ± 1° for 24 hr. The volume of the vegetative inoculum was increased to 250 ml contained in one litre conical flasks by transferring seed cultures from a 250 ml conical flask aseptically. The volume of inoculum was further scaled upto 5 l in 10 l glass-stainless steel fermenter for pilot plant gum fermentation in 50 and 100 l stirred fermenters.

Fermentation medium. The fermentation medium consisting of (g/l) sucrose, 30.0; K_2HPO_4, 8.0; $MgSO_4.7H_2O$, 0.25; $(NH_4)_2HPO_4$, 1.5 and $CaCO_3$, 1.0 was used for xanthan gum production. The medium was divided into three parts for sterilization at 121° for 15 min., (a) sucrose 20%, w/v solution, (b) salt solution, and (c) $CaCO_3$ suspension. These solutions were transferred aseptically in the fermenter. In some experiments, $(NH_4)_2HPO_4$ was supplemented or substituted by "proflo" in water at 100° for 30 min. The hot suspension was then centrifuged at 3000 rpm for 15 min. The extract of 2.0 g "proflo" was added per litre culture medium.

Fermentation technique. Xanthan gum fermentation was carried out in 10, 50 and 100 l. capacity glass-stainless steel fermenters, designed and fabricated by the Engineering Section of the Lahore Laboratories. The working volumes of the fermenters were 6, 30 and 60 litres respectively. The glass pipes were obtained from QVF, UK Stainless steel plates were attached at both ends of the pipes and all parts in contact with the glass plates were of stain-

* This work has been conducted under the Pakistan Science Foundation Grant No. PSF/RES/PCSIR/Chem (103).

less steel. The fermenters were equipped with an agitator of disc turbine system entering from the top (10 and 50 l) or bottom (100 l) plates, cooling coils, baffles, air inlet and outlet, medium transfer lines and sampling devices. Two disc turbine impellers in 50 l fermenters, however were attached with the shaft. The agitators were rotated at 200 r.p.m. by differential pulleys and the temperature of the culture medium was kept at 30 ± 1°. Air was sterilized using glass wool tubes and air flow, in general, was kept at v/v/min. The fermenters while empty were steam sterilized for an hour before the transfer of sterilized production medium aseptically.

Analytical. The viscosity of the fermented broth after appropriate dilutions were determined by the Ostwald viscometer at 30°. The residual sugar was estimated by the method of Lo and Garcean [11]. The xanthan gum and dry cell mass were determined by the methods of Jeans, [4] and Morain Rogovin [2] respectively.

RESULTS

Xanthan gum fermentation.

(a) 10 litre glass-stainless steel fermenter. The pattern of xanthan gum fermentation in stirred fermenter with $(NH_4)_2.HPO_4$, as a source of nitrogen, is shown in Fig. 1. The residual sugar, 48 hr. after inoculation, with 24 hr. old seed culture, was 6.0 g/l and the amount of exocellular polysaccharide produced was about 17.5 g/l. The percentage yield of xanthan gum, on the basis of the sugar added, was about 60.6%. The pH of the fermented broth at the end of fermentation was 6.0. Both viscosity and xanthan gum production increased in proportion to the bacterial cell mass formation. The viscosity of the fermented mash, even 24 hr. after inoculation, was greatly increased, thus affecting the oxygen supply (or aeration) to the culture as observed visually. The addition of cottonseed meal as "proflo extract" to the culture medium increased both the cell mass and the rate of gum formation. The fermentation period was shortened by adding "proflo extract" to the culture medium.

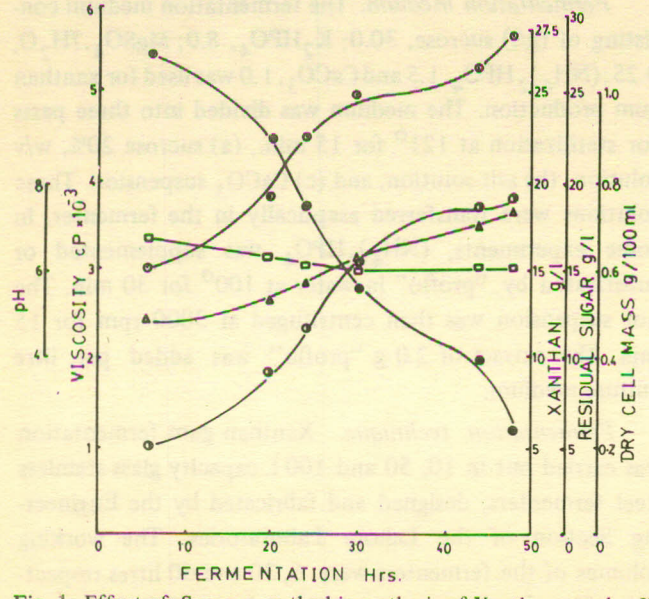

Fig. 1. Effect of Sucrose on the biosynthesis of Xanthan gum by *X. cucurbitae* PCSIR-52 in 10 liter glass-stainless steel fermenter. (○-○, Viscosity, △-△, xanthan, ◪-◪, pH; ●-●, Residual sugar; ●-●, Dry cell mass).

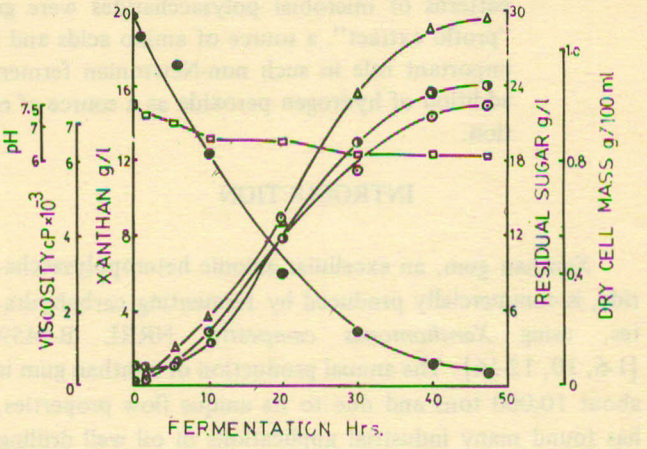

Fig. 2. Effect of di-ammonium hydrogen phosphate and Proflo Extract on the production of Xanthan gum by *X. cucurbitae* PCSIR-52 in 10 liter glass-stainless steel fermenter. (⊙-⊙, Viscosity; ◑-◑, Dry cell mass, ◪-◪, pH, △-△, Xanthan; ●-●, Residual sugar)

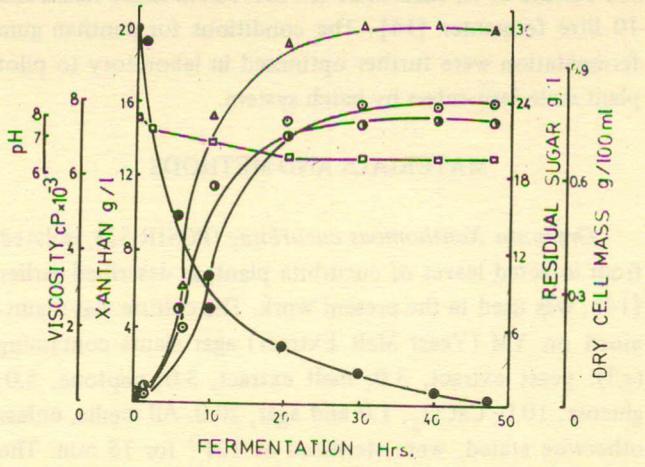

Fig. 3. Production of Xanthan gum by *X. cucurbitae* PCSIR-52 in 50 liter glass-stainless steel fermenter. (⊙-⊙, Viscosity; ◑-◑, Dry cell mass; ◪-◪, ph; △-△, Xanthan; ●-●, Residual sugar)

(b) 50 litres glass-stainless steel fermenter. Scale-up studies of xanthan gum fermentation using sucrose-salt medium with "proflo extract" as a source of nitrogen were also carried out *(Fig. 3).* The rate of blopolymer synthesis, as compared with shake flasks and 10-litres glass stainless steel fermenter, was greatly increased. The main reason was the improved supply of oxygen due to better agitation and aeration systems than in 10-litres stirred fermenter or shake flasks. The cell mass formation hence xanthan gum fermentation reached maximum 30 hr. after inoculation as compared with 48 hr. and 72 hr. in 10 litres stirred fermenter and shake flask respectively. The amount of sugar consumed and the percentage yield of exocellular polysaccharide were higher than in control studies described earlier.

(c) 100-litre glass stainless steel fermenter. Fig-4 shows the fermentation patterns of xanthan gum in a 100-litre stirred fermenter by *X-cucurbitae* (PCSIR-52) using sucrose-salt medium with "proflo extract". The cell mass formation was about the same as determined in 10 and 50-litre stirred fermenters but the conversion of sugar to biopolymers was greatly affected; that is, the amount of gum produced decreased by 33.0%. The main reason for the poor yield of xanthan gum in the fermenter was that the agitation system was not efficient as in the 10-or 50 litre fermenter. Thus the aeration or supply of oxygen to the bacterium in non-Newtonian fermentations plays significant role in the biosynthesis of biopolymers.

Effect of oxygen supply on xanthan gum fermentation limited. The oxygen supply in non-Newtonian fermentations is thus, affecting the conversion of substrate to both the cell mass and metabolites. The effect of oxygen supply

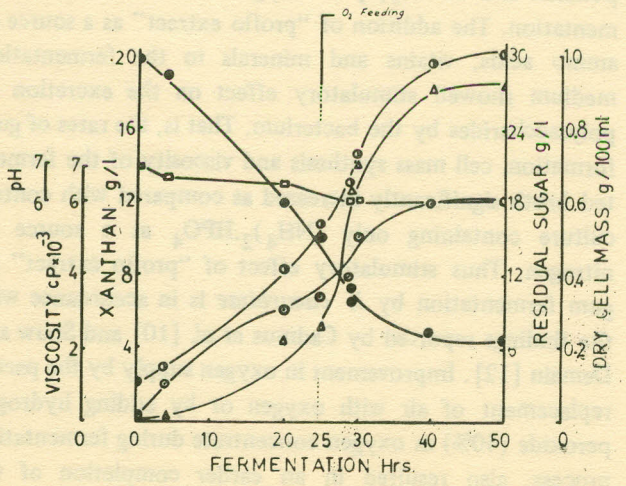

Fig. 4. Effect of partial replacement of air by oxygen on the production of Xanthan gum by *X. cucurbitae* PCSIR-52 in glass-stainless steel fermenter. (⊙⊙, Viscosity; ◑◑, Dry cell mass; ◪◪, pH; △△ Xanthan; ●●, Residual sugar).

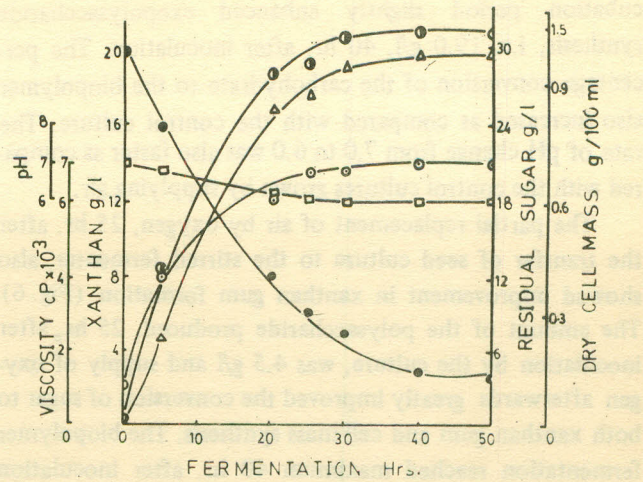

Fig. 5. Effect of partial replacement of air by oxygen (5:1) on the production of Xanthan gum by *X. cucurbitae* PCSIR-52 in 10 liter glass stainless steel fermenter. (⊙⊙, Viscosity; ◑◑, Dry cell mass; ◪◪, pH; △△, Xanthan; ●●, Residual sugar).

to the culture medium, in a 10-litre stirred fermenter, by partially replacing air with oxygen was investigated. Oxygenation was started at 0 and 24 hr. after inoculation (Fig. 4 5). The rate of aeration was kept at 1/1/min. and the ratio of air to oxygen was 5 : 1. The partial replacement of air by oxygen, started at 0 hr., increased the rates of fermentation parameters such as cell synthesis, conversion of sugar to biopolymer and viscosity of the fermented broth. The biopolymer formation reached maximum, 30 hr. after inoculation as compared with 48 hr. as described earlier in the control culture using air (Fig. 1). Further increase in the in-

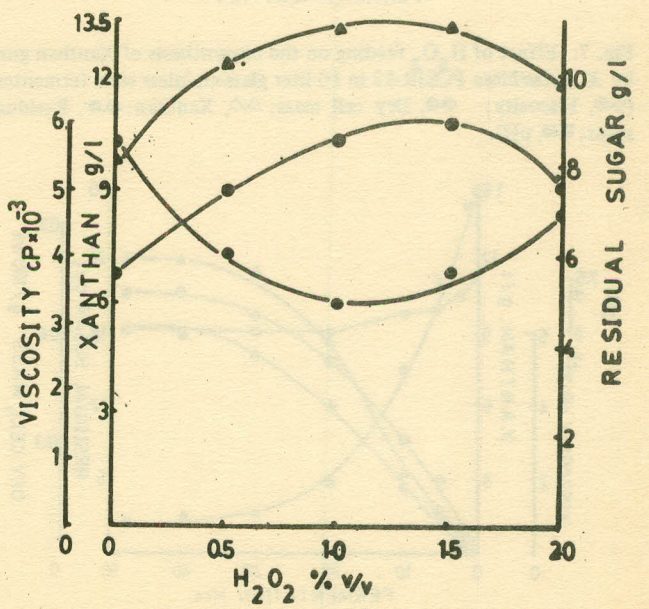

Fig. 6. Effect of H_2O_2 added 24 hr., after inoculation, on the production of Xanthan gum in sucrose-salt medium by *X. cucurbitae* PCSIR-52 in shake flasks. ⊙⊙, Viscosity; △△, Xanthan; ●●, Residual sugar).

cubation period slightly enhanced exopolysaccharide synthesis, i.e. 19.0 g/l, 40 hr. after inoculation. The percentage conversion of the carbohydrate to the biopolymer also increased as compared with the control culture. The rate of pH change from 7.0 to 6.0 was also faster as compared with the control cultures grown by supplying air.

The partial replacement of air by oxygen, 25 hr. after the transfer of seed culture to the stirred fermenter, also showed improvement in xanthan gum formation (Fig. 6). The amount of the polysaccharide produced, 25 hr. after inoculation by the culture, was 4.5 g/l and supply of oxygen afterwards greatly improved the conversion of sugar to both xanthan gum and cell mass synthesis. The biopolymer fermentation reached maximum 40 hr. after inoculation and further increase in the incubation period did not show any improvement in sugar utilization and gum synthesis.

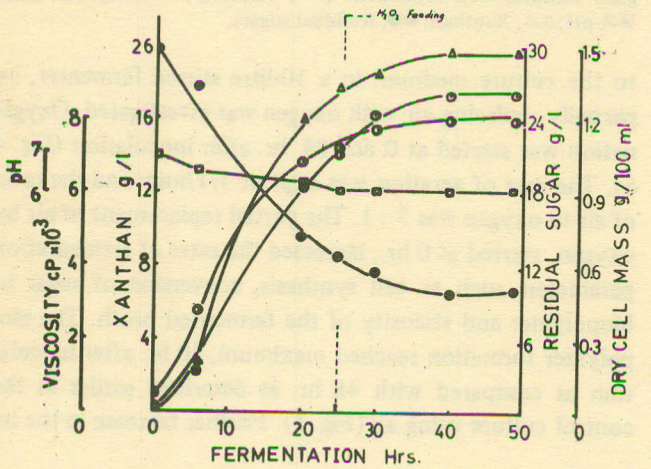

Fig. 7. Effect of H_2O_2 feeding on the biosynthesis of Xanthan gum by *X. cucurbitae* PCSIR-52 in 10 liter glass-stainless steel fermenter. (○-○, Viscosity; ●-●, Dry cell mass; △-△, Xanthan; ●-●', Residual sugar; ■-■, pH).

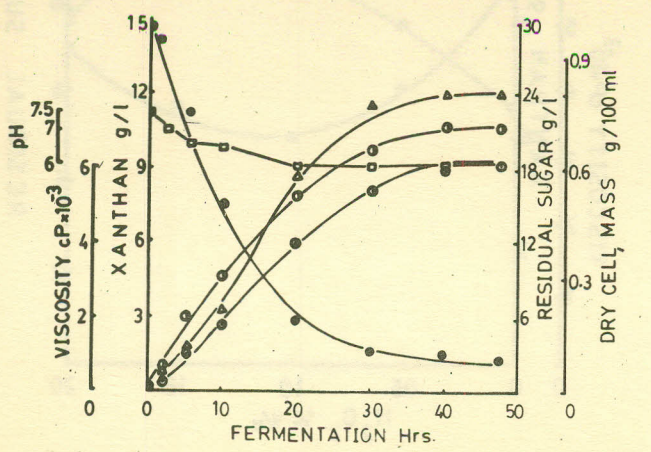

Fig. 8. Xanthan gum production by *X. cucurbitae* PCSIR-52 in 100 liter glass-stainless steel fermenter. (○-○, Viscosity; ●-●, Dry cell mass; ■-■, pH; △-△, Xanthan; ●-● Residual sugar).

Further effect of oxygen supply by adding hydrogen peroxide (40% solution) to the fermentation medium was also studied in both shake flasks and in 10-litre stirred fermenter (Fig. 6-7). The optimum amount of hydrogen peroxide was determined by adding it to the shake flask cultures 24 hr. after inoculation. The addition of hydrogen peroxide to the shake flasks culture stimulated xanthan gum formation, that is, the amount of xanthan gum produced in control cultures, 72 hr. after inoculation, was 10 g/l but in the presence of 1.0% v/v of hydrogen peroxide, the gum formation was 13.0 g/l. Further effect of feeding hydrogen peroxide solution (12 ml/hr) to the stirred fermenter was also investigated. Xanthan gum fermentation reached maximum 40 hr. after inoculation (19.8 g/l) instead of 48 hr. in control cultures (Fig. 1). The percentage conversion of carbohydrate to gum synthesis also increased as residual sugar was about 11.0 g/l instead of 3.0 g/l in control cultures. The cell formation was also greater in the presence of hydrogen peroxide.

DISCUSSION

The present work describes xanthan gum fermentation by an independently isolated culture of *X. cucurbitae* in sucrose-salt medium in stirred fermenters of 10, 50 and 100 litre capacity. The culture of *X. cucurbitae* was quite efficient in converting carbohydrate to extracellular polysaccharide as compared with other cultures of *Xanthomonas* species reported in the literature (Leach *et al.* [5]; Lilly, *et al.* [6]; Silman and Rogovin [15]; Silman and Rogovin [16] and Morain and Rogovin [3]). The important factors responsible for maximum gum production are the medium composition and the supply of oxygen to non-Newtonian fermentation. The addition of "proflo extract" as a source of amino acids, vitains and minerals to the fermentation medium showed stimulatory effect on the excretion of polysaccharides by the bacterium. That is, the rates of gum formation, cell mass synthesis and viscosity of the fermented broth significantly increased as compared with control culture containing only $(NH_4)_2.HPO_4$ as a source of nitrogen. Thus stimulatory effect of "proflo extract" on gum fermentation by *X cucurbitae* is in accordance with the findings reported by Cadmus *et al.* [10] and Souw and Demain [12]. Improvement in oxygen supply by the partial replacement of air with oxygen or by adding hydrogen peroxide (40%) as oxygen concentrate during fermentation process, also resulted in an earlier completion of the fermentation, i.e. 30 hr. as compared with 48 hr. under control conditions. The stimulatory effect of hydrogen peroxide is due to the availability of free oxygen by its

enzymic decomposition. Geiger [9] used hydrogen peroxide catalase system to overcome oxygen limitations in non-Newtonian fermentation in which *Aspergillus oryzae* was used for the production of enzyme proteases. Thus energy saving can be achieved by incorporating nitrogenous compounds in the culture medium or by improving oxygen supply during fermentation process.

Acknowledgements. The authors thank Messers Makhdoon Ahmad, Rehm Din and Muhammad Abbas for their technical assistance throughout the investigations.

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