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MICROBIAL PRODUCTION OF XANTHAN GUM FOR INDUSTRIAL USE

FOOD TECHNOLOGY AND FERMENTATION DIVISION
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C O N T E N T S

ABSTRACT

Xanthan gum fermentation was studied by a locally isolated culture of Xanthomonas cucurbitae PCSIR-52. The extracellular polysaccharide produced by the bacterium was identical to xanthan gum produced by Xanthomonas cambestris as shown by the infrared spectra of both gums. The isolated culture was found very efficient as compared with industrially used Xanthomonas campestris NRRL B-1459. Nutritional studies of the isolated culture were carried out in shake flasks before scale up production in stirred fermenters of 10, 50, 100 and 250 liter capacity by batch process. Evaluation of carbohydrates, nitrogen sources, both inorganic and organic and carboxylic acids for gum fermentation, were carried out in shake flasks. Of all carbohydrates, sucrose was found to be an ideal substrate for the production of xanthan gum and its optimum level was 3.0 percent. The production of xanthan biopolymer was also encouraging in cane juice, and increased in the order of cheese whey, beet molasses, hydrol or defatted rice bran when used as raw carbon sources. The pH control near neutrality was maintained by adding calcium carbonate (0.1%).

The inorganic nitrogen sources evaluated were diammonium hydrogen phosphate, sodium nitrate, disodium hydrogen phosphate, ammonium phosphate, and ammonium sulphate. The biopolymer production was about the same in the presence of di-ammonium hydrogen phosphate, disodium hydrogen phosphate or ammonium phosphate, the

concentration of nitrogen was from 0.30 to 0.40 g/l. The addition of organic nitrogen sources, however, greatly improved the conversion of sugar into extracellular-polysaccharide. The organic nitrogen sources tested were urea, thiourea, cornsteep liquor, cabbage extract, penicillin waste mycelium or cotton seed meal (local and imported from U.S.A., i.e. Proflo and Pharmamedia). The production of xanthan gum, however, was greatly stimulated in the presence of corn-steep liquor, cabbage extract or Proflo in the culture medium. Maximum xanthan gum formation however, was found by adding "Proflo extract" to the basal medium containing di-ammonium hydrogen phosphate. The amount of pyruvate was doubled in the presence of "Proflo extract" or corn-steep liquor as compared with the inorganic nitrogen sources and the viscosity of the fermented broth was greatly increased than that obtained by adding inorganic nitrogen sources.

The addition of carboxylic acids or their salts such as sodium pyruvate, potassium citrate, oxalic acid, sodium acetate, DL-malic acid, succinic acid, or tartaric acid increased the conversion of sugar to biopolymer in comparison with the control culture. The production of biopolymer was maximum in the presence of sodium pyruvate or potassium citrate.

Effect of improving oxygen supply by (i) partial replacement of air with oxygen, or (ii) by the addition of hydrogen peroxide solution as oxygen concentrate on the production of biopolymer was studied in 10 liter stirred fermenter. The rate of gum formation

was enhanced as compared with simple aeration system. Studies of biopolymer fermentation by both batch and repeated-batch processes showed that the efficiency of the bacterium was little affected during its continuous growth for 150 hrs in three cycles (each cycle of 50 hrs.). Production of xanthan gum was greater in sucrose-salt medium than that obtained in simple cane juice. The design of the stirred culture vessel plays significant role in the conversion of substrate to metabolites and cell mass. The multiple impeller system (disc turbine) resulted in high yield of xanthan gum due to better agitation and aeration (Oxygen supply).

The parameters such as growth rate (Rx), rate of product formation (Rp) and specific substrate consumption rate or metabolic quotient (q) were also determined from the data obtained in fermenter studied. The rate of gum production, cell growth and metabolic quotient were maximum in 50 liter fermenter.

INTRODUCTION

INTRODUCTION

Background:

The microorganisms capable of producing extracellular polysaccharides are widely distributed in the soil or natural environments. The microbial polysaccharides are complex and of varying composition and conformation and perform one or more of the following functions: (a) act as binders between the organism and soil particles or other substrates, (b) form protective envelops that retain water to prevent dehydration of the organism, (c) create physical barriers to viral infections, (d) provide the adhesive matrix between plant pathogenic cells and plant cell surfaces on which invaders colonize. There polysaccharide coatings are synthesized continuously and are not bound to the organism's cell wall by covalent linkages, rather they are excreted into the liquid medium. High concentration of polysaccharide, therefore, is obtained during culture under controlled conditions. This characteristic of polysaccharide excretion from the bacterial cell has resulted in the development of a new fermentation industry for the production of biopolymers from renewable resources. The manufacture of polysaccharides by fermentation process instead of their extraction from plant sources such as seeds, tree exudates or sea weeds and animal source is not seasonal rather they can be produced in an unlimited amount using sugary or starchy materials round the year. The polysaccharides or gums with noval and unique flow properties are in

great demand in food processing and a large number of industrial operations.

The biopolymers of great commercial significance are the extracellular polysaccharides such as dextran, xanthan, pullulans, curdlan and zanflo synthesized by Leuconostic mesenteroids, Xanthomonas campestris, Pullularia pullulan, Alcaligenes faecalis var. myxogenes and Erwina tathitica respectively (Murphy and Whistler, 1973; Cately and Whelan, 1971; and Lowson and Sutherland, 1972).

The commercial usefulness of the polysaccharides is based on their ability to change the rheological properties of water. Of all these biopolymers, xanthan gum particularly, is finding extensive uses in large number of industrial perations such as oil drilling, textile printing, ceramic glazing and in food preparations and developments are forecast which could open unlimited potentials (Anonymous, 1976 ; Kohn, 1976; Wernau, 1978; Anderson, 1977 and Sadvik and Maerker, 1977). The production of microbial gum is now a multimillion dollar industry because of their stability to heat, pH, cations and divalent ions associated to their pseudoplastic behaviour under conditions of high shear. The polysaccharide, xanthan gum is preferred for lubrication of bentonite muds used in tertiary oil recovery from existing oil wells (Anderson, 1977, and Sandvik and Mearker, 1977). During 1975, about 1,8000 tons of xanthan gum were used in drilling operations with potential usage of 3,000 tons predicted by 1980 (Arnold, 1976).

Composition:

Xanthan gum is a heteropolysaccharide with a molecular weight of more than one million (Leach et al. 1957) and consists of mannose, glucose and glucuronic acid. Each repeating block contains fine sugar units: two glucose, two mannose, and one glucuronic acid with the molar ratios of 2.8:3:2.0 respectively (Slonekar et al. 1964). The main chain of xanthan gum is built up of β-D-glucose units linked through 1- and 4-positions (identical to the chemical structure of cellulose). The side chain consists of the two mannose units and the glucuronic acid unit. The terminal β-D-mannose unit is linked glycosidically to sugar side chain linked to the 3-position of every other glucose residue in the main chain. About half of the terminal D-mannose residues contain a pyruric acid residue linked via the keto group to the 4- and 6-positions (Fig.1). The distribution of these pyruvate groups is unknown. The non-terminal D-mannose unit in the side chain contain an acetyl group at position 6(Janson, et al. 1975 and Moorehouse et al. 1977). Xanthan gum is unusual in that it contains pyruvate, 3% of the total weight and attached to a single unit glucose side chain by a beta linkage. It is partially acetylated without 4.7% acelyl.

Production:

Xanthan gum is industrially produced by fermentation process using a pure culture of Xanthomonas species. Stolp and Starr (1964) isolated more than 100

species of Xanthomonas capable of producing biopolymer using carbohydrates as substrate. Of all, these species, Xanthomonas campestris. X. phaseoli, X. malvacearum and X. carotae were found to be the best producer of extracellular-polysaccharide (Lilly et al. 1958). The nutritional requirements of Xanthomonas species were first studied by the Starr (1946) and it was found necessary to supply adequate air and control of pH near neutrality (6.8) in the fermentation process. A simple medium containing glucose, ammonium chloride, phosphate buffer, magnesium sulphate met the minimal growth requirements. The addition of growth factors in the form of casein hydrolysate gave much better growth than the control medium. Lilly et al. (1958) further carried out nutritional studies of X. phaseoli and X. campestris for better yield of extracellular-polysaccharides. These workers determined optimum glucose concentrations ranging from 1-5 per cent and incubation temperature of 28°C. The conversion of glucose to biopolymer was poor at higher levels of glucose added. Other carbohydrates such as sucrose, starch or its hydrolyzate were found to be useful as substrates. The nitrogen sources such as hydrolyzed casein or soybean protein, starch, sugar beat juice, sorghum, kenf juice and sorghum flour were found to be useful for optimum production of xanthan gum (Lindblom et al. 1965; Cadmus et al. 1971, and Bretschneinder, 1976). Souw and Demain (1979) found that carbon, nitrogen, sulphur and phosphorous have significant effects on the biosynthesis of xanthan gum by Xanthomonas compestris NRRL B-1459. The addition of certain organic acids of Kreb's cycle greatly stimulated the microbial growth and

be scaled up. However, the maintenance of aseptic cultural conditions for a commercial fermenter installations involve a greater risk with basal medium used in the manufacture of xanthan gum. A few studies have been reported regarding the production of xanthan gum by repeated-batch process using cheese whey as carbon source (Charles & Radjai, 1977). There was little change in fermentation process from cycle to cycle in the repeated-batch operations.

Much work has been reported in the literature regarding the various species of genus Xanthomonas as a plant pathogen (Cobb, 1895; Chardon; 1926; Cook, 1928; Ashby, 1929; Cook, 1932; Cook, 1935; Starr, 1946; Nayudu & Walker, 1961; Kotasthane, et al. 1965; Reddy et al. 1972; Wallis et al. 1973; Joneo, 1973; Murata et al. 1973; Sirgh & Verma, 1973 and Prasad & Pandey, 1977). However, no report appear in literature describing the production of extracellular-polysaccharides by the local isolates of Xanthomonas species.

PLAN OF WORK

The present study describes the synthesis of xanthan biopolymer by locally isolated cultures of Xanthomonas species and the plan of work is briefly described as follows:

a) Isolation and identification of Xanthomonas species from infected plant leaves/materials that produce gums.

b) Screening and selection of bacterial cultures capable of producing xanthan gum in shake flasks and X. cuccurbitae PCSIR-52 was selected.

c) Nutritional studies of X. cuccurbitae PCSIR-52 for maximum gum synthesis in shake flasks using synthetic carbohydrate salt medium and evaluation of various agro industrial bye-products as carbon and nitrogeneous sources.

d) Effect of adding salts of the acids of Kreb's cycle or plant extract (as stimulant) on the synthesis of biopolymer.

e) Effect of different nitrogen sources on the pyruvic acid content of xanthan gum.

f) Effect of oxygenation on gum formation by (i) partially replacing air with oxygen and (ii) by adding H_2O_2 as oxygen concentrate in the stirred fermenter.

g) Scale up studies of gum fermentation in 10, 50, and 100 glass-stainless steel fermentor and 250 liter stainless steel fermentor.

h) Xanthan gum fermentation of simple cane or synthetic sucrose salt medium by repeated batch process.

i) Kinetic of xanthan gum fermentation by the bacterium in the fermenter.

ISOLATION AND MAINTENANCE OF ORGANISM

The infected parts of the selected diseased plants were washed with 0.5 per cent sodium hypochlorite solutions 1:9 for two minutes. The washed material was then swirlled thoroughly in sterilized distilled water and macerated to obtain desired organism. The macerated material was then suspended in sterilized distilled water and spreaded onto Yeast-Malt extract agar plates (Table-1) according to the methods recommended by Kiraly et al. (1974). The isolated mucoid bacterial colonies were examined microscopically for their morphological and biochemical characterization (Table 3,-5-).

Viable mucoid cultures of Xanthomonas species were maintained on Yeast-Malt extract agar slants. The subculturing was made after every two weeks and the cultures were incubated at 30 $\pm$ 0.1^{o}C for 24 hrs. and then kept in refregerator.

TABLE-1

Composition of Yeast-Malt Agar Medium.

Constituents	g/l
Yeast extract	3.0
Malt extract	3.0
Peptone	5.0
Glucose	10.0
$CaCO_3$	1.0
Agar	25.0
pH	6.5-7.0

INOCULUM DEVELOPMENT

The inoculum development was carried out in stages as described by Burton et al. (1976).

First Stage: The fresh cells from 24 hrs old Yeast-Malt extract agar slants were inoculated into 50 ml (YM) broth in 300 ml. conical flask and placed on a rotary shaker rotating at 150 rpm. The shake flasks cultures were incubated at 30 ± 0.1°C for 24 hrs.

Second Stage: 250 ml of fermentation medium contained in one liter conical flask was inoculated by active vegetative cells at the rate of 5% v/v. The shake flask cultures were incubated at 30 ± 0.1°C for 24 hrs on a rotary shaker rotating at 150 rpm. The seed culture developed was used for both shake flasks and fermenter studies.

Final Stage: Seed culture development was carried out in 10 liter glass-stainless steel fermenter for the production of xanthan gum on semi-pilot scale in 50 and 100 liter fermenters. The size of inoculum, 24 hrs old, was kept at 5 per cent v/v throughout the investigations.

FERMENTATION TECHNIQUE

a) Shake Flask Studies:

Xanthan gum production was carried out in one liter cotton wool pluged conical flask containing 100 ml fermentation medium. (Table-2). The fermentation medium was divided into three parts for sterlization:

i) Sugar solution (10% at pH 6.0);
ii) Salts and,
iii) Calcium carbonate suspension.

All solutions were autoclaved separately at 121°C for 15 minutes, and after cooling they were mixed aseptically before inoculation. Shake flask cultures were incubated at 30 ± 0.1°C for 72 hrs. The rotary shaker designed and fabricated by Research Industrialization Division, PCSIR Laboratories, Lahore, was rotated at 150 rpm.

Fermenter Studies:

The scale up studies of xanthan gum fermentation was carried out in 10,50 and 100 liter glass-stainless steel fermenters (using QVF pipes with SS plates at both ends) and 250 liter stainless steel fermenter, designed and fabricated by "Research Industrialization Division, PCSIR Laboratories, Lahore". The fermenters were provided with stirring shafts at the top plate for agitation, aeration and coils for heating and cooling. In 100 liter glass-stainless fermenter, however, the stirring shaft was attached with the bottom plate. The temperature with fermenters was maintained by circulating water, maintained at desired temperature in an electric waterbath, by feeding pump. The rates of agitation and aeration were kept at 200 rpm and v/v/minute respectively. The fermentation medium for 10,50 and 100 liter fermenter was sterilized in aspirators as described earlier in shake flask studies. In 250 liter stainless steel fermenter, however, the culture medium was sterilized in the fermenter by passing steam in the coils and the temperature was maintained at 110 °C for 45 min. The empty glass-stainless steel fermenters were sterilized by steaming for one hour. Air was sterilized by passing through sterile glass wool tubes. The sterilized medium was

poured in the fermenters (10,50 and 100 liter) by gravity and inoculated by using 24 hr old vegetative inoculum developed in shake flasks in a 10 liter stirred fermenter. The size of inoculum used was 5.0 per cent throughout the studies.

TABLE-2

Composition of fermentation medium

Constituents	g/l
Sucrose (or Other-carbohydrates)	30.0
KH_2PO_4	8.0
$(NH_4)_2HPO_4$	1.5
$MgSO_4.7H_2O$	0.2
$CaCO_3$	1.0

All ingredients were dissolved in tap water, and corn oil was used as antifoam during fermentation.

ANALYTICAL

1. ESTIMATION OF SUGARS.

a) Glucose (or Sucrose):

Glucose or Sucrose was estimated according to the method described by Lo et al. (1975). All chemicals used were of analytical grade.

i) Preparation of Anthrone Reagent: The sulphuric acid solution was prepared by adding concentrated sulphuric acid (Specific gravity 1.84) to 50 ml. of redistilled water and the volume was made up to one liter in a volumetric flask. The 0.2% W/V anthrone reagent (Seifter et al. 1950) stabilized by thiourea (Roe, 1955), was made by dissolving 0.2 g anthrone and 1.0 g of thiourea in 100 ml of H_2SO_4 solution.

The mixture was warmed to 80-90^{o}C with occasional shaking. The reagent was stored in a refrigerator at about 0^{o}C and freshly prepared after every two weeks.

ii) Stock Solution: Stock solution of glucose (or Sucrose) of analytical grade, dried at 100^{o}C in an oven overnight, was prepared by dissolving 100 mg. of sugar in 100 ml of saturated benzoic acid solution (0.2% W/V). The stock solution containing 1.0 mg/ml sugar was kept in the refrigerator.

iii) Procedure: To each test tube containing glucose (or Sucrose) solution of different concentrations, 5 ml of Anthrone reagent was added followed by chilling for 10 minutes. The test tubes were loosely stoppered and heated in a water bath for 6 minutes at 100^{o}C to allow colour development.

Standard curves were plotted between the concentrations and absorbance using HELENA S-ER Sr. Spectrophotometer-Enzyme Analyzer. The absorbance was recorded at 570 and 480 nm for glucose and sucrose respectively.

Calculations:

$$\frac{Du}{DS} \times 0.1 \times \text{dilution of sample} \times 100 = \text{mg sugar/100 ml}$$

Where

Du = Optical density of unknown solution.

DS = Optical density of standard solution.

0.1 = mg of glucose or sucrose in 1 ml of standard solution.

b) Lactose:

The lactose was estimated by the method of Lawrence et al. (1968). To 1.0 ml of aliquot sample after appropriate dilutions in a stoppered test tubes, was added 1.0 ml of 5.0% aqueous phenol solution and mixed thoroughly. From a rapid burrette 5.0 ml of concentrated H_2SO_4 was added. Mixed the both solutions and allowed to stand at room temperature for cooling to develop colour that remained stable for at least one hour.

The optical density was measured at 480 nm using HELENA S-ER Jr. Spectrophotometer-Enzyme Analyzer. A standard graph of lactose was also plotted.

c) Estimation of Sugars in the Fermented Broth:

The fermented broth was diluted to reduce the viscosity and a few drops of 2NHCl were added to dissolve insoluble salts. The broth was then centrifuged at 5,000 rpm for 10 minutes and the supernatant was used for sugar estimation.

ESTIMATION OF TOTAL NITROGEN

Total nitrogen in the organic nitrogen sources (Solid material or extracts) was estimated according to the method described in A.O.A.C. (1955) using

$(CuSO_4\text{-}K_2SO_4\text{-}SeO_2$) as catalyst to digest the samples in
$1 : 9 : 0.02$)

A.R. sulphuric acid.

BACTERIAL CELL MASS DETERMINATION

Bacterial cell mass was measured by determining the optical density of the fermented broth at 600 nm using EEL 194 SPECTRA, Colorimeter, by the method of Morain et al. (1971). The uninoculated medium was used as blank

Broth samples were diluted with distilled water and the optical density was caliberated against cell concentration determined gravimetrically as reported by Morgain and Rogovin (1966). Broth samples after measuring the optical density were centrifuged in 50 ml plastic tubes at 10,000 rpm (using MSE refrigerated centrifuge) for 30 minutes. After the supernatant was discarded, the cell mass was washed again with diluted HCl (Ca.2N) to dissolve inorganic minerals and centrifuged at 10,000 rpm to recover cells. Again the supernatant was discarded. Finally, the cells were washed with distilled water and centrifuged at 10,000 rpm., the supernatant was discarded and the cells settled in the tubes were dried at $90^{o}C$ to constant weight.

DETERMINATION OF XANTHAN GUM

The Xanthan gum was determined gravimetrically according to the method described by Morain and Rogovin (1966). Broth samples were diluted with distilled water to a viscosity below 1000 cP and KCl (2% W/V) was added. Eethanol was then added slowly while mixing to precipitate the polymer. The precipitate was washed after centrifugation with ethanol containing

0.05% KCl into glass tubes, centrifuged again to recover the washed precipitates, and dried in the tared glass tubes at 60°C to constant weight. Weight of the cells determined separately was substracted from the weight of the precipitate to give the next polymer weight.

INFRARED SPECTROSCOPY OF XANTHAN GUM

The spectra of purified Xanthan gum produced by Xanthomonas cucurbitae and xanthomonas campestris NRRL B-1459 were obtained on a Perkin-Elmer model 1320, double beam recording infrared Spectrophotometer. Purified samples of Xanthan gum were studied by "mull" technique as described by Rao (1963). The mull was made by grinding a sample of (1-5 mg) in a few drops of Nujol and thoroughly homogenized in Pestel-mortar to destroy the orientation of the crystal structure to eliminate the adanger of the strengthening and diminishing of certain bands. A thin film of mull was placed between two sodium chloride plates to obtain spectra of two polysaccharides over the range of 2.5-7 microns (M).

ESTIMATION OF PYRUVIC ACID

The pyruvic acid content of Xanthan gum was determined by the method of Sloneker and Orentas (1962).

A 0.2-0.4 per cent solution of polysaccharide was hydrolysed at 100°C in 1 N hydrochloric acid for 3 hours. A 2 ml aliquot was removed and was mixed with 1 ml of a 2,4-dinitrophenyl hydrazine reagent (0.5 per cent in 2 N hydrochloric acid) for 5 minutes. The reaction

mixture was extracted with 5 ml of ethyl acetate, and the aqueous layer was discarded. Ethyl acetate layer was extracted with three 5 ml portions of 10 per cent sodium carbonate, the extract was diluted to 25 ml with additional 10 per cent sodium carbonate. Concentration of pyruric acid was determined by measuring optical density of the sodium carbonate solution at 400 mu using Helena S-ER Jr. Spectrophotometer.

MEASUREMENT OF VISCOSITY

The viscosity of the fermented broth was measured by "Ostwald Viscometer"., The fermented broth after appropriate dilution was centrifuged at 2,000 rpm to remove isoluble and other suspended particles. The viscometer was placed in a thermostatically controlled water bath at 30 $\pm$ 0.5^{o}C for 3 minutes to attain temperature. The liquid was then sucked up through capillary until the surface of the liquid "A" was above the upper gradation line. It was then allowed to flow down and the time required for the miniscus to pass from upper to lower gradation line, was recorded. The apparatus was then thoroughly cleaned with chromic acid and washed with distilled water, and again equal volume of liquid "B" was filled in it and the flow time of this liquid was also recorded.

The viscosity was calculated in "Centipoise". Water was taken "liquid" 'A' as reference standard.

Calculations:

$$\frac{\eta B}{\eta A} = \frac{tB \times dB}{tA \times dA} = \text{Poise}$$

Where

ηB, ηA = Viscosity of liquid B and A respectively.

tB, tA = the time taken for flow of liquid B and A respectively.

dB, dA = the density of liquid B and A respectively.

ISOLATION AND IDENTIFICATION OF XANTHOMONAS SP.

The Xanthomonas species, plant pathogens, were isolated from the infected leaves of different plants. The infected parts of selected leaves were separated and washed with sterilized distilled water before the maceration of diseased tissues as reported by Kiraly et al. (1974). The macerated tissues were suspended in 10 ml of sterilized distilled water and further dilutions were made for the isolation of Xanthomonas sp. by pour plate method. The isolated mucoid colonies on agar plate were further purified and identified morphologically and physiologically, using criteria of Bergay's Mannual of Determinative Bacteriology. (Table-3-5).

SCREENING AND SELECTION OF XANTHOMONAS SP.

The isolated four cultures of Xanthomonas Sp. and Xanthomonas campestris NRRL B-1459 were screened for the biosynthesis of xanthan gum in shake flasks (Table-6). The shake flasks cultures were analysed 68 hr after inoculation with 24 hr old seed cultures developed in shake flasks. Of all strains, however, Xanthomonas cucurbitae PCSIR-52 produced maximum xanthan biopolymer (8.1 g/l). The viscosity of fermentation broth was 1.55×10^{-3}Cp. The production of gum by other isolates of the genus Xanthomonas was also satisfactory. Xanthomonas cucurbitae PCSIR-52 therefore, was selected for the production of biopolymer. The amount of gum produced by Xanthomonas campestris NRRL B-1459 however, was lesser than the locally isolated cultures.

TABLE-3

Morphological characteristics of locally isolated Xanthomonas species.

Organisms	Source	Phase of growth	ASAR colonies			Morphology				
			Form	Margins	Surface	Form and arrangement	Diameter (microns)	Gram's reaction	Flageua	Capsule
X. campestris (PCSIR-51)	Cabbage	mucoid	circular	entire	smooth	rods	0.2 - 0.4 by 0.5 - 1.0	-	Monotrichous	Present
X. cucurbitas (PCSIR-52)	Cucurbite leaves	"	"	"	"	"	0.3 - 0.5 by 0.5 - 1.0	-	"	"
X. ricinicola (PCSIR-53)	Ricinus cumunis	"	"	"	"	"	0.4 - 0.7 by 1.0 - 1.5	-	"	"
X. citri (PCSIR-54)	Citrus leaves	"	"	"	"	"	0.5 - 6.0 by 1.3 - 1.5	-	"	"

TABLE-4

Physiological characteristics of locally isolated Xanthomonas species.

Organisms	Biological relationship	Relation to free oxygen	Nitrate reduction	Chromogenesis	Indol	Hydrogen sulfide	Protein liquification		Indicator reduction	
							Geletin	Casein	Litmus	Methylene blue
X. campestris (PCSIR-51)	Plant pathogen	aerobic	+	Light yellow	+	+	+	+	+	+
X. cucurbitae (PCSIR-52)	"	"	+	"	+	±	+	+	+	+
X. ricinicola (PCSIR-53)	"	"	+	Strong yellow	+	±	+	+	+	+
X. citri (PCSIR-54)	"	""	+	Yellow	+	±	+	+	+	+

TABLE-5

Biochemical characteristics of locally isolated Xanthomonas species.

Organisms	(CARBOHYDRATES FERMENTATION)										
	Monosaccharides				Disaccharides			Polysaccharides		Alcohols	
	Glucose	Galactose	Mannose	Xylose	Sorbose	Lactose	Maltose	Starch	Reffinose	Manitol	Sorbitol
X. campestris (PCSIR-51)	+	+	+	+	+	+	+	+	+	+	+
X. cucurbitae (PCSIR-52)	+	+	+	+	+	+	+	+	+	+	+
X. ricinicola (PCSIR-53)	+	+	+	+	-	+	-	+	+	+	+
X. citri (PCSIR-54)	+	+	+	+	+	+	+	+	+	+	+

TABLE-6

Screening of Xanthomonas Sp. capable of producing Xanthan gum.

Isolates	*Viscosity cP x 10^{-3}	Xanthan g/l
X. campestris (PCSIR-51)	1.03	6.0
X. cucurbitae (PCSIR-52)	1.55	8.1
X. ricinicola (PCSIR-53)	1.2	7.1
X. citri (PCSIR-54)	1.1	6.2
X. campestris NRRL-B-1459	0.81	4.1

* Viscosity at $30^{o}C$

EFFECT OF INOCULUM

Kinetics of the fermentation process are greatly influenced by the size and age of inoculum. Fig.1 shows the effect of the age of inoculum on polysaccharide synthesis and sugar utilization by Xanthomonas cucurbitae PCSIR-52. The amount of Xanthan gum produced was maximum (15.0 g/l) when the age of inoculum was 12 hr. Further increase in the age of inoculum resulted in less sugar consumption, hence low biopolymer formation and viscosity of the fermented broth.

Fig.2 shows the effect of the size of inoculum on the sugar consumption, xanthan production and viscosity of the fermented broth. The production of xanthan biopolymer was slightly higher in the presence of 7.5% V/V inoculum than that obtained by adding 5% inoculum. The size of inoculum selected for subsequent experiments, however was 5.0% V/V.

EFFECT OF TEMPERATURE

Fig.3 shows the effect of different incubating temperatures on the biosynthesis of xanthan gum by the bacterium in shake flasks. The optimum temperature of selected organism was found to be 30°C. The temperature variations greatly affected both the gum formation and sugar utilization by Xanthomonas cucurbitae PCSIR-52.

NUTRITIONAL STUDIES

1. CARBOHYDRATES:

The data of Fig.4 indicates the effect of various carbohydrates such as xylose, glucose, fructose, sorbose, mannose, galactose, sucrose, maltose, lactose or starch on

the biosynthesis of xanthan gum by the organism in shake flasks. The polysaccharide formation was maximum in the presence of sucrose, i.e. 15.0 g/l, and decreased in the order of lactose, sorbose, glucose, fructose, mannose, maltose, galactose, starch and xylose. The production of polysaccharide was better in the presence of disaccharides as compared with monosaccharides.

Fig.5 shows the synthesis xanthan biopolymer in cane juice, cheese whey, beet molasses, hydrol or defatted rice bran. The amount of polysaccharide produced was maximum (2.7 g/l) in the presence of cane juice, a rich source of sucrose and nutrients, and decreased in the order of cheese whey, beet molasses, hydrol and defatted rice bran. For subsequent experiments, however, sucrose was used throughout as substrate for the production of Xanthan gum.

The sugar concentration in non-Newtonian fermentation is of great significance for both cells and exocellular-polysaccharide production. Effect of the concentration of sugar (1-5% W/V) on Xanthan gum formation by the bacterium was studied (Fig.6). The optimum level of sugar was about 3.0% and further increase in its concentration resulted in the decrease of Xanthan gum formation by the organism. In subsequent experiments, therefore, sugar concentration was kept at 3.0% throughout the present study.

2. CALCIUM CARBONATE:

The metabolic activities of the microbes are greatly influenced by the pH of culture medium. The biopolymer formation is very sensitive to hydrogen ion concentration of the fermentation broth, that is, pH near

neutrality is desirable for maximum conversion of sugar to polysaccharide. Effect of varying concentration of $CaCO_3$ (0-2.5 g/l) on the sugar consumption, gum formation, viscosity and pH were determined in shake flasks. The optimum level of calcium carbonate was found to be 1.0 g/l and the amount of gum produced 17.2 g/l. Further increase in the concentration of $CaCO_3$ affected the fermentation process (Fig.7).

3. NITROGEN SOURCES

a) INORGANIC:

Selection of inorganic nitrogen sources for xanthan gum fermentation was carried out by adding different salts of nitrogen to the basal medium (Fig.8). The level of the nitrogen was kept at 0.32 g/l. The production of extracellular-biopolymer was maximum in the presence of $(NH_4)_2HPO_4$, $(NH_4)_3PO_4$ and $NaNO_3$, i.e. 16 g/l. The residual sugar was about 7.8 g/l indicating that 77% of the added sugar was consumed for metabolites, cell synthesis and respiratory CO_2. Xanthan gum formation in the presence of $(NH_4)_2SO_4$ and NH_4NO_3, however, was 10.0 g/l. The effect of varying concentration of nitrogen sources such as $(NH_4)_3PO_4$, $NaNO_3$, NH_4NO_3 and $(NH_4)_2SO_4$ on xanthan gum fermentation was further carried out (Fig.9-12). The optimum levels of nitrogen were found to be 0.039, 0.032, 0.042 and 0.033% in the presence ammonium phosphates, sodium nitrate ammonium nitrate, and ammonium sulphate, respectively. Maximum amount of xanthan produced was 16.0 g/l in the presence of ammonium phosphate or sodium nitrate. However, on the addition of ammonium nitrate or ammonium sulphate its production was about 10.0 g/l. Further increase in the concentration of nitrogen beyond optimum level resulted in lowering the conversion of carbohydrate to the biopolymer. The precise control of nitrogen in the medium, therefore, was necessary for maximum gum production.

ii) ORGANIC:

a) Urea: Fig.13 shows the effect of addition of urea (0-0.14%) to the culture medium on the synthesis of exocellular-polysaccharide by the Xanthomonas cucurabitae

PCSIR-52, residual sugar and viscosity of the fermented broth. The residual sugar in the presence of urea (0.06% or 0.08%) was about 2.0 g/l indicating that sugar consumption was more than 96% of the added carbohydrate. The amount of xanthan gum produced was 12.0 g/l and viscosity of the fermented broth was 5.5×10^{-3} cP. Further increase in the concentration of urea increased residual sugar or decreased sugar consumption, gum formation and viscosity of the fermented broth. The optimum level of nitrogen was found to be 0.028%.

b) Thiourea: Effect of thiourea, a sulphur containing nitrogen source, on xanthan gum fermentation was also studied (fig.14). The nitrogen concentration varied from 0-0.126%. The optimum level of nitrogen was 0.108%, the amount of biopolymer produced 14.0 g/l; the residual sugar 6.0 g/l and viscosity of the fermented broth was 4.4×10^{-3} cP. The production of xanthan gum, sugar utilization and consequent viscosity of the fermented broth were reduced by further increasing the concentration of thiourea.

c) Cabbage Extract: The plant pathogens capable of producing exocellular-polysaccharide are usually found on the leaves of vegetables. Effect of the addition of cabbage extract on the biosynthesis of xanthan gum was investigated in order to determine any factor that can stimulate the synthesis of biopolymer by the bacterium. The cabbage extract was prepared by refluxing 100 g of crushed cabbage leaves in one liter tap water. The extract was centrifuged at 3000 rpm for 15 minutes and the clear supernatant was added to the fermentation medium (Fig.15). The production of gum in control culture was

2.0 g/l and it increased with the increase in the concentration of cabbage extract. The optimum level of the extract was 40 ml/l. The amount of exocellular-polysaccharide produced was 15 g/l and residual sugar 4.0 g/l. The sugar consumption was more than 86.0%. Further increase in the concentration of cabbage extract did not show any increase in gum formation indicating that sugar consumption was reduced by increasing cabbage extract. It may be due to an increase in viscosity of the fermented mash.

d) Corn-Steep Liquor: Corn-steep liquor (CSL), a by-product of maize starch industry is a rich source of nitrogenous compounds, minerals and other growth factors. Effect of its addition to the basal medium was studied on xanthan gum fermentation by Xanthomonas cucurbitae PCSIR-52. Corn-steep liquor was obtained as dilute effluent from "Rafhan Maize Products, Faisalabad". It was concentrated in an open steam jacketed pan with a moisture level of about 7%. The amount of corn-steep liquor added to the basal medium varied from 0-1.0 g/l and the concentration of total nitrogen ranged from 0-1.28%.

Visual observation showed that the growth of the bacterium was very fast after the addition of CSL. Xanthan gum production in control culture was 1 g/l and residual sugar 20 g/l. The production of biopolymer sugar utilization were greatly stimulated by adding corn-steep liquor at the rate of 0.1% in the medium (Fig.16). Further increase in the concentration of CSL did not show much improvement in gum synthesis except at 0.6% level. The amount of total nitrogen in

the basal medium was 0.32%. The amount of xanthan gum formation was 18.0 g/l and residual sugar about 2% indicating that more than 93% of the added sugar was consumed in gum formation, cell mass and CO_2 production. The viscosity of the fermented mash reduced the sugar consumption. Corn-steep liquor, however, was found to be a good source of nitrogen in the gum synthesis but colour of the fermented broth was slightly darkish.

e) Penicillin Waste Mycelium: Fig.17 shows the effect of the addition of penicillin waste mycellium, a by-product of penicillin plant, Daudkhel, on biopolymer fermentation. The mycelium contained about 3.8% nitrogen. The substitution of waste mycelium greatly increased the conversion of added sugar to xanthan gum hence viscosity of the fermented broth as compared with control culture. Xanthan gum production was about 12.5 g/l in the presence of 0.2% waste mycelium and it remained about the same with the increase in nitrogen level in mycelial form. The residual sugar was negligible in the presence of optimum amount of mycelium indicating that the consumption of sugar by the bacterium was more than 98%.

f) Cotton-seed Meal: Cotton-seed meal, after alcoholic treatments for the removal of gossypol, was also evaluated as a nitrogen source in the fermentation medium. Its nitrogen content was 0.30%. The addition of cotton seed meal greatly improved the metabolic system and a large amount of sugar was converted into xanthan gum. The optimum concentration of cotton seed meal was 0.1-0.3% and further increase in its amount reduced biopolymer formation. It follows that carbon-

nitrogen ratio in the fermentation medium is very important for the maximum conversion of sugar to exocellular-polysaccharide production. Viscosity of the fermented broth was in proportion to the production of xanthan gum (Fig.18).

Effect of adding cotton seed meal extract, with and without di-ammonium hydrogen phosphate in the culture medium for gum fermentation was also investigated. The cotton-seed meal extract was prepared by refluxing 10 g of cotton seed meal with 100 ml distilled water at 100^{o}C for 30 minutes and centrifuged at 3000 r.p.m. The clear supernatant was used as nitrogen source (2.0-10% V/V) for gum synthesis (Fig.19). The production of exocellular-polysaccharide was stimulated by increasing nitrogen concentration. The amount of xanthan gum produced was 4.5 g/l in the presence of 4% V/V cotton seed meal extract and showed very little further increase by increasing its concentration. The addition of di-ammonium hydrogen phosphate to the fermentation medium with cotton seed meal extract, however, greatly increased sugar conversion to biopolymer (Fig.20). The amount of xanthan gum produced was about 11.5 g/l in the presence 2-4% cotton seed meal extract with di-ammonium hydrogen phosphate. Sugar utilization did not increase by increasing nitrogen but conversion of sugar to gum decreased with the increase in the concentration of nitrogen.

g) Pharmamedia: Pharmamedia, a cotton seed meal fluor manufactured by "Traders Protein Division", of Traders Oil Mill Co. Texas, USA, was also evaluated as a source of nitrogen (0-0.5% W/V) in xanthan gum fermentation (Fig.21). The maximum amount of gum produced was 8.0 g/l in the presence of 0.2% W/V pharmamedia, and

the residual sugar was 2.0 g/l indicating that the utilization of sugar by the bacterium was 94% but its conversion to exocellular-polysaccharide quite low. Further increase in nitrogen level by adding pharmamedia to the culture medium did not show any stimulatory effect on xanthan gum production.

h) Proflo: It is also manufactured by "Traders Protein, Traders Oil Mill Co. Taxus, USA", from cotton seed embryo. Fig.22 shows the effect of the addition of "Proflo" to the basal medium on the synthesis of biopolymer by Xanthomonas cucurbitae PCSIR-52. The concentration of "Proflo" varied from 0-0.5 g/l. The optimum level of "Proflo" was 0.1% providing 0.036% nitrogen, and xanthan gum formation was 7.0 g/l. Further increase in its concentration, however, caused decline in gum synthesis. Residual sugar, (i.e. sugar consumption) however, remained about the same. The viscosity of the fermented broth was also little affected by increasing "Proflo" concentration.

Xanthan gum fermentation was further studied in the presence of "Proflo extract" prepared like cotton seed meal extract described earlier. The basal medium was prepared without adding di-ammonium hydrogen phosphate. The level of nitrogen as "Proflo extract" was 0-0.25%. The maximum amount of gum produced in the presence of 0.02% nitrogen was 11.5 g/l and residual sugar (3.0 g/l) indicating that 90% of the added carbohydrate was consumed for gum formation, cell synthesis and other metabolites. These parameters of gum formation

4. EFFECT OF NITROGEN SOURCES ON THE PYRUVIC ACID CONTENT OF XANTHAN GUM.

The rheological properties of xanthan biopolymer are greatly influenced by the amount of pyruvic acid in the exopolysaccharide molecule. The concentration of pyruvic acid, however, depends upon the bacterial culture and composition of fermentation medium. The data of Fig.25 shows the effect of various nitrogen sources on pyruvate content of xanthan gum produced by Xanthomonas cucurbitae PCSIR-52 in shake flasks and its relationship with the viscosity of the fermented broth. The maximum amount of pyruvate in the biopolymer was 4.5 and 4.3% in the presence of "Proflo extract" and cornsteep liquor respectively. The viscosity of fermented broth was proportionate to the amount of pyruvate in the gum molecule. Thus organic nitrogen sources such as "Proflo extract" and corn steep liquor were found to be good nitrogen sources for the production of xanthan gum with higher pyruvate content. Inorganic nitrogen sources, in general excreted polysaccharide with low concentration of pyruvic acid.

5. EFFECT OF CARBOXYLIC ACIDS

Acids of Kreb's cycle play an important role in the metabolism of sugar to synthesize metabolities, CO_2 and cell mass. Attempts were made to study the effects of adding acids of Kreb cycle (or their salts) on the sugar consumption, gum formation and viscosity of the fermented broth.(Fig.26). Sodium pyruvate shows the effect of adding sodium pyruvate (0-1% W/V) to the basal medium on gum formation. Tho amount of xanthan gum produced in control culture was 1.8 g/l and the residual sugar 2.58 g/l. Thus conversion of sugar to polysaccharide was very low. The addition of sodium pyruvate, however, greatly stimulated the conversion of sugar to biopolymer. Xanthan gum production started increasing (16.0 g/l) on addition of 0.2% sodium pyruvate. The biosynthesis of polysaccharide, however, was maximum (18.0 g/l) in the presence of 0.8% sodium pyruvate. The fermented broth was highly viscous and further addition of sodium pyruvate did not increase gum formation. The residual sugar was also found to be negligible in the fermented broth.

b) Potassium Citrate:

Effect of the addition of potassium-citrate (0-1.0%) on the production of xanthan gum, residual sugar and viscosity of fermented broth was also investigated (Fig.27). The biopolymer formation increased with the increase in the concentration of potassium citrate. The amount of xanthan gum excreted in the medium was 16.0 g/l on addition of 0.8% potassium citrate to the culture medium and the residual sugar was 1.5 g/l. However, the amount of gum produced decreased with further addition of the salt.

c) Sodium Acetate:

The addition of sodium acetate (0-1.0%) to the basal medium greatly improved the conversion of sugar to xanthan gum formation (Fig.28). The amount of biopolymer produced, in the presence of Na-acetate (0.2%), was 12.5 g/l while residual sugar was about 1.25 g/l. The synthesis of marobial gum, however, was slightly increased with the increase in the concentration of sodium acetate. The optimum level of the salt was 0.8% xanthan formation about 15.0 g/l and sugar consumption about 99% since residual sugar was 1.0 g/l. The viscosity of the fermented broth also increased in proportion to gum synthesis.

d) DL-malic Acid:

Fig.29 shows that the production of xanthan gum by the bacterium also increased by adding DL-mallic acid 0-1.0% to the fermentation medium. The optimum level of DL-mallic acids was 0.2% and the production of xanthan gum 13.5 g/l. Further increase in the concentration of DL-mallic acid, however, did not show any increase in the synthesis of polysaccharide. The residual sugar slightly increased when the level of DL-malic acid was 0.8 and 1.0% w/v in the fermentation medium.

e) Succinic Acid:

Effect of the addition of succinic acid (0-1.0%) on the biosynthesis of xanthan gum, viscosity and residual sugar was studied (Fig.30). The conversion of sugar to biopolymer increased by increasing the level

of succinic acid in the basal medium. Maximum gum formation was found to be about 14.8 or 15.0 g/l in the presence of 0.6 or 0.8% succinic acid respectively. The residual sugar was about 1.75 g/l. That is, sugar consumption was about 94%. The viscosity of the fermented broth was more than $6x10^{-3}$cP.

f) Oxalic Acid:

Effect of adding oxalic acid on xanthan gum fermentation was also studied (Fig.31). The optimum level of oxalic acid was 0.2% w/v and the amount of gum produced was 15.0 g/l and its formation decreased with the increase in the concentration of oxalic acid. Extracellular-polysaccharide production was reduced to 7.0 g/l in the presence of 1.0% oxalic acid. The visvosity of the fermented broth was also affected with the increase in acid concentration.

g) Tartaric Acid:

The amount of polysaccharide formation was maximum (13.0 g/l) in the presence of 0.6% tartaric acid (Fig.32). The biopolymer synthesis, however, was reduced by further increase in the concentration. Both residual sugar and viscosity of the fermented broth decreased by increasing the concentration of tartaric acid in the fermentation medium.

XANTHAN GUM FERMENTATION IN STIRRED CULTURE VESSELS

Xanthan gum fermentation was scaled up in 10, 50 and 100 liter glass-stainless steel fermenters after shake flask studies. The fermenters were steam sterlized by steaming continuously for 45-60 minutes. The culture medium separately sterilized were aseptically poured by gravity in the fermenters before inoculation with actively growing cells as reported in materials and methods. In general, the rate of cell mass formation, sugar utilization and product formation were faster than in shake flasks.

a) 10 liter Glass Stainless Steel Fermenter:

Figs.(33-35) show the comparison of xanthan gum fermentation by Xanthomonas cucurbitae PCSIR-52 using glucose, sucrose, or lactose as carbon source respectively. Di-ammonium hydrogen phosphate was used as a source of nitrogen in these experiments. The parameters studied were xanthan gum synthesis, residual sugar, dry cell mass, viscosity and pH changes in the course of fermentation. The residual sugar, 48 hr after inoculation, was about 6.0 g/l, (i.e. 80% sugar consumption) in the presence of these sugars but further incubation did not increase sugar utilization. The amount of gum produced decreased in the order of sucrose, lactose, and glucose, i.e. 17.5, 16.0 and 13.0 g/l respectively. The pH of the basal medium during fermentation varied from 7.0-6.0 in presence of these sugars. The cell mass synthesis was 0.79, 0.82 and 0.7 g/100 ml in the presence of sucrose, lactrose and glucose respectively. The percentage yield of exocellular-polysaccharide, on the basis of sucrose added, was about 60.0 per cent.

For further studies, therefore, sucrose was used as substrate for gum production.

Effect of the supplementation of sucrose-salt medium with corn-steep liquor was studied on the biopolymer synthesis by Xanthomonas cucurbitae PCSIR-52 (Fig.36). Xanthan gum formation, and residual sugar were 16.0 g/l and 5.5 g/l respectively, but the cell mass slightly increased (0.95 g/100 ml) 48 hrs after inoculation.

The addition of "Proflo extract" to basal medium also resulted in increasing the conversion of sugar added to biopolymer formation (Fig.37). The residual sugar, 40 hrs after inoculation was 1.0 g/l and the amount of gum synthesis and cell mass were 19.0 g/l and 0.95 g/100 ml respectively. Further increase in the incubation time did not show any significant increase in xanthan gum production, sugar utilization and cell mass. The pH of the fermented broth was varied from 7.5 to 6.0 40 hrs. after inoculation and it remained unchanged with the increase in the incubation period. Thus, "Proflo extract" (Cotton seed meal) was found to be a better source of protein that enhanced the rate of gum formation.

b) 50 liter Glass Stainless Steel Fermenter:

Xanthan gum fermentation was further scaled up in 50 liter stirred fermenter using sucrose-salt medium supplemented with "Proflo extract" (Fig.38). All parameters such as rate of gum formation, sugar consumption, cell mass and viscosity of the fermented broth were greater than those obtained in shake flasks and 10 liter stirred culture vessel. The amount of gum produced 20 and 30 hrs after inoculation with seed culture was

19.0 and 20.0 g/l respectively. Further incubation however, did not show any increase in gum production. The fermented broth became highly viscous and viscosity, 30 hrs after the incubation was 7.5×10^{-3} cP. The residual declined sugar in the basal medium was negligible and the pH 20 hrs after inoculation from 7.5 to 6.0. In general, however, the gum fermentation reached maximum 20 to 30 hrs after inoculation.

c) 100 liter Stainless Steel Fermenter:

The production of xanthan gum by Xanthomonas cucurbitae PCSIR-52 was further carried out on semi pilot plant scale in 100 liter stirred fermenter (Fig.39). The basal medium of sucrose-salt was supplemented with "Proflo extract". The agitator was attached at the bottom of fermenter. The conversion of sugar to cell mass and xanthan gum hence viscosity of the fermented broth were lesser as compared with the results obtained in 10 and 50 liter culture vessels described earlier. The amount of gum produced 30 hrs after inoculation was 12.0 g/l and further incubation did not increase biopolymer synthesis. Contrarily, gum production decreased by 33.0%. The main reason for poor yield of the xanthan gum was the agitation system, as it was not efficient like that in 10 and 50 liter culture vessels. Thus, aeration or oxygen supply to the bacterium in non-Newtonian fermentation, plays a significant role in the biosynthesis of biopolymers.

d) 250 liter Stainless Steel Fermenter:

Xanthan gum fermentation was also studied on semi pilot plant scale in 250 l stirred fermentor (Fig.39^{b}). Sucrose-salt medium suplemented with "Proflo extract" was sterilized in the fermenter at 115^{o}C for 45 minutes. Calcium carbonate suspension, however, was separately sterilized before its addition to the fermenter. The rate of exocellularpolysaccharide formation was slightly lesser than that obtained in 50 liter glass-stainless steel fermenter as described earlier. The amount of gum produced, 50 hrs after inoculation, was 17.2 g/l and sugar consumption was about 28.0 g/l. The bacterial cell was 0.9 g/100 ml and pH was changed from 7.2 to 6.0 during fermentation. The viscosity of the fermented mash was 5.0x10^{-3}cP, at the end of the fermentation.

EFFECT OF OXYGEN SUPPLY ON XANTHAN GUM FERMENTATION

The supply of oxygen in non-Newtonian fermentation is limited due to viscosity thus affecting the conversion of substrate to both cell mass and metabolites. A study of oxygen supply to the culture medium, by partially replacing air with oxygen was made in 10 liter stirred fermenter. Effect of oxygenation started at 10 hrs and 25 hrs after inoculation was investigated (Fig.40 & 41). The rate of aeration was kept at 1 l/l minute and the ratio of air to oxygen was 5:1. The partial replacement of air by oxygen started at 0 hr and stimulated the rates of various fermentation parameters such as cell synthesis, conversion of sugar to biopolymer and viscosity of the fermented broth. The biopolyer formation was maximum in 30 hrs. after inoculation as compared with 48 hrs reported earlier using only air (Fig.37). Further increase in the incubation period slightly enhanced exopolysaccharide synthesis, i.e. 19.0 g/l in 40 hrs after inoculation. The percentage conversion of carbohydrate to biopolymer also increased as compared with the aerated control culture. The rate of pH change during oxygenation from 7.0 to 6.0 was faster as compared with the control cultures grown by supplying air.

Xanthan gum production was also improved by partial replacement of air with oxygen 25 hrs. after the transfer of seed culture to the stirred fermenter (Fig.43). The amount of polysaccharide produced was 4.5 g/l 25 hrs after inoculation by the bacterium and supply of oxygen afterwards greatly improved the conversion of sugar to both xanthan gum and cell mass formation. The production

of biopolymer was maximum in 40 hrs and further increase in incubation period did not increase sugar utilization and biopolymer synthesis.

Further effect of oxygen supply by adding hydrogen peroxide (40% solution) to the optimum level of hydrogen peroxide was determined by adding it, 24 hrs after inoculation, to shake flasks or by feeding at the rate of 12 ml/hr in 10 l stirred culture vessel. In general, addition of H_2O_2 enhanced the rate of gum formation by the bacterium and conversion of sugar to biopolymer was also increased as compared with the control cultures. Xanthan gum fermentation reached maximum in 40 hrs after inoculation by adding H_2O_2 instead of 48 hrs in the control cultures. The sugar consumption in control cultures was 27.0 g/l as compared with 29.0 g/l in culture containing H_2O_2 but the amount of gum produced was 18.0 to 19.8 g/l respectively. Thus percentage conversion of sugar to polysaccharide was greater in the presence of H_2O_2. The cell formation was also increased by feeding hydrogen peroxide.

XANTHAN GUM FERMENTATION BY REPEATED-BATCH PROCESS

After batch-wise study in stirred culture vessel, xanthan gum fermentation was also carried out by repeated-batch process in 10 liter fermenter (Fig.44-46). The fermented broth (80%) was replaced 50 hrs after inoculation by fresh sterilized medium. Three cycles of 50 hrs each, were run continuously, for 150 hrs. Sucrose-salt medium, with and without "Proflo extract" and simple cane juice were evaluated for gum formation.

Calcium carbonate, however, was added to all media to control the pH near neutrality.

The rate of xanthan gum fermentation in cane juice or synthetic sucrose-salt medium was greatly enhanced in stirred fermenter (10 liter capacity) as compared in shake flasks described earlier. Maximum biopolymer synthesis took 72 hrs in shake flasks and 30 hrs in stirred fermenter after inoculation. The conversion of sugar to xanthan gum was almost doubled in the fermenter (20 g/l) than that obtained in shake flask (7 g/l). The viscosity of the fermented broth in the culture vessel was also greatly increased. The reasons for earlier completion of exocellular-polysaccharide fermentation and improved conversion of sugar to gum in the stirred fermenter may be better supply of oxygen due to agitation and aeration of culture medium.

The parameters studied in repeated-batch system were, gum formation, cell mass synthesis, residual sugar, viscosity of the fermented broth and pH during fermentation in all cycles. The efficiency of the bacterium was little affected in three cycles during repeated-batch process. In cane juice medium, biopolymer formation was 14 g/l in 40 hrs after inoculation. The amount of xanthan produced in synthetic sucrose-salt medium, however, was greater (20 g/l) than in cane juice medium indicating that conversion of sugar to gum formation was greater in synthetic sucrose-salt medium.

It follows that sugar was not completely metabolized by the organism in nutrionally deficient synthetic sucrose-salt medium. The addition of "Proflo extract" in synthetic sucrose-salt medium slightly enhanced the rate of extracellular-polysaccharide synthesis in the stirred fermenters.

KINETIC STUDIES ON XANTHAN GUM FERMENTATION

The kinetic parameters such as the growth rate (Rx), metabolic quotient or specific rate of substrate consumption (q) and rate of polysaccharide formation (Rp) were calculated from experimental data according to the formulae described by Tsao, (1979).

The rate of production of biomass (Rx) is given by the equation:

$$dX = Rx.dt$$

or

$$Rx = \frac{dX}{dt} \quad \ldots\ldots\ldots\ldots\ldots \text{(i)}$$

In written equation (i) microbial cell lysis and other processes that destroy the biomass are assumed to be absent, i.e. the rate of consumption of biomass is zero.

Growth yield is defined by the quotient:

$$\frac{\Delta X}{\Delta S} \quad Yx/S \quad \ldots\ldots\ldots\ldots\ldots \text{(ii)}$$

Where X is the increase in biomass, and ΔS, is the consumption of substrate.

As ΔS approaches zero

$$Y_{x/S} = \frac{dX}{dS} = \frac{dx/dt}{ds/dt} = \frac{Rx}{Rs} \quad \ldots\ldots \text{(iii)}$$

Where Rs is the rate of substrate consumption. Instead of total biomass, the yield can also be defined as specific product. If p is a specific product, then

$$Y_{P/S} = \frac{dP}{dS} = \frac{dP/dt}{dS/dt} = \frac{Rp}{Rs} \quad \ldots\ldots \text{(iv)}$$

Where Rp is the rate of P formation.

Metabolic product formation can be similarly related to nutrient consumption. Furthermore, product formation can not occur without the presence of cells.

The Kinetic relationship between growth and product formation depends on the role of the product in cell metabolism. A common pattern is that where growth initially occurs without product formation but after some period, the product begins to appear while growth continues.

The specific rate of product formation may be defined as

$$Rp = \frac{1}{X} \frac{dp}{dt} \quad \ldots\ldots\ldots\ldots \quad (v)$$

The specific consumption rate of substrate (q) is also known as metabolic quotient of the substrate.

$$q = \left(\frac{1}{X}\right)\left(\frac{dS}{dt}\right) \quad \ldots\ldots\ldots \quad (vi)$$

Metabolic quotient, like the yield factor, is a useful factor to evaluate the fermentation process.

A. BATCH FERMENTATION

1) Culture Vessels: The Kinetics of xanthan gum, (Rx) of bacterium, metabolic quotient (q) and rate of product (polysaccharide) formation (Rp) in 10, 50 and 100 liter capacity glass-stainless steel fermenters of different agitation system were compared. Of all the culture vessels used, the results were quite encouraging in 50 liter fermenter. It follows that the designing of equipment used has great influence on the rates of fermentation process. The fermenter was provided with multiple impleller system, thus both agitation and aeration were better than rest of the culture vessels.

Fig.47 shows the comparison of growth rate (Rx) in different fermenters. The growth rate of Xanthomonas cucurbitae- PCSIR-52 in 50 liter fermenter was significantly increased (1.0 g/l h^{-1}) and further decreased in the order 0.18 , 0.03 g/l h^{-1} in 10 and 100 liter fermenter respectively. The metabolic quotient or rate of specific substrate (sugar) consumption(q) in biopolymer fermentation process was also affected the culture vessels by their agitation system. Thus, the values of 'q' were decreased in the order 2.25 , 1.0, 0.45 g/l h^{-1} in 50, 100 and 10 liter fermenter respectively (Fig.48). The rate of xanthan biopolymer production (Rp) showed great variations in different culture vessels (Fig.49). The values of Rp, however, were increased in the order 0.3 , 1.0 , 1.12 g/l h^{-1} in 10, 100 and 50 liter capacity fermenter respectively.

2. Effect of Oxygenation: The effect of oxygenation on growth rate (Rx), metabolic quotient (q) and rate of product formation (Rp) were also studied in 10 liter glass stainless steel fermenter. The feeding of H_2O_2 greatly enhanced the rate of bacterial growth (0.6 g/l h^{-1}). The maximum value of Rx was 0.37 g/l h^{-1} by partially replacing air with oxygen started 25 hrs after inoculation and it was decreased to 0.27 g/l h^{-1} when oxygen sparging started 0 hr after inoculation (Fig.50).

The metabolic quotient was affected by feeding H_2O_2 or supplying oxygen gas and air to the culture medium (Fig.51). The values of 'q' was maximum (0.48 g/l h^{-1}) when supply of oxygen-air mixture started 0 hr after inoculation, and decreased from 0.15 to 0.11 g/l h^{-1} by sparging air with oxygen gas or feeding hydrogen peroxide started 25 hrs after inoculation.

Oxygenation by the mixture of air and oxygen gas enhanced the rate of exocellular-polysaccharide production (0.20 g/l h^{-1}), 25 hrs after inoculation. The values of Rp, however, decreased in the order 0.17, 0.10 g/l h^{-1} by partially replacing air with oxygen gas or feeding H_2O_2 respectively (Fig.52).

B. REPEATED-BATCH PROCESS.

Comparison of growth rate (Rx) of the bacterium was studied in 10 liter stirred fermenter by repeated-batch process in sucrose-salt, with and without "Proflo extract" or cane juice media. In general

growth rate showed great variation in different growth cycles. The growth rate in sucrose-salt medium was 0.18 g/l h^{-1} in first cycle and it increased to 0.5 g/l h^{-1} in second and third cycle (Fig.53). This higher growth rate might have been caused by heavy inoculum. The addition of "Proflo extract" almost doubled the growth rate only in the first cycle (0.475 g/l h^{-1}), with no significant improvement (0.4 g/l h^{-1}) in second and third (0.64 g/l h^{-1})cycle (Fig.54). The values of Rx in cane juice medium was 0.54 g/l h^{-1}, in first cycle and varied from 0.4 and 0.3 g/l h^{-1} in second and third cycle respectively (Fig.55). Metabolic quotient (q) is an essential parameter to asses, the biochemical activities of bacterium in a fermentation process. Xanthomonas cucurbitae PCSIR-52 was sensitive to culture media. Fig.56 shows metabolic quotient or specific substrate composition rate in three cyclic repeated-batch process, using sucrose-salt medium. The rate of sugar consumption was decreased in the order 0.4 , 0.35 , 0.15 g/l h^{-1}, in first, second and third cycle of the process. The addition of "Proflo extract" slightly enhanced (0.5 g/l h^{-1}), the metabolic quotient in the first cycle of the process. The values of 'q' (0.28 g/l h^{-1}) remained almost the same in the second and third cycles. The bacterial activities in the presence of "Proflo extract" were faster in the first cycle as compared to second or third cycle (Fig.57). It may be due to the metabolites produced in the medium. Cane juice is a rich source of carbohydrates, proteins, macro and

micro-nutrients and other growth supporting substances. Metabolic quotient (q) in cane juice medium was little affected in three cycles, indicated by 'q' values of 0.3, 0.22 and 0.25 g/l h^{-1} in first, second and third cycle respectively (Fig.58).

Fig.59 indicates the rate of xanthan biopolymer production (Rp) by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in repeated-batch process. The values of Rp in first, second and third cycles of the process were 0.168, 0.132 and 0.121 g/l h^{-1} respectively. The addition of "Proflo extract" in sucrose-salt medium, however, greatly increased the rate of biopolymer formation (0.22-0.24 g/l h^{-1}) in all cycles (Fig.60). Xanthan gum production was also studied in repeated-batch process using cane juice (Fig.61). The rate of gum production (Rp) in simple cane juice medium was 0.149 g/l h^{-1} in the first cycle and it was slightly affected in other two cycles.

COMPARISON OF XANTHAN GUM BY INFRARED SPECTROSCOPY

Infrared spectroscopy was used to identify the functional groups in the locally produced gum by Xanthomonas cucurbitae PCSIR-52 and to compare it with xanthan gum (Kelco Co. U.S.A.) recorded in Nujol. A Perkin-Elmer (Model-1320) Infrared Spectrophotometer was used for this purpose.

Fig.62 shows the spectra of xanthan gum produced by Xanthomonas cucurbitae PCSIR-52 and the imported xanthan gum (Kelco Co. U.S.A.), which had been produced by X. campestris NRRL B-1459. The concentration of both the samples in Nujol is different.

Both the spectra are nearly overlaping. The obsorption band at 3600-3000 cm^{-1} appears as a broad shoulder and is attributed to the free and bonded O-H groups. The shape of this band indicates that nearly all the O-H groups are present in the bonded form. The second characteristic peak of O-H group which mostly indicates the configuration of this group appears between 1050 and 1075 cm^{-1}. As a large number of O-H groups are present in the gums, it was not possible to determine the configuration of these groups. However, these peaks are so identical in both the samples that about some type of O-H groups are present in both the gums.

The C-H stretching bands of the saturated compounds appear between 3000 and 2800 cm^{-1}. Both

the spectra also exhibit very strong C-H stretching. There is no indication of unsaturation in both the spectra. A small additional peak at 1600 cm^{-1} which appears in spectrum of the gum produced by Xanthomonas cucurbitae PCSIR-52 may be due to the presence of some aromatic compound in the gum. This may be an impurity in the sample. However, the presence of aromatic compounds is easily detected in the ultraviolet spectrum. The CH_2 and CH_3 stretching appear in both the gums at 1460 and 1365 cm^{-1} respectively. The peak at 1365 cm^{-1} shows the branching of carbon chain. In these two spectra, both the peaks are of the same type and show that least branching occurs in both the gums. A medium sized peak at 720 cm^{-1} is present in both the spectra. This peak is attributed to $(CH_2)_n$ where 'n' is greater than 4. As the intensity of this peak in both the spectra is the same, no straight chain compounds are present in either of the gums. This peak in both the spectra is due to Nujol.

A shoulder exists at 1730 cm^{-1} in both the spectra, however, the intensity of the peak in the locally produced gum is slightly higher than in the imported gum. This region of the infrared spectra contains peaks due to carbonyl functions. These peaks are usually of high intensity. Such a small peak in this region may be due to some carbonyl compound which gets formed due to aerial oxidation.

From the foregoing discussion it is evident that both the gums are nearly the same in infrared Spectroscopy.

EVALUATION OF XANTHAN GUM

The fermented broth containing xanthan gum was evaluated in non-food industrial processes after giving heat shock at 90°C for 5 minutes in order to kill the non-spore former bacterium, Xanthomonas cucurbitae. KCl (1.0%) was added to the fermented broth prior to heat shock, and after cooling to room temperature, formaldehyde was added at the rate of 0.2% v/v. The gum solution was stored in plastic containers and viscosity of the fermented broth was determined after different intervals (Fig.). The viscosity of the solution was little affected during 30 days in the plastic containers and further increase in the storage resulted in decreasing the viscosity of the broth. The viscosity of gum solution, 60 days after storing, was reduced by 28%. The stability of the fermented broth for one month without significant loss in the viscosity was encouraging for its use in the crude form in various non-food industrial processes.

a) Ceramic Glazing: The fermented broth was used after its dilution to the desired level in the ceramic glazing operations in callaboration with "Glass and Ceramic" Division of PCSIR Laboratories, Lahore. The glazing material was suspended in gum solution and was applied to ceramic wares. The results were highly encouraging as compared with the conventional methods followed in Pakistan using starch suspension.

b) Textile Printing: The gum solution in the crude form was also evaluated in textile printing in Kohinoor Textile Mills, Faisalabad. This study still needs

further investigation since the prints using crude xanthan gum was not sharp as in case of sodium alginate or guar gum. The isolation and purification of the gums from the broth is being followed for textile printing.

c) Oil Drilling: The gum solution has not been evaluated in the oil drilling operation since its production in the laboratory fermenter is not sufficient for the process.

D I S C U S S I O N

The present study describes the synthesis of xanthan gum, a microbial extracellular-polysaccharide, by locally isolated culture of Xanthomonas cucurbitae PCSIR-52 from infected cucurbita plants. Four locally isolated Xanthomonas sp. and Xanthomonas campestris NRRL B-1459 were evaluated for gum formation. Of all these strains, however, Xanthomonas cucurbitae PCSIR-52 produced maximum biopolymer in synthetic sucrose-salt medium in shake flasks. Nutritional requirements of the selected culture of Xanthomonas cucurbitae PCSIR-52 were further studied for maximum gum formation in shake flasks before scale up production in stirred fermenters of both laboratory and semi-pilot scale (10, 50 and 100 liter).

The metabolic system of Xanthomonas sp. was very sensitive to physio-chemical conditions such as temperature, pH, size and age of inoculum, carbohydrate and nitrogen sources. The optimum temperature of the selected culture was about 29°C instead of 26°C for Xanthomonas compestris NRRL B-1459 as reported in the literature (Leach et al. 1957). The sugars such as sucrose, starch hydrolyzates, i.e. glucose, maltose and hydrol, xylose, mannose, galactose, beet melasses or lactose (or cheese whey) were evaluated for xanthan gum production (Fig. 4&5). Of all these sugars, however, sucrose gave maximum production of polysaccharide. This is in accordance with the findings reported earlier by Leach et al. (1957); Wallis et al. (1973); Berhrens et al. (1973); and Souw and Demain (1979) using Xanthomonas campestris NRRL B-1459.

The concentration of sugar in the basal medium was very critical for xanthan gum synthesis. The increase in viscosity due to the polysaccharide formation affected oxygen supply to the culture hence sugar utilization by the bacterium. The optimum level of sucrose was found to be 3.0% and further increase in its concentration did not show any increase in the yield of biopolymer. The pH of the fermented broth was lowered because of acidogenic nature of xanthan gum and its by-products which affected the conversion of sugar to polysaccharide. The maintenance of pH near neutrality, therefore, was essential for maximum gum synthesis. Thus, addition of $CaCO_3$ was made to control the pH near neutrality during fermentation.

Nitrogen sources, both inorganic and organic including plant extracts were evaluated for the synthesis of biopolymer in shake flasks. Of all the inorganic nitrogen sources, di-ammonium hydrogen phosphate, ammonium phosphate or sodium nitrate gave maximum production of biopolymer. Souw and Demain (1979), had also shown maximum gum formation by Xanthomonas campestris NRRL B-1459 in the presence of these inorganic salts in the fermentation medium. The optimum concentration of nitrogen was about 0.32 g/l. Further effect of varying concentrations of these nitroben sources was studied on gum formation by Xanthomonas cucurbitae PCSIR-52. The optimum level of nitrogen was about 0.32 to 0.42 g/l. The addition of urea or thiourea to the basal medium also showed an increase in the synthesis of xanthan gum (Fig.13 & 14). The conversion of sugar to biopolymer in the presence

of these nitrogen sources (at optimum level) was about 90.0 and 87.0% respectively. It is interesting to note that both ammonium sulphate and thiourea are sulphur containing nitrogen compounds but sugar consumption hence gum formation were greater in the presence of thiourea than that obtained by adding ammonium sulphate. It may be due to the lowering of physiological pH by the release of sulphate ions after the use of ammonium ions by the bacterium. The addition of the extract of cabbage leaves, a source of certain growth hormones, amino acids and vitamins, slightly increased gum formation.

Effect of industrial by-product such as corn-steep liquor, pencillin waste mycelium, cotton-seed meal as (locally produced or imported such as Proflo or Pharmamedia) sources of nitrogen was also studied on the gum production. The biopolymer synthesis was greater in the presence of corn-steep liquor or cotton-seed meal (Fig.16&18) as compared with inorganic nitrogen sources, cabbage leaves extract or pencillin waste mycelium. The production of extracellular-pslysaccharide was not significantly increased by the addition of locally available cotton-seed meal powder as compared with Proflo or Pharmamedia in the fermentation medium. The addition of the extract of premium quality cotton-seed meal (Proflo), however, greatly increased the bioconversion of sugar into biopolymer by Xanthomonas cucurbitae PCSIR-52. The soluble amino acids or minerals in the extract may be responsible for the enhancement of gum formation. The

Clarke and Meadow, 1959; Souw and Demain, 1979 and 1980). Both these organims find application in the manufacture of biopolymers of clinical and commercial importance. Similarly, Scharamm et al. (1957) observed that citrate, acetate, succinate, pyruvate and alpha-ketglutarate were oxidized to CO_2 without cellulose production by washed cells of Acetobacter xylinum. Dudman (1959) and Hamilton and Dawes (1959) working with Acetabacter acetogenum and Psendomonas aeruginosa respectively, found that citrate and other organic acids such as succinate, malate and acetate, are utilized in preference to glucose as carbon source in a medium containing both glucose and organic acids. Effect of various carboxylic acids or their salts such as sodium pyruvate, potassium citrate, sodium acetate, D-malic acid, succinic acid, oxalic acid or Tartaric acid, was studied on the synthesis of biopolymer by Xanthomonas cucurbitae PCSIR-52. Of all these carboxylic acid, the production of xanthan gum was maximum in pyruvate in the order citrate > acetate > malate > succinate > oxalate > tartarate. Similar work has also been reported by Souw and Demain (1979 and 1980). The actual mechanism of stimulation by carboxylic acids on the bioconversion of sugar into polysaccharide is obscure. It may be due to the (i) chelation of metal ions present in the basal medium, (ii) acting as a source of carbon in preference to sugar, or (iii) provide pH pattern favourable to the synthesis of biopolymer.

Scale up studies of xanthan gum fermentation in stirred fermenters showed that rates of cell synthesis, sugar consumption and gum formation hence viscosity were greater than that obtained in shake flasks. The percentage conversion of sugar to biopolymer also increased

availability of oxygen by its enzymic decomposition to water and free oxygen. Geiger (1976) also studied hydrogen peroxide-catalase system to over come oxygen limitations in non-Newtonian fermentation in which Aspergillus oryzae was used for the production of enzymes proteases.

The design of the culture vessel particularly agitation and aeration systems also play an important role in supplying oxygen to the growing cultures during fermentation. The results of gum fermentation in 50 liter stirred fermenters with multiple impeller system, were quite encouraging. That is, the amount of xanthan gum produced was 20 g/l in 30 hr after inoculation with 24 hr old seed culture. In 100 liter fermenter, agitation system was provided with single six-bladded disc-turbine impeller attached at the bottom, did not show encouraging results of biopolymer production as compared with that obtained in 10 or 50 liter culture vessels.

Xanthan gum fermentation by repeated-batch process using simple cane juice or synthetic sucrose-salt media in 10 liter fermenter was also carried out. The amount of gum produced was greater in synthetic sucrose-salt medium than that obtained in simple cane juice. Similar studies of xanthan gum production by repeated-batch has also been reported by Charles and Radjai, (1978) using acid or cheese whey. The nutritionally deficient conditions in synthetic sucrose-salt medium, such as minerals, vitamins and growth factors favoured the accumulation of xanthan gum instead of cell mass or carbon dioxide through Kreb's cycle. The efficiency of the bacterium was little affected during

repeated-batch process. The culture was continuously grown for 150 hr in three cycles. Each cycle was terminated 50 hrs after inoculation. The bacterium did not show any degeneration in three cycles of a repeated-batch process. Thus, period of lag phase or preparation of fresh seed culture for each fermentation run can be eliminated by the repeated-batch process.

The results of xanthan gum fermentation obtained in batch and repeated-batch process, were also evaluated methematically. Parameters studied were growth rate (Rx), metabolic quotient or specific substrate consumption (q) and rate of polysaccharide (product) formation (Rp).

The design of a culture vessel has great influence on the Kinetic parameters of non-Newtonian fermentation. Maximum values of Rx, q and Rp were observed in 50 liter fermenter equipped with multiple impeller (disc turbine) system. The reason may be the better agitation hence increase supply of oxygen, than rest of the culture vessels. The oxygenation of the culture medium by adding hydrogen peroxide or partial replacement of air with oxygen showed increase in growth rate (Rx), metabolic quotient (q) and rate of polysaccharide formation (Rp) in 10 liter fermenter. The addition of H_2O_2 provided molecular oxygen to increase the cell mass hence rate of biopolymer formation. The partial replacement of air with oxygen gas, 0 hr after inoculation, enhanced the rate of product formation (Rp). The values of Rp, however, was decreased in the order of 0.17 and 0.10 g/l h^{-1} by partially replacing air with oxygen and or feeding H_2O_2 respectively.

The Kinetic parameters were also studied in repeated-batch process, in three cycles of 150 hrs (each cycle was terminated 50 hrs after inoculation). The supplementation of "Proflo extract" greatly enhanced the Rx values in sucrose-salt medium. There were slight variations in Rx values using cane juice medium. The rate of sugar consumption or metabolic quotient remained about the same in each cycle of repeated-batch process in the presence of synthetic sucrose-salt or cane juice medium. The rate of product formation (Rp) was greatly enhanced by adding "Proflo extract" in synthetic sucrose salt medium in each cycle. The values of Rp were also unaffected in three cycles of repeated-batch process. It follows that the activities of Xanthomonas cucurbitae PCSIR-52 were unaffected in three cycles.

ECONOMICS OF THE PROCESS

The typical average yield of crude xanthan gum as 2.0% W/V solution based on laboratory to pilot plant fermentation studies is as follows:

a) Raw Materials:

Cane sugar	=	1000 Kg
Salts (NH_4, K, Mg & Ca)	=	6.0 Kg
Water		40,000 liters

b) Products:

Xanthan gum (2.0% crude solution)	=	600 Kg
Total Vol.	=	40,000 liters

c) Cost of Raw Materials:

Cane sugar @ Rs.10/Kg	=	10,000
Salts		600
Total cost	=	Rs.10,600
		Rs.12,000

d) Cost of the product (2% W/V crude solution) = Rs. 0.40/liter

e) Manufacturing cost = Rs. 0.60/liter

Total cost of 2% Xanthan gum solution = Rs. 1.0/liter

List of Scientists and supporting staff

1. Mr. Shahjahan Baig ..... Aug. 1980 - 1981
   (Assistant Research Officer)
2. Mr. Farooq Latif ..... May, 1983 - March 1984
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3. Miss Bushra Naheed ..... March, 1984- May, 1984
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5. Mr. Mohammad Abbas ..... May, 1983 - May, 1984

List of Publications:

1. Microbial Synthesis of Xanthan Gum by locally isolated Xanthomonas cucurbitae.
   Pak.J.Sci.Ind.Res. 25, 134-38 (1982).
2. Scale up studies of Xanthomonas cucurbitae in stirred fermenter.
   Pak.J.Sci.Ind.Res. (Accepted).
3. Microbial Synthesis of Xanthan Gum in Repeated-batch process.
   Pak.J.Sci.Ind.Res. (submitted).
4. Xanthan Gum fermentation of cane juice by Repeated-batch process. (Presented in Vth International Congress on culture collection). 1984 (ICCE-V) Bangkok-Thailand.
5. Studies on Xanthan Gum fermentation by Xanthamonas cueurbitae. Presented in "International Symposium on Biologically Active Macromolecules", held at Univ. Baluchistan, Quetta (March 30-April 1985).
6. Ph.D. thesis entitled "Biosynthesis of Xanthan Gum by locally isolated Xanthomonas species has been submitted to the University of Punjab by Mr. Shahjahan Baig.

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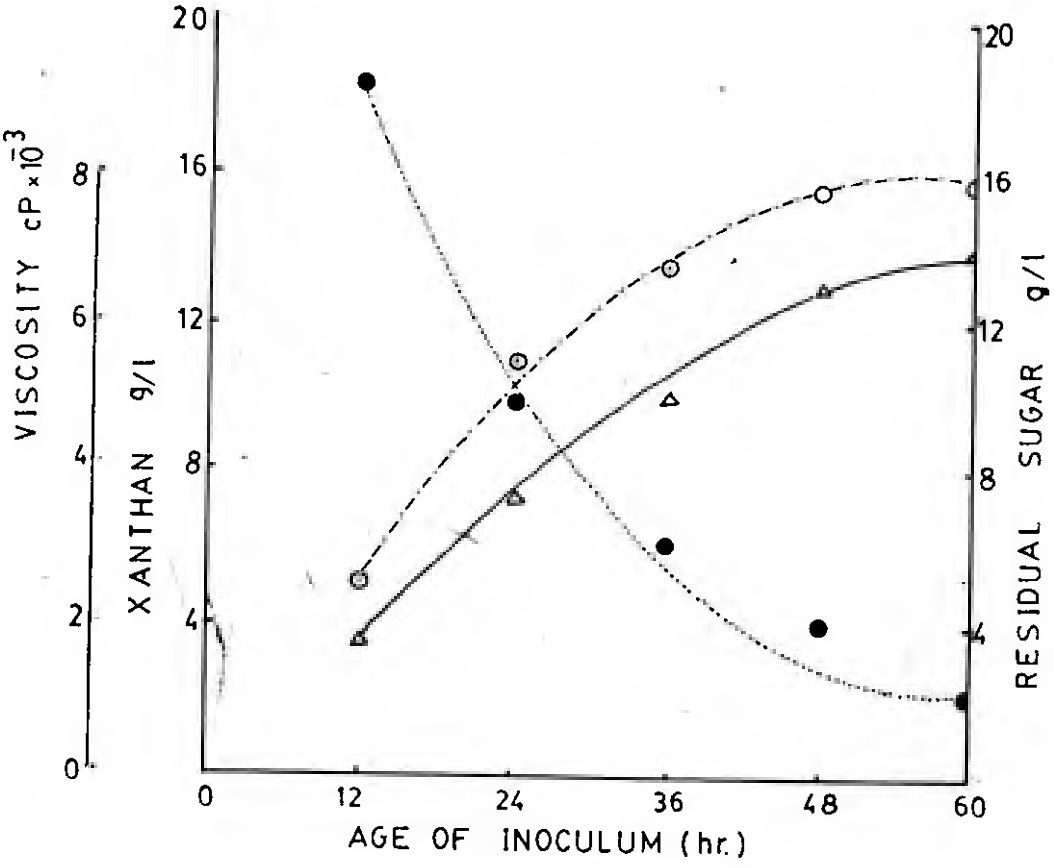


Fig. 1 - Effect of age of inoculum on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Residual sugar ●—● Xanthan △—△ Viscosity ○-○)

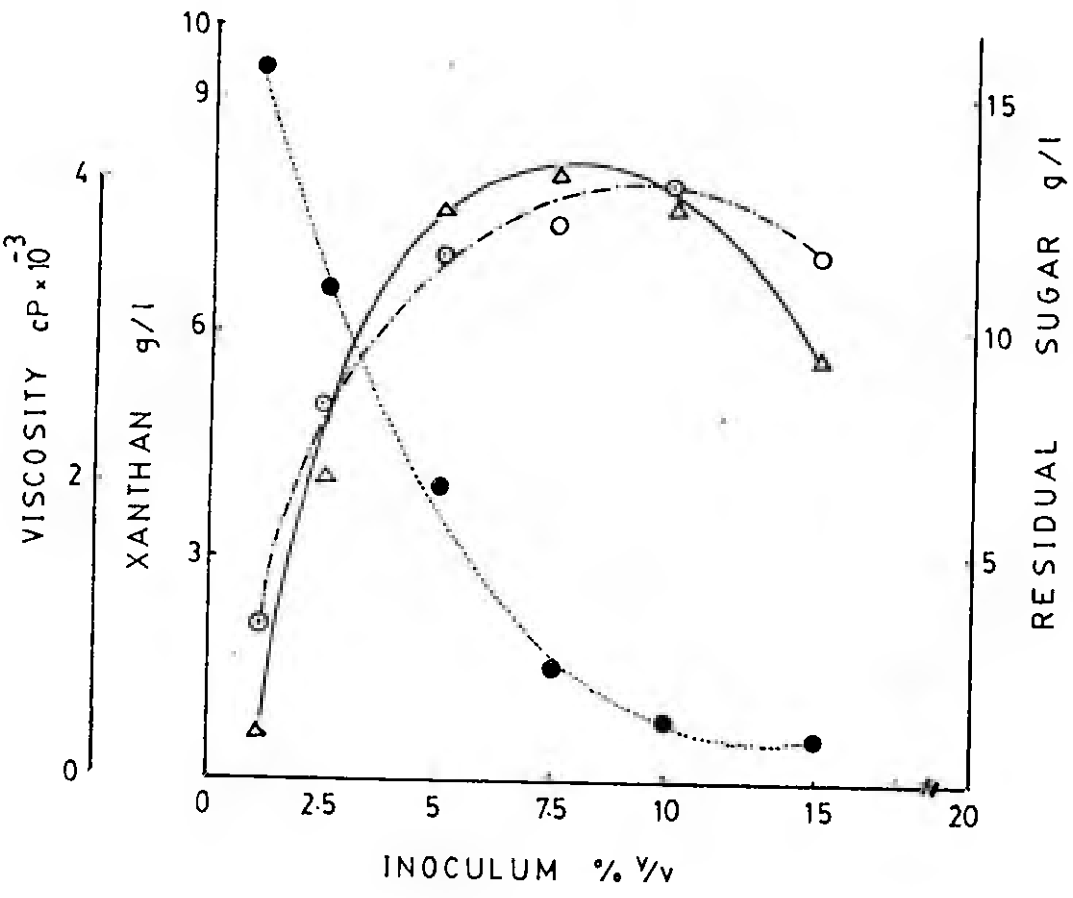


Fig.2 - Effect of size of inoculum on the production of Xanthan biopolymer by Xanthomonas cucurbitae PCSIR-52 using sucrose–salt medium in 1 liter conical shake flasks.

(Residual sugar ●---● ; Xanthan Δ—Δ ; Viscosity O--O)

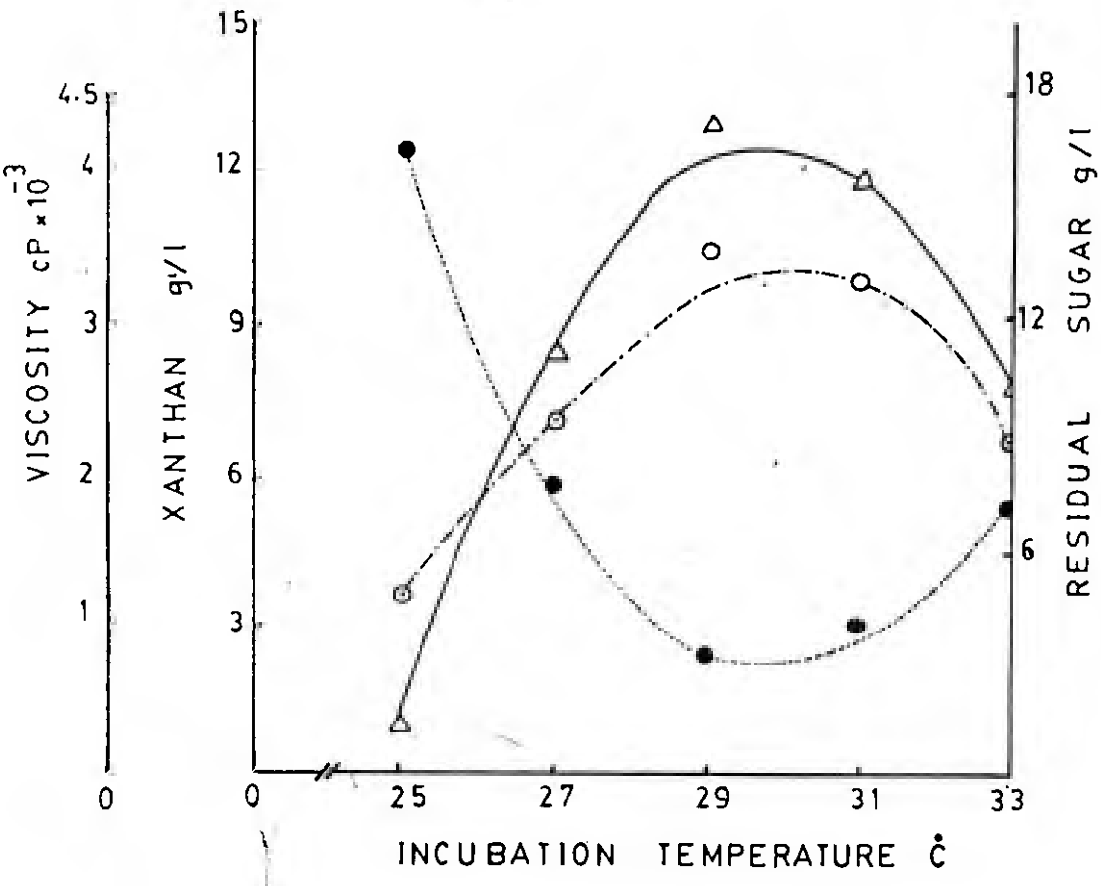


Fig. 3 - Effect of temperature on the biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose salt medium in 1 liter conical shake flasks.

(Viscosity ⊙--⊙ , Residual sugar ●--● , Xanthan Δ—Δ)

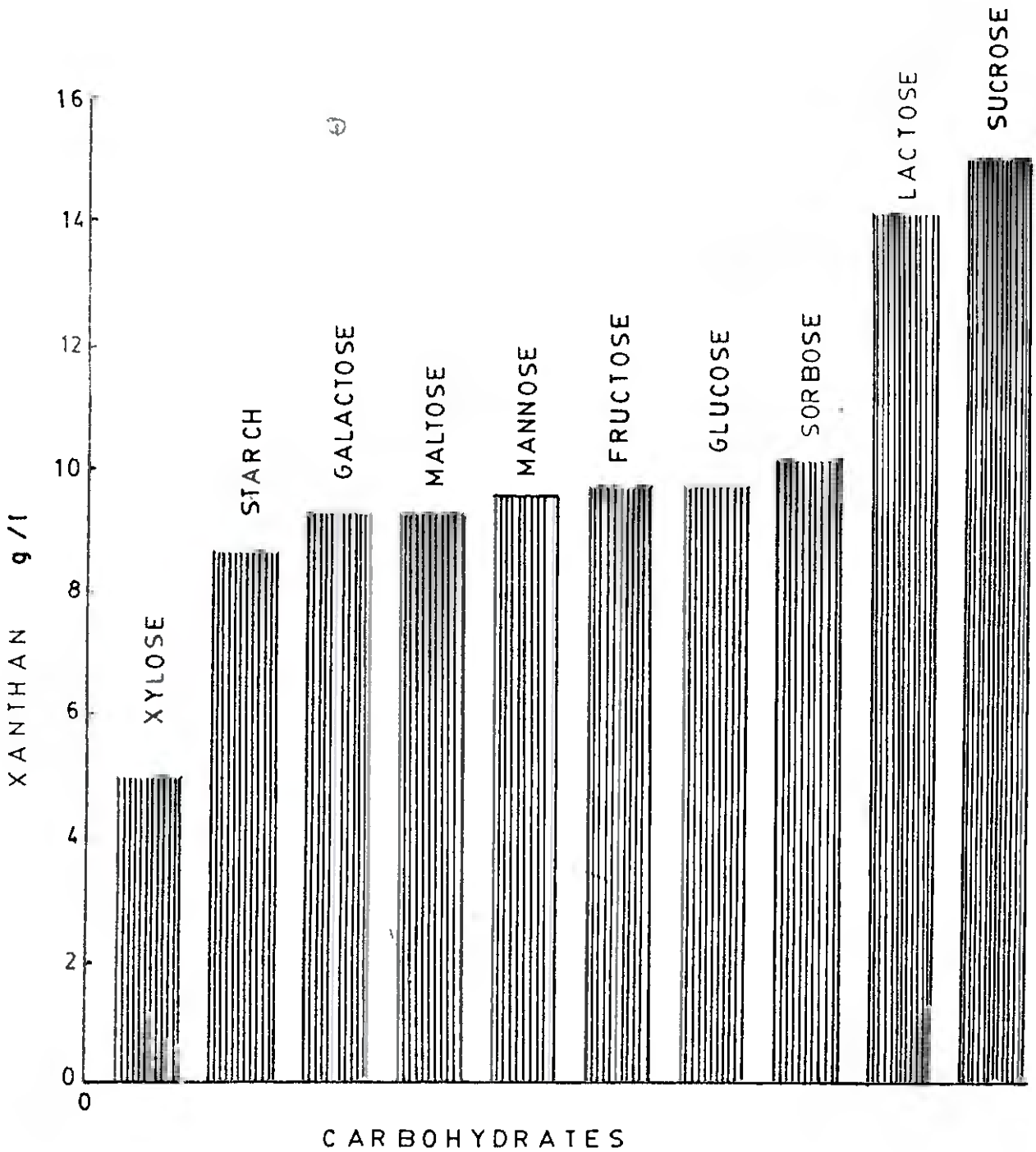


Fig. 4 - Effect of Carbohydrates on the biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in shake flasks.

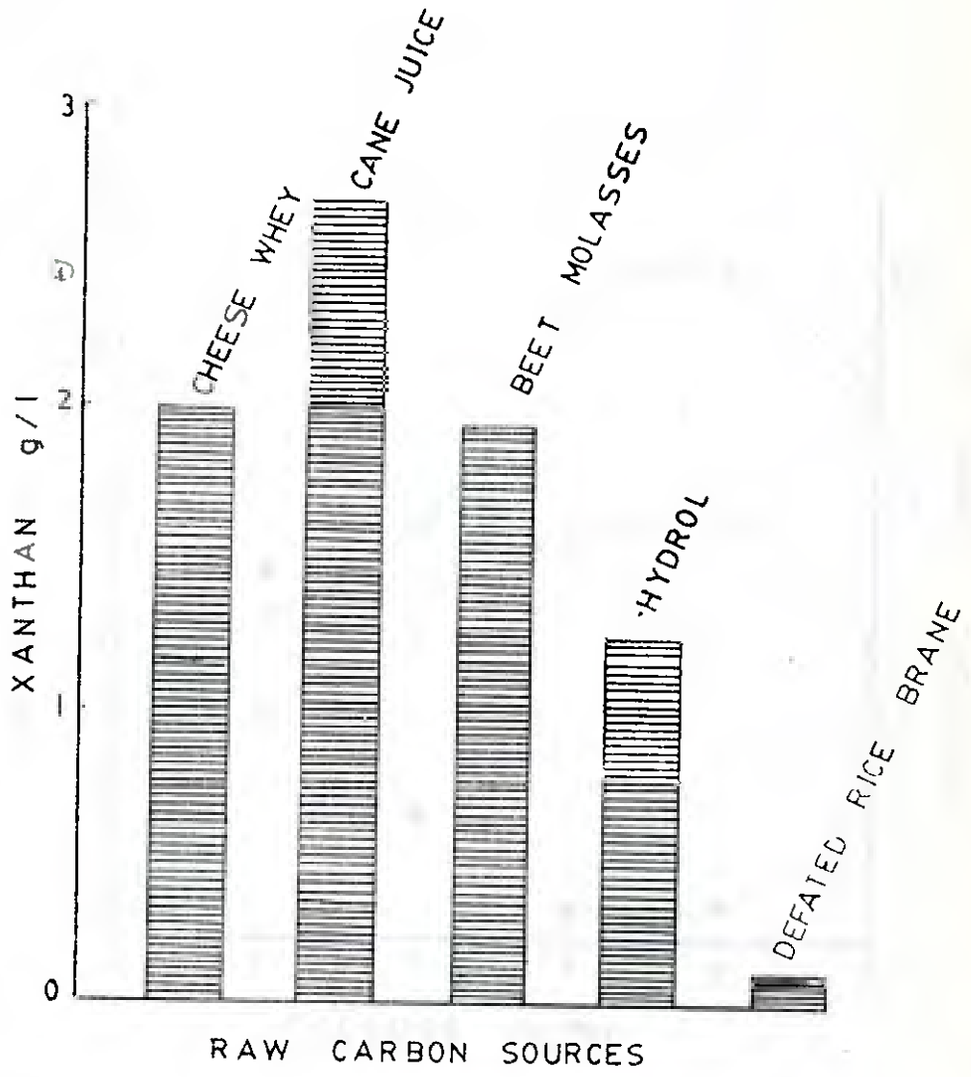


Fig. 5 - Effect of Raw Carbon sources on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in 1 liter conical shake flasks.

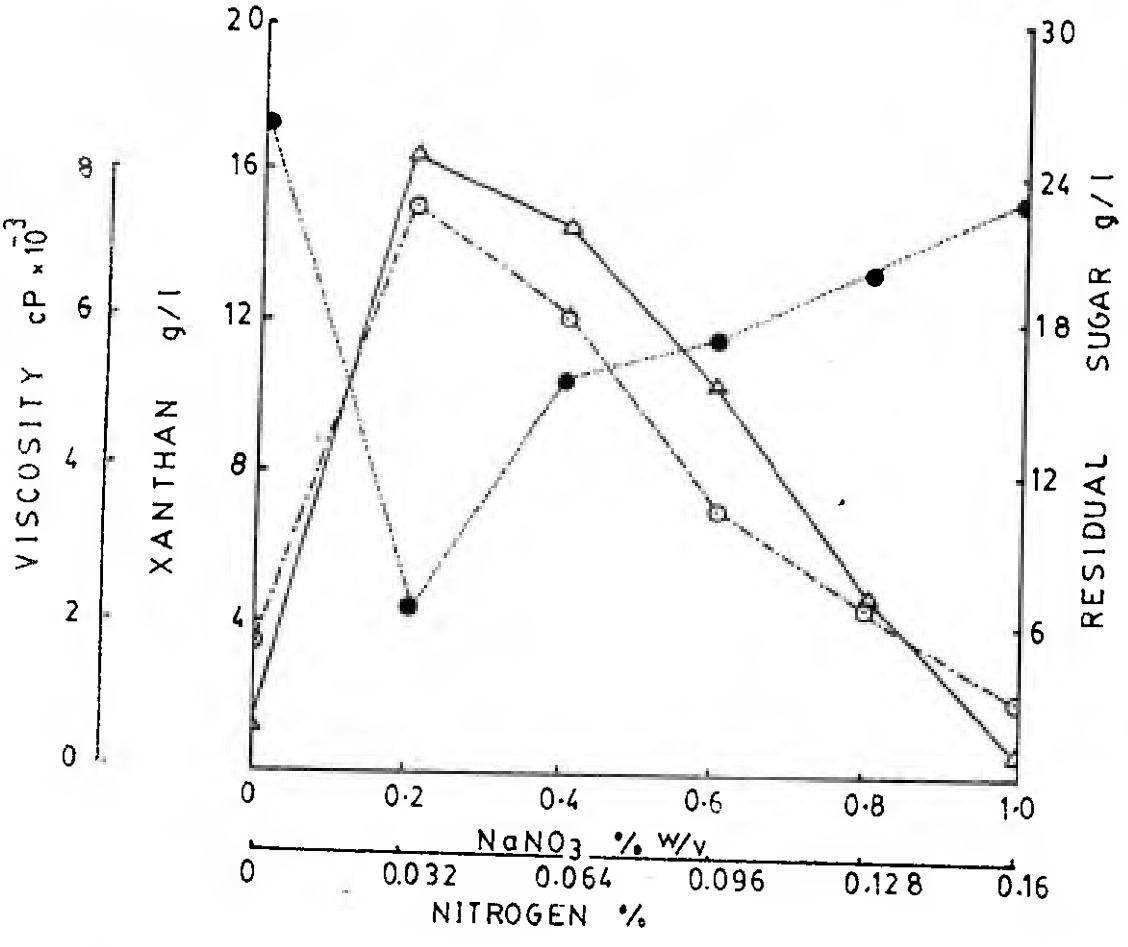


Fig.10 - Effect of Sodium nitrate on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Residual sugar ●—● , Xanthan Δ—Δ , Viscosity ⊙-⊙)

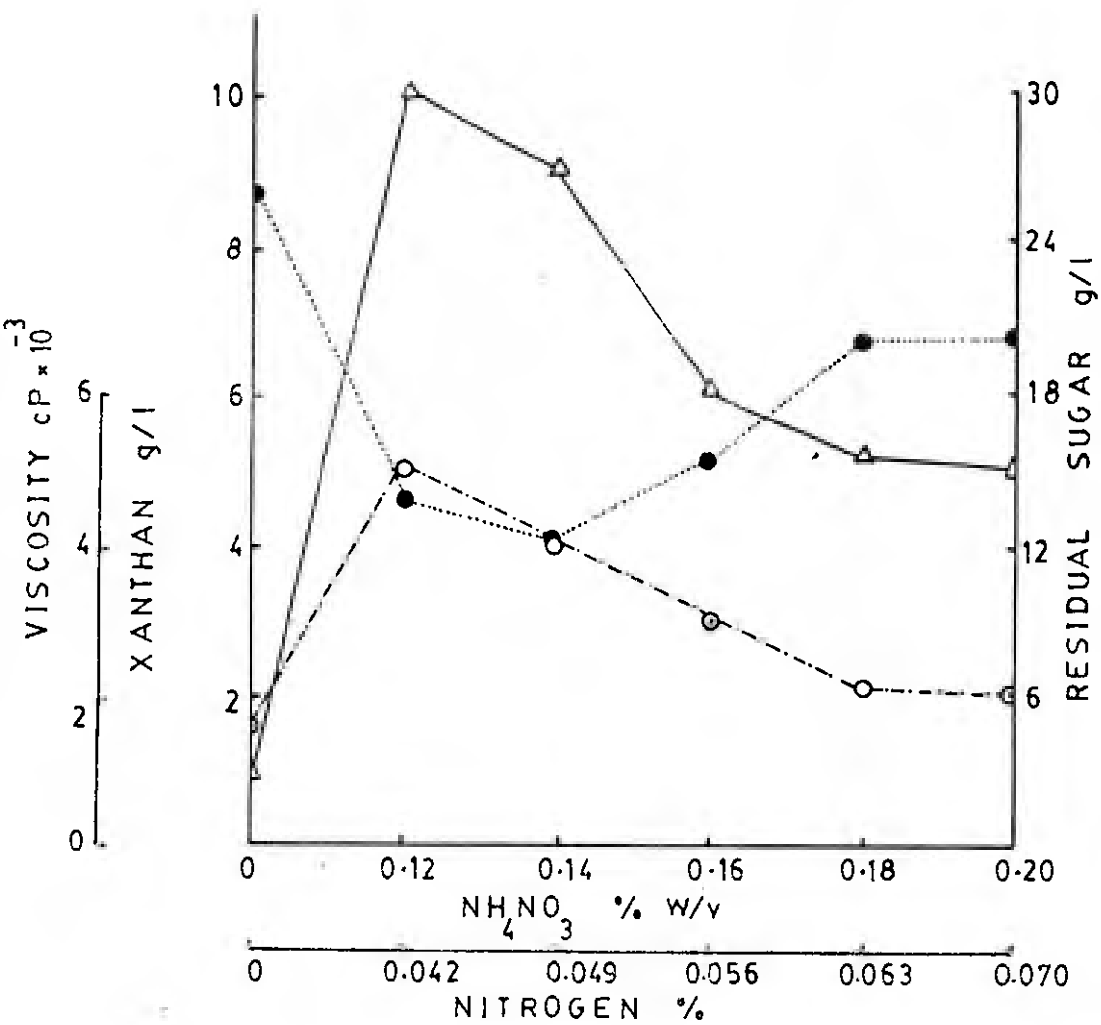


Fig. 11 - Effect of ammonium nitrate on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in sucrose-salt medium in 1 liter conical shake flasks.

(Residual sugar ●---●, Xanthan Δ—Δ, Viscosity ○-·-○)

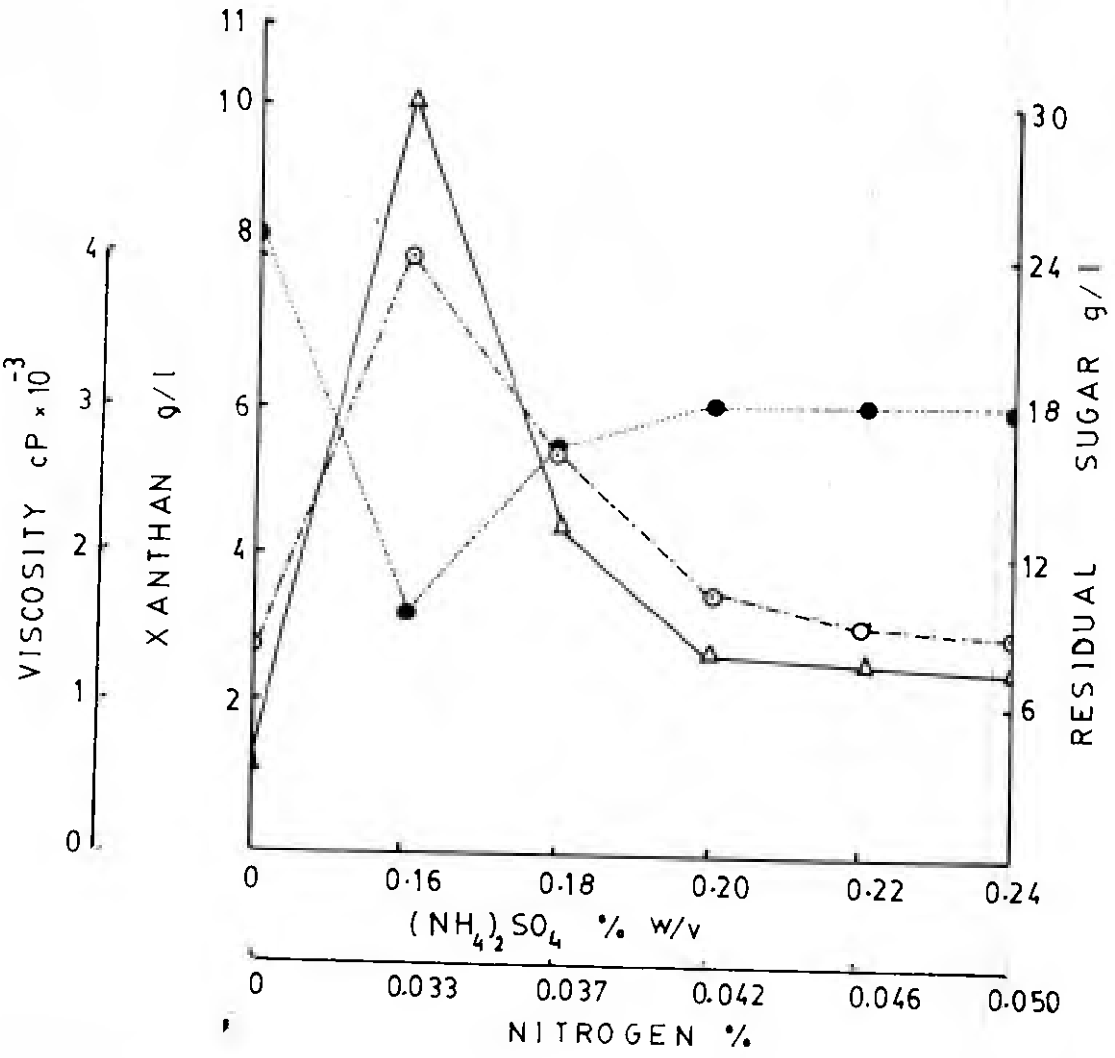


Fig. 12 - Effect of ammonium sulfate on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in sucrose-salt medium in 1 liter conical shake flasks.

(Viscosity O—O , Xanthan Δ—Δ , Residual sugar ●—●)

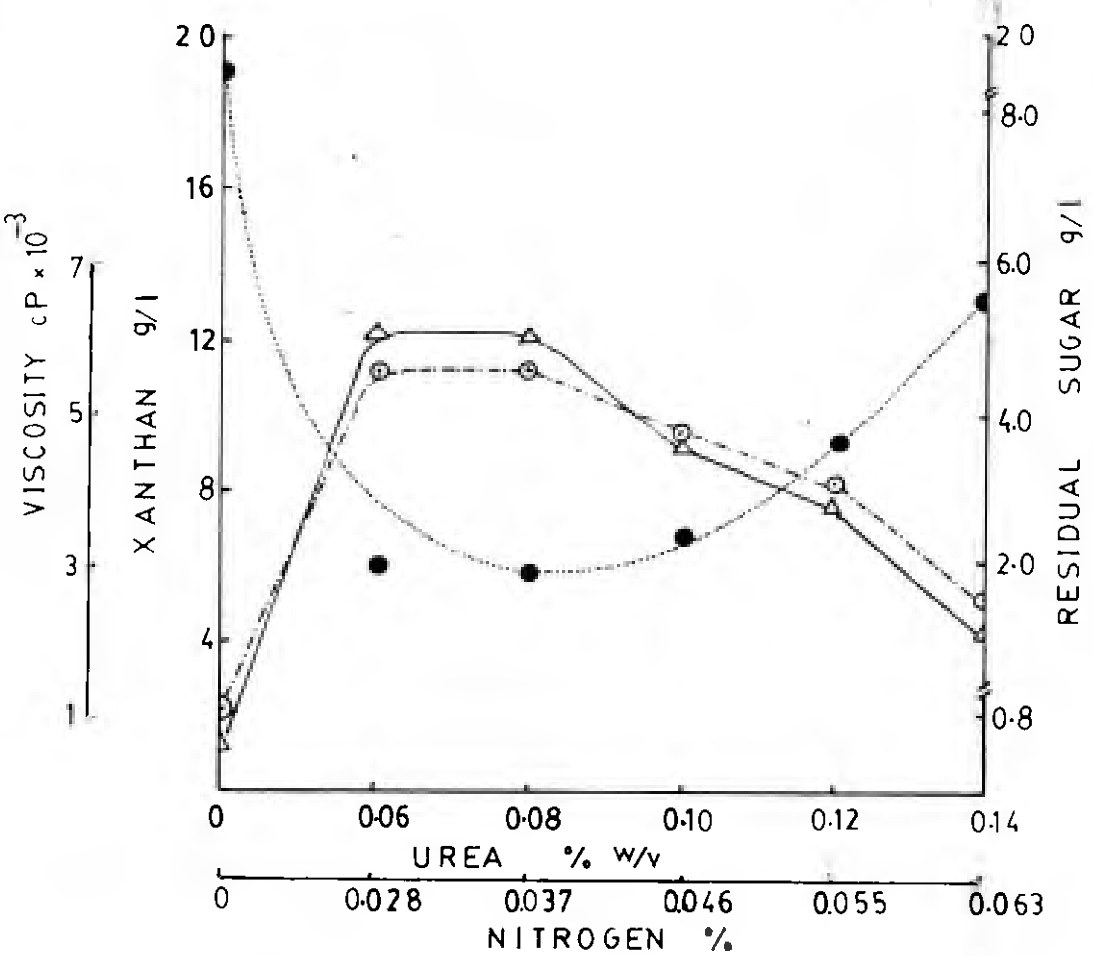


Fig. 13 - Effect of Urea on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks

(Residual sugar ●···● , Xanthan Δ—Δ , Viscosity ○-·-○)

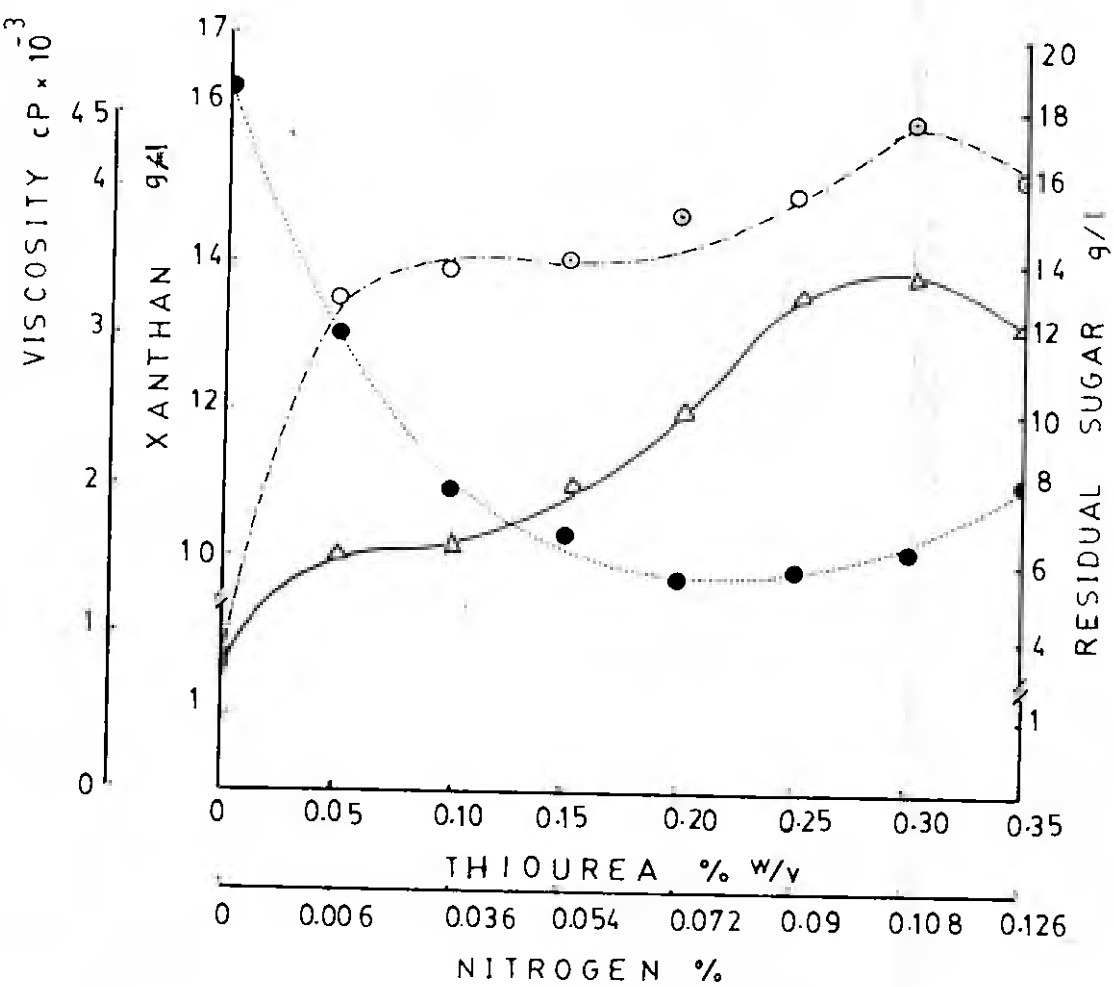


Fig.14 - Effect of Thiourea on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Residual sugar ● ● ; Xanthan △—△ ; Viscosity ⊙—⊙)

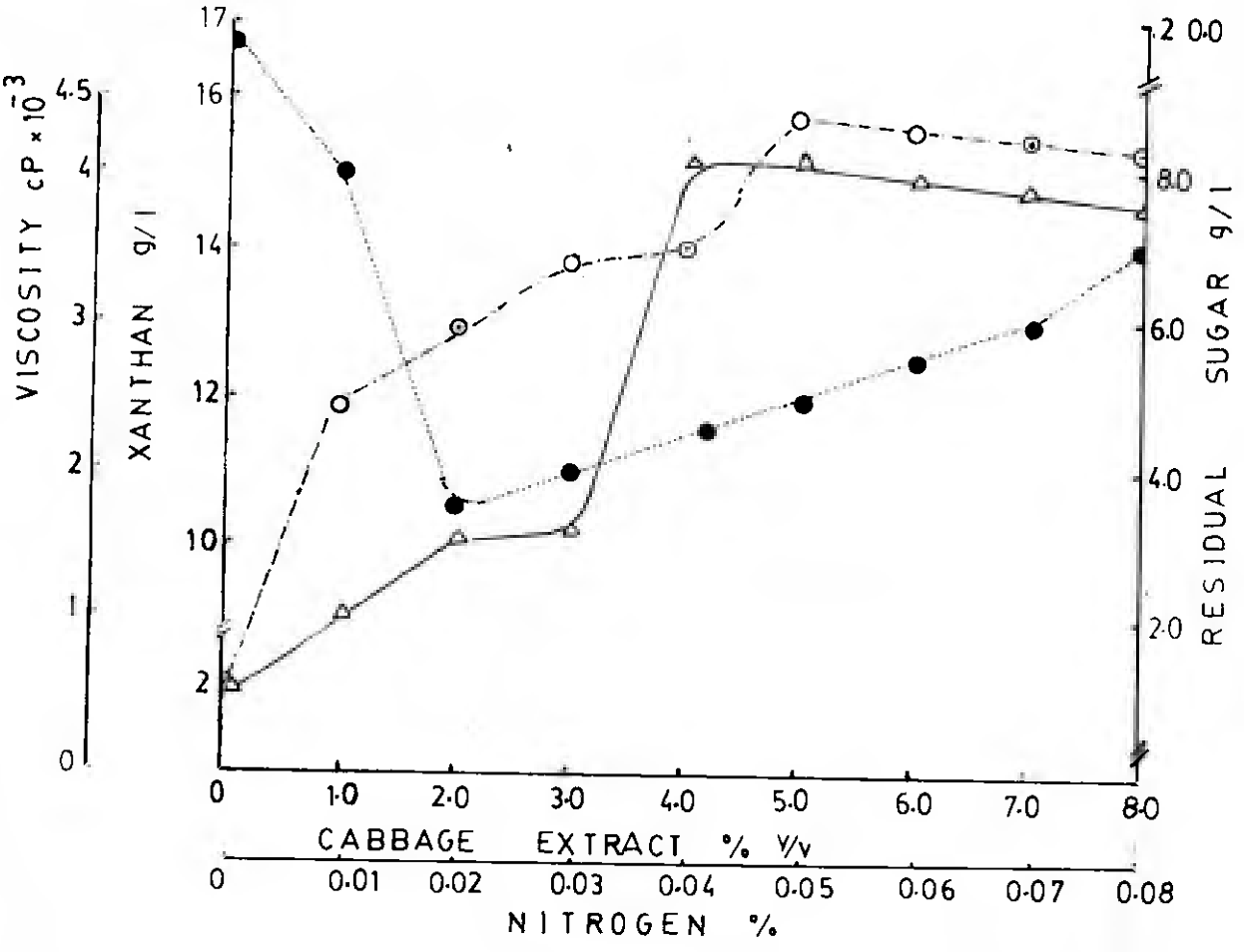
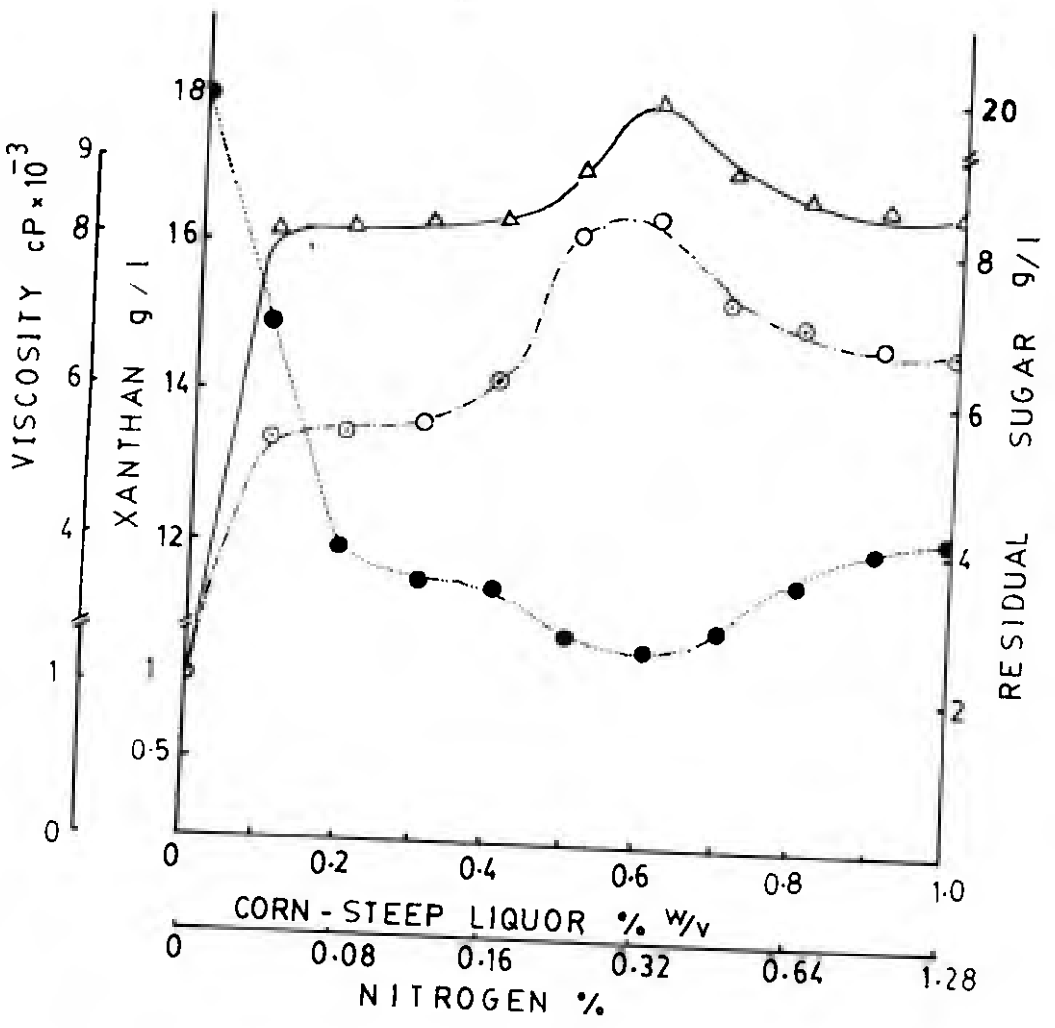


Fig.15 – Effect of Cabbage extract on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Residual sugar ● ● , Xanthan Δ Δ , Viscosity ⊙ ⊙)



Fig·16 - Effect of Corn - Steep Liquor on the biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose - salt medium in 1 liter conical shake flasks.

(Residual sugar ●—● , Xanthan △—△ , Viscosity ○—○)

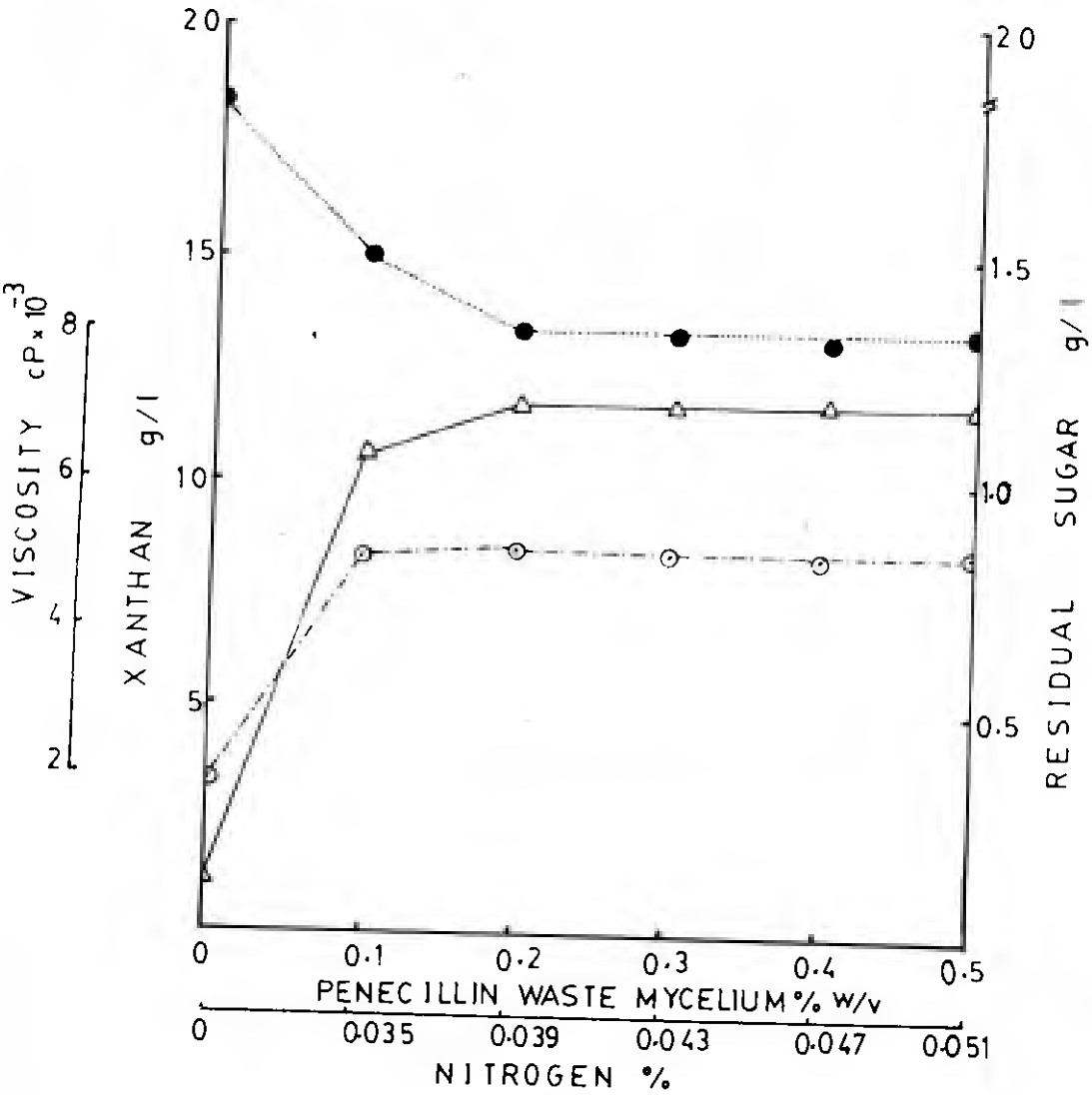


Fig.17- Effect of Penecillin waste mycelium on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Xanthan Δ—Δ ,Viscosity ⊙—⊙ ,Residual sugar ● ●)

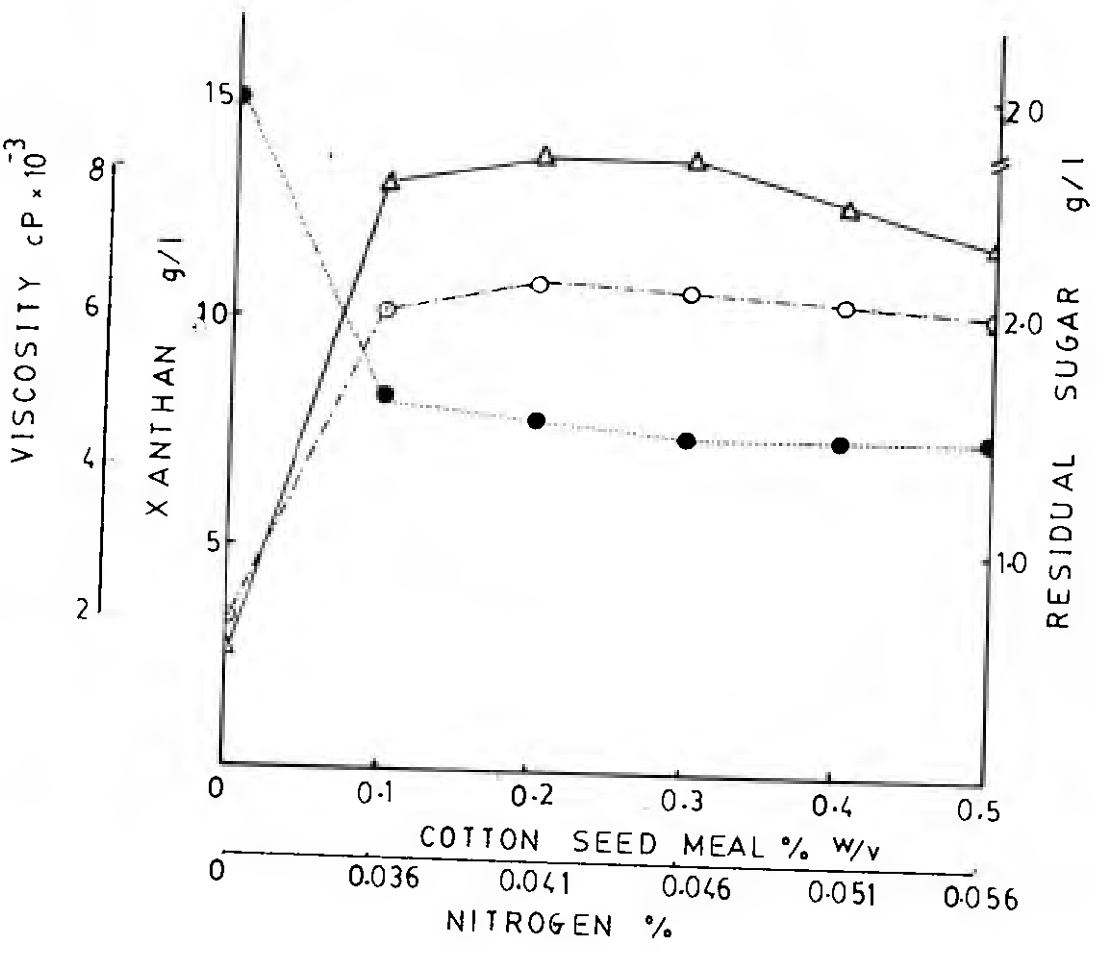


Fig.18 - Effect of Cotton seed meal on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Residual sugar ● — ●, Xanthan △ — △, Viscosity ○ — ○)

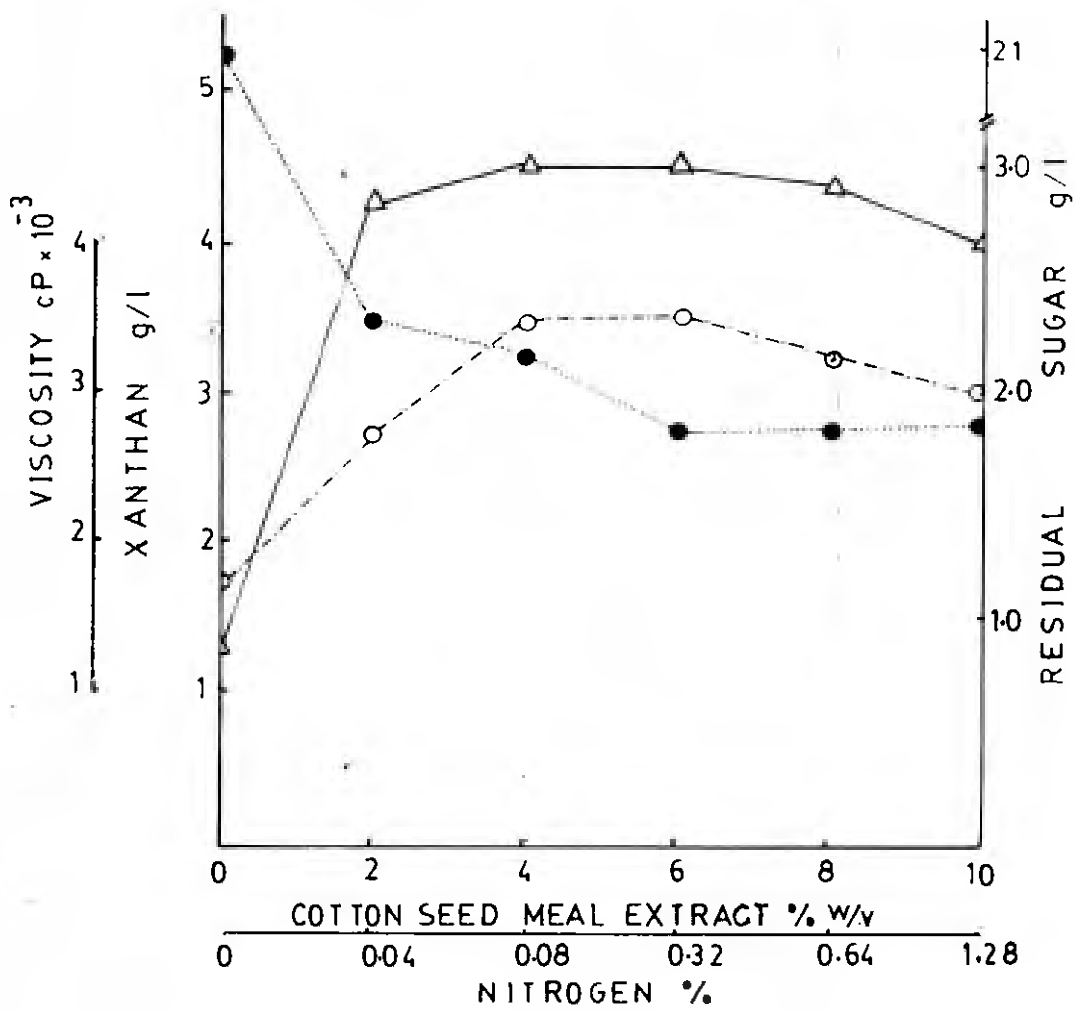


Fig.19 - Effect of Cotten seed meal extract (without di-ammonium hydrogen phosphate) on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in one liter conical shake flasks

(Residual sugar ●—●, Xanthan △—△, Viscosity ○—○)

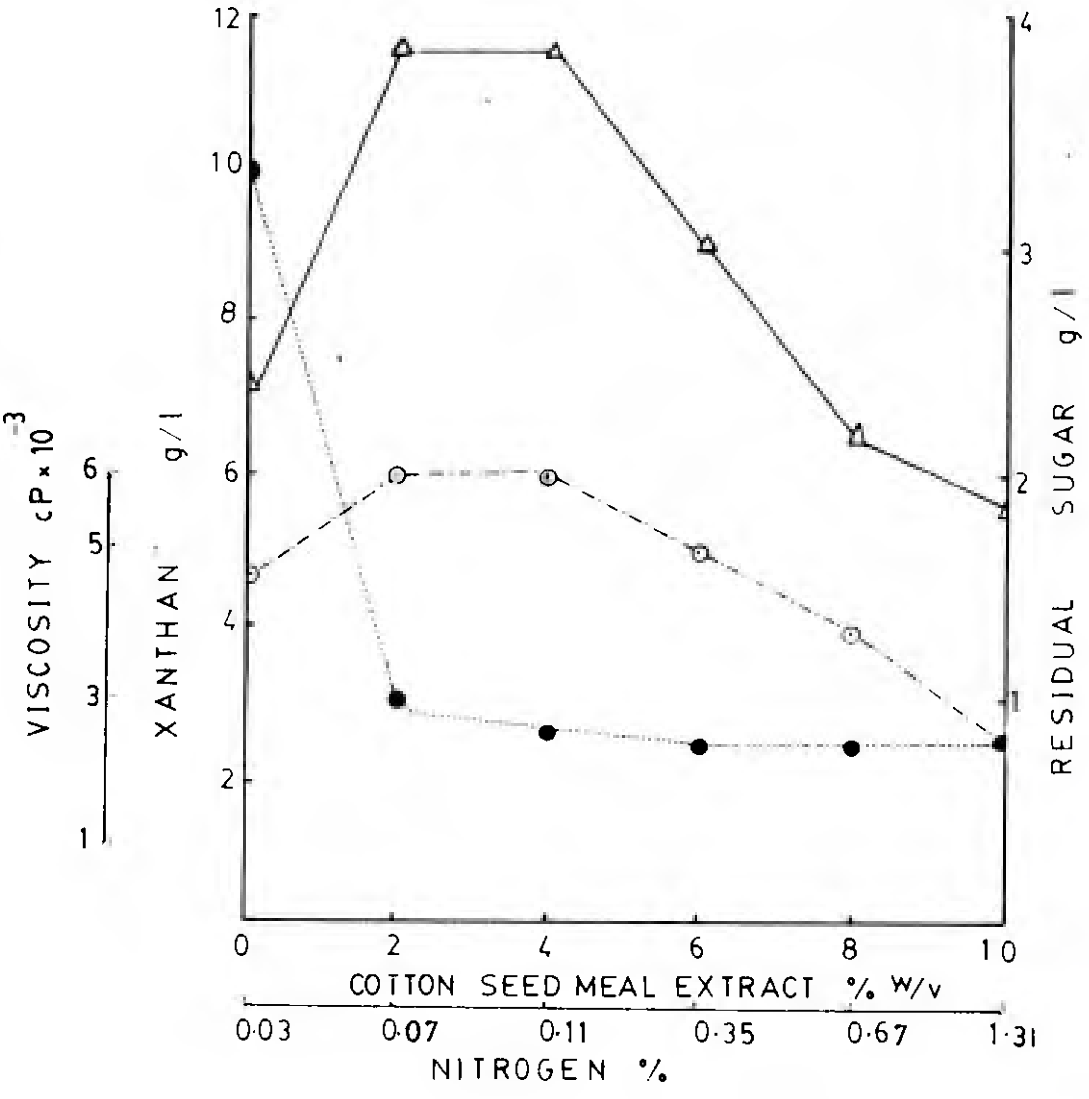


Fig. 20 - Effect of Cotton seed meal extract (with di-ammonium hydrogen phosphate) on the biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Residual sugar ● - ● , Xanthan Δ—Δ , Viscosity o- -o)

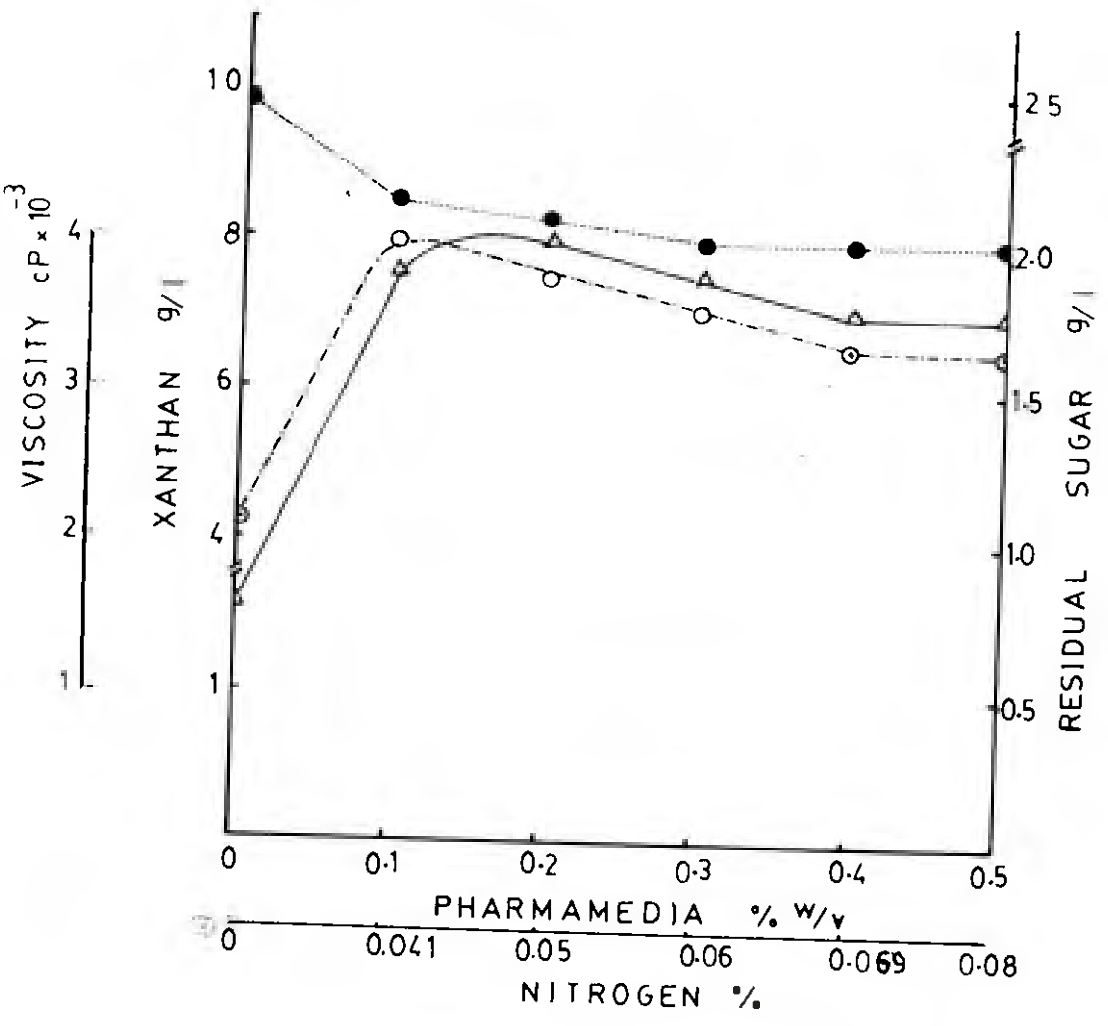


Fig.21 - Effect of Pharmamedia on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Residual sugar ● — ●, Xanthan Δ — Δ, Viscosity ○ - ○)

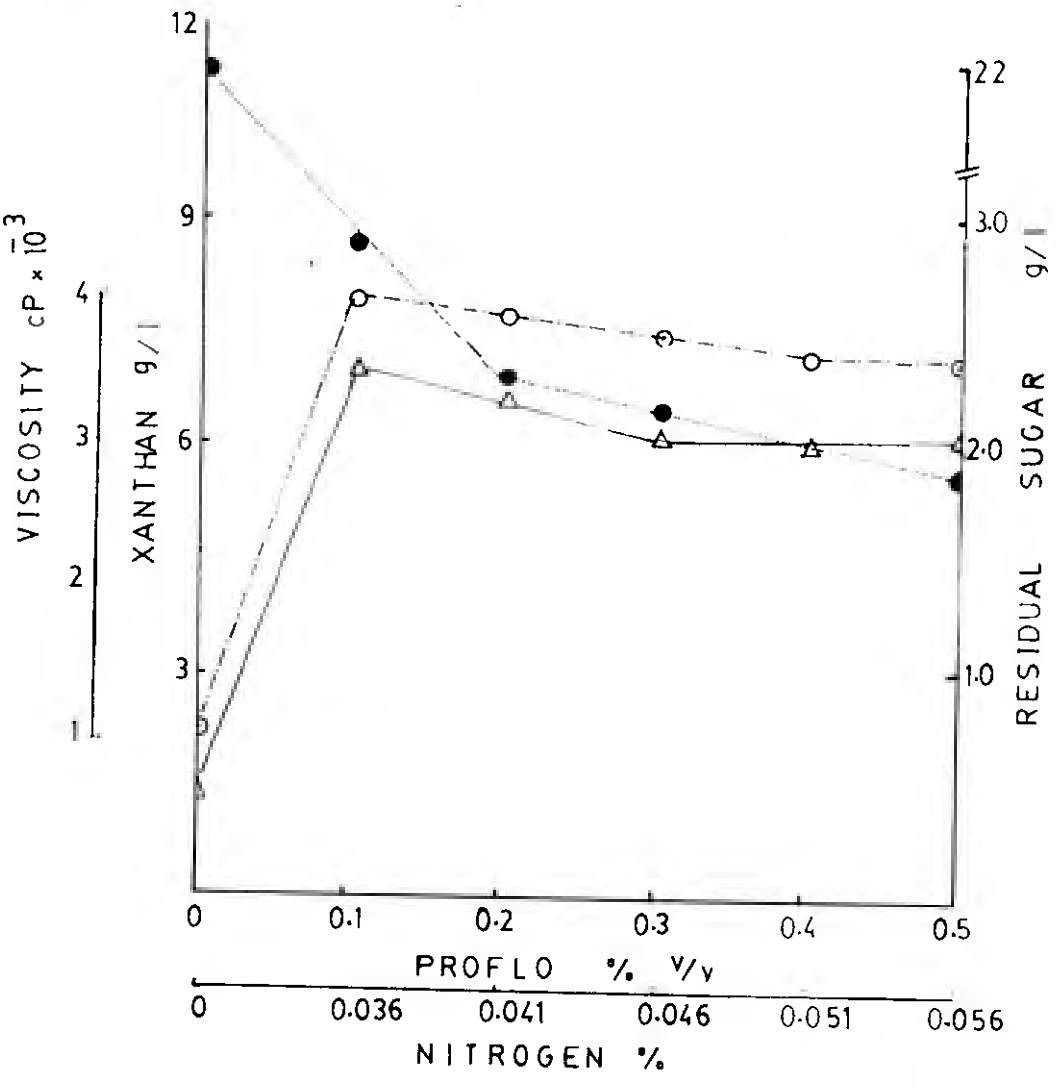


Fig.22 - Effect of Proflo on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Residiual sugar ● ● , Xanthan Δ Δ , Viscosity ⊖ ⊖)

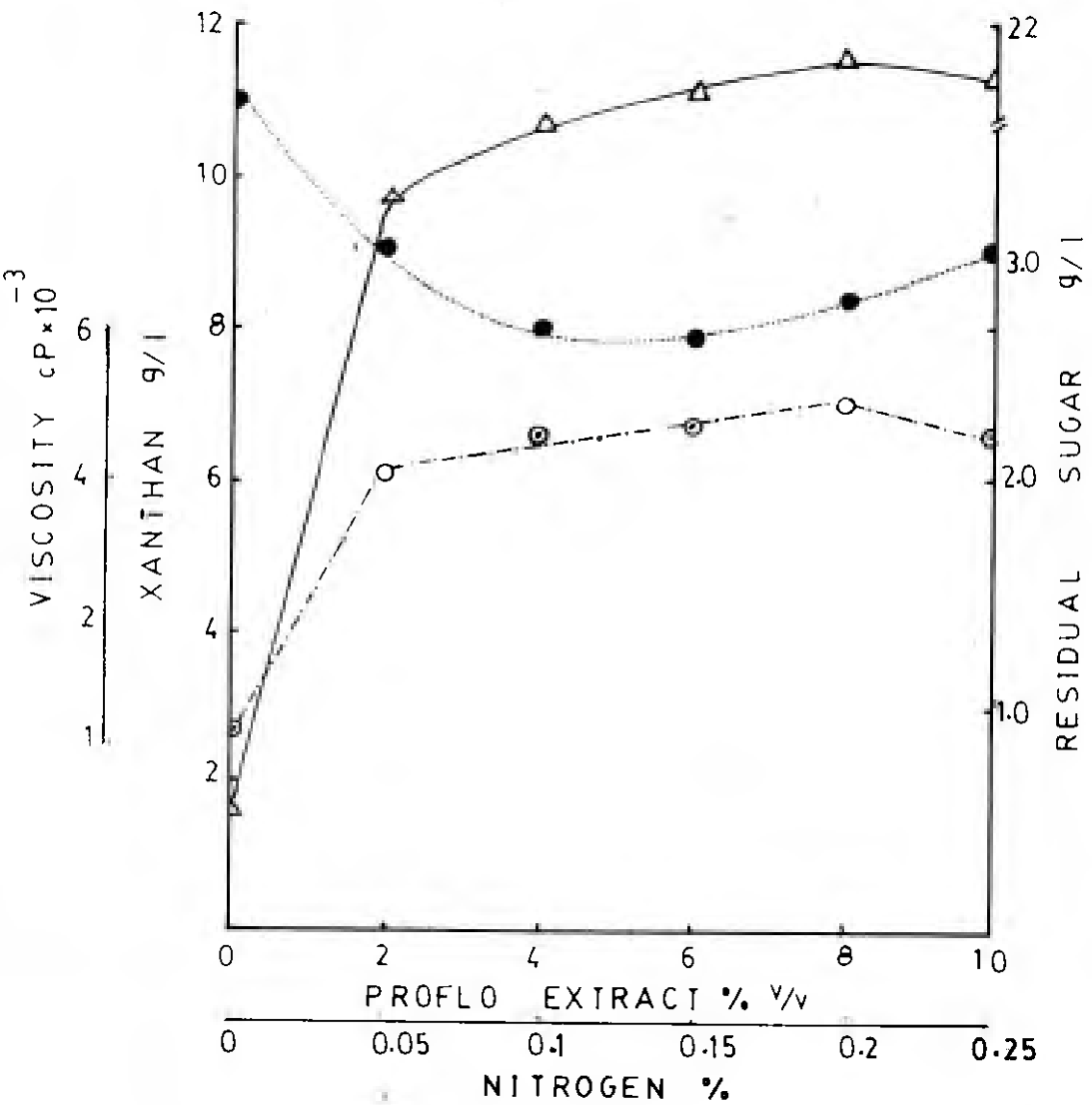


Fig.23 - Effect of Proflo extract (without di-ammonium hydrogen phosphate) on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Residiual sugar ● ● Xanthan Δ—Δ Viscosity o-·-o)

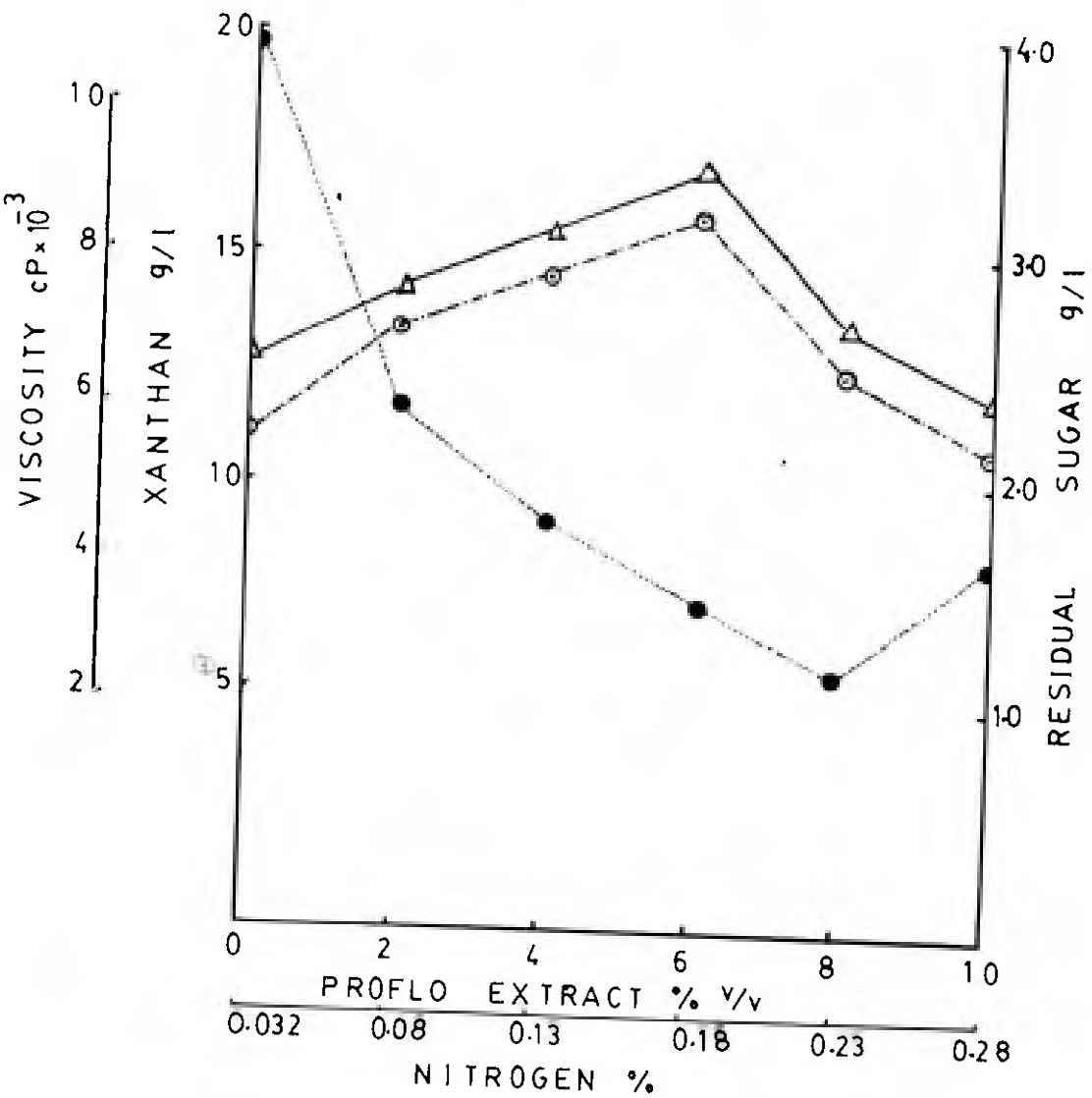


Fig.24- Effect of Proflo extract (with di-ammonium hydrogenphosphate) on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose salt medium in 1 liter conical shake flasks.

(Residiual sugar ●—● Xanthan △—△ Viscosity ○--○)

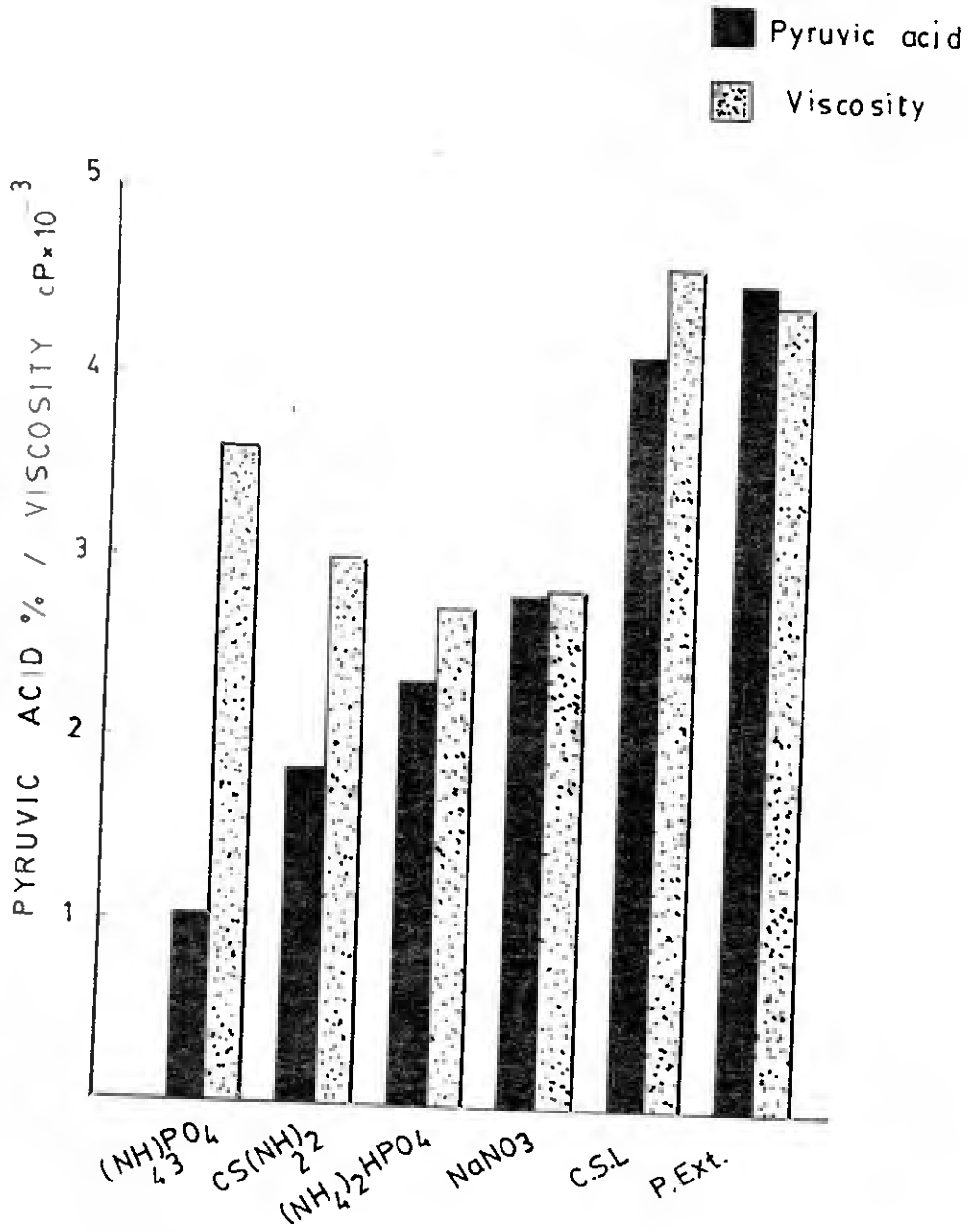


Fig.25 - Effect of nitrogen sources on the pyruvic acid content of Xanthan gum produced by Xanthomonas cucurbitae PCSIR-52 in shake flasks.

[C.S.L, corn steep liquor; P. Ext, proflo extract] Total nitrogen was 0.038 %

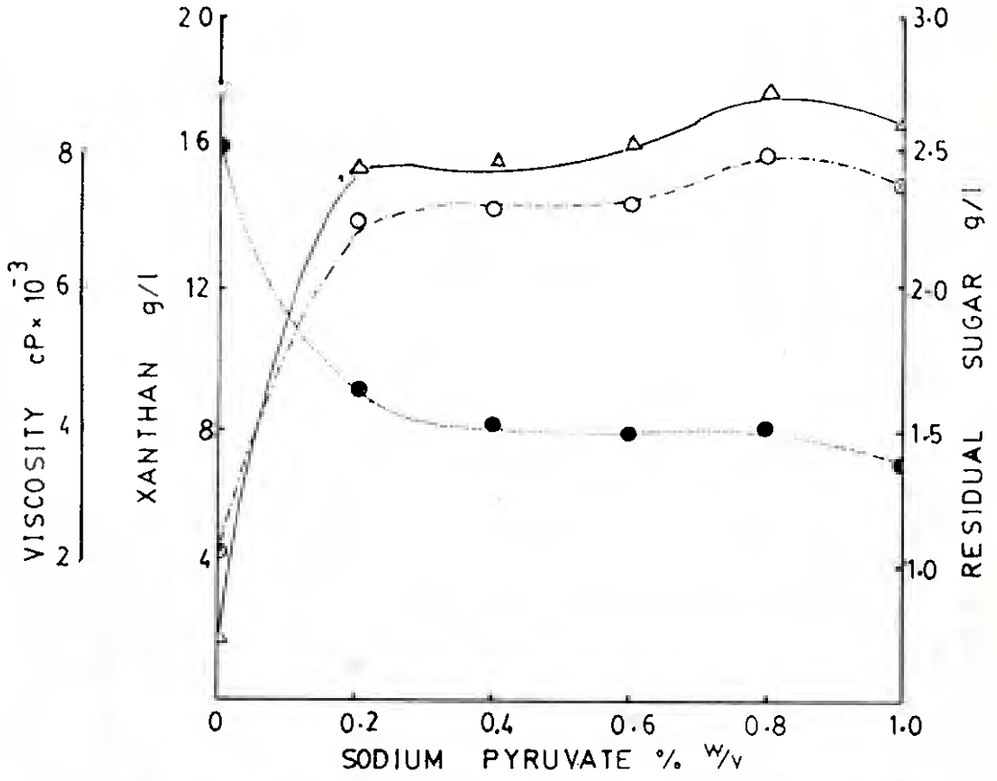


Fig.26 - Effect of Sodium pyruvate on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks

(Residual sugar ● ● , Xanthan Δ—Δ , Viscosity O- -O)

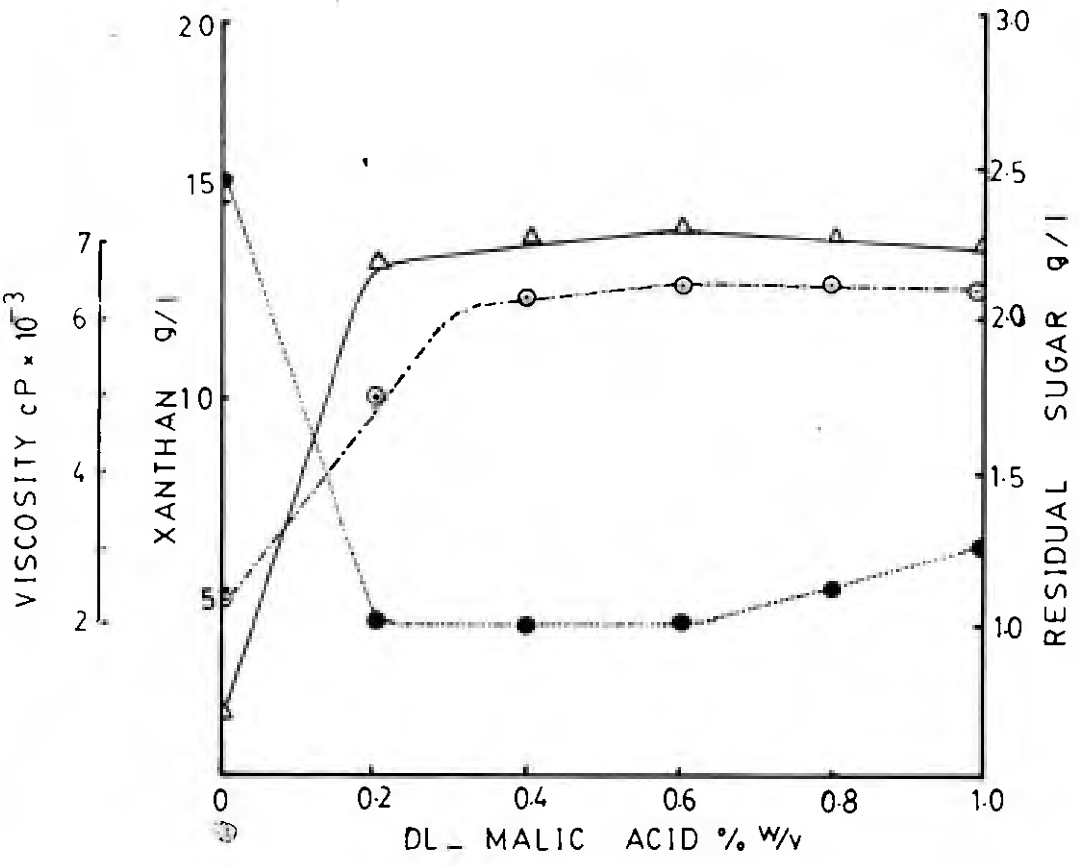


Fig-29 - Effect of DL - Malic acid on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose - salt medium in 1 liter conical shake flasks

(Residual sugar ●---● , Xanthan Δ—Δ ; Viscosity ○—◉)

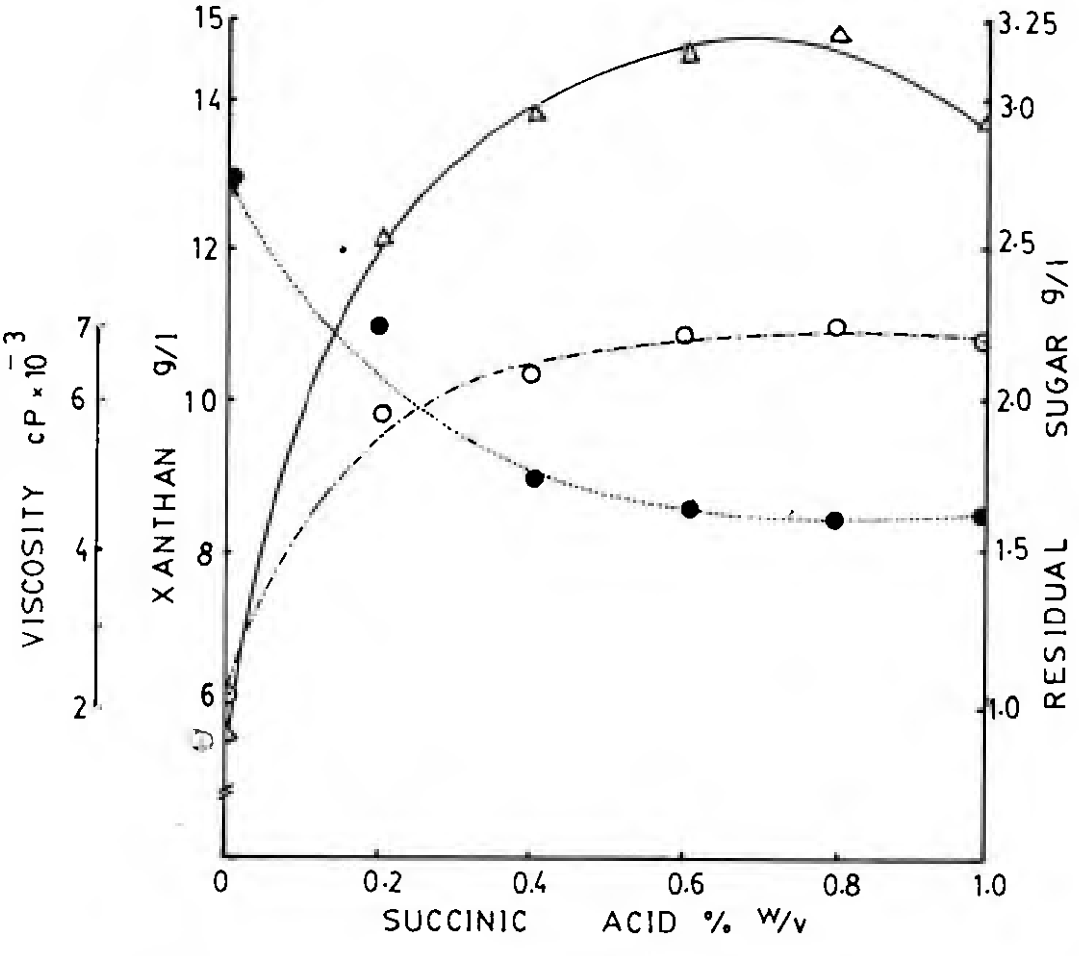


Fig.30 - Effect of Succinic acid on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Residual sugar ●····● ; Xanthan Δ—Δ ; Viscosity ⊙—○)

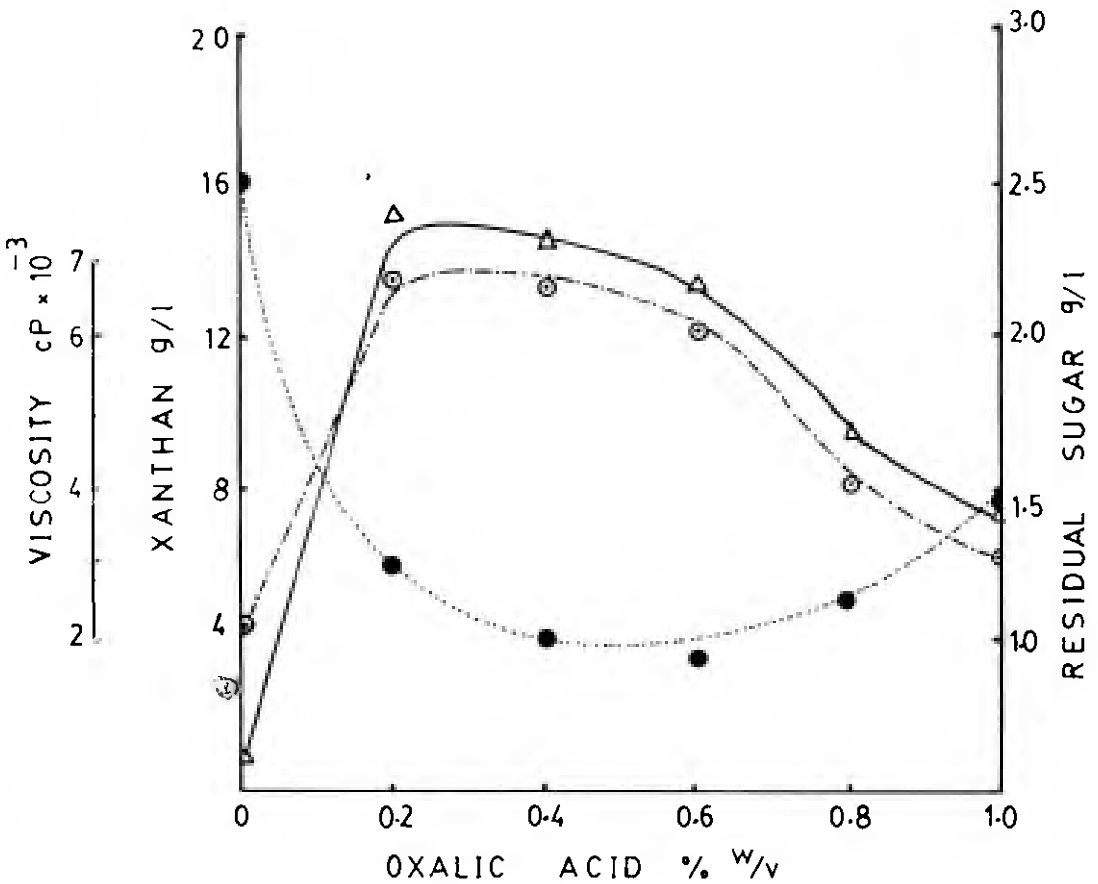


Fig.31 - Effect of Oxalic acid on the biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Viscosity ⊙—⊙ , Xanthan Δ—Δ , Residual sugar ● ●)

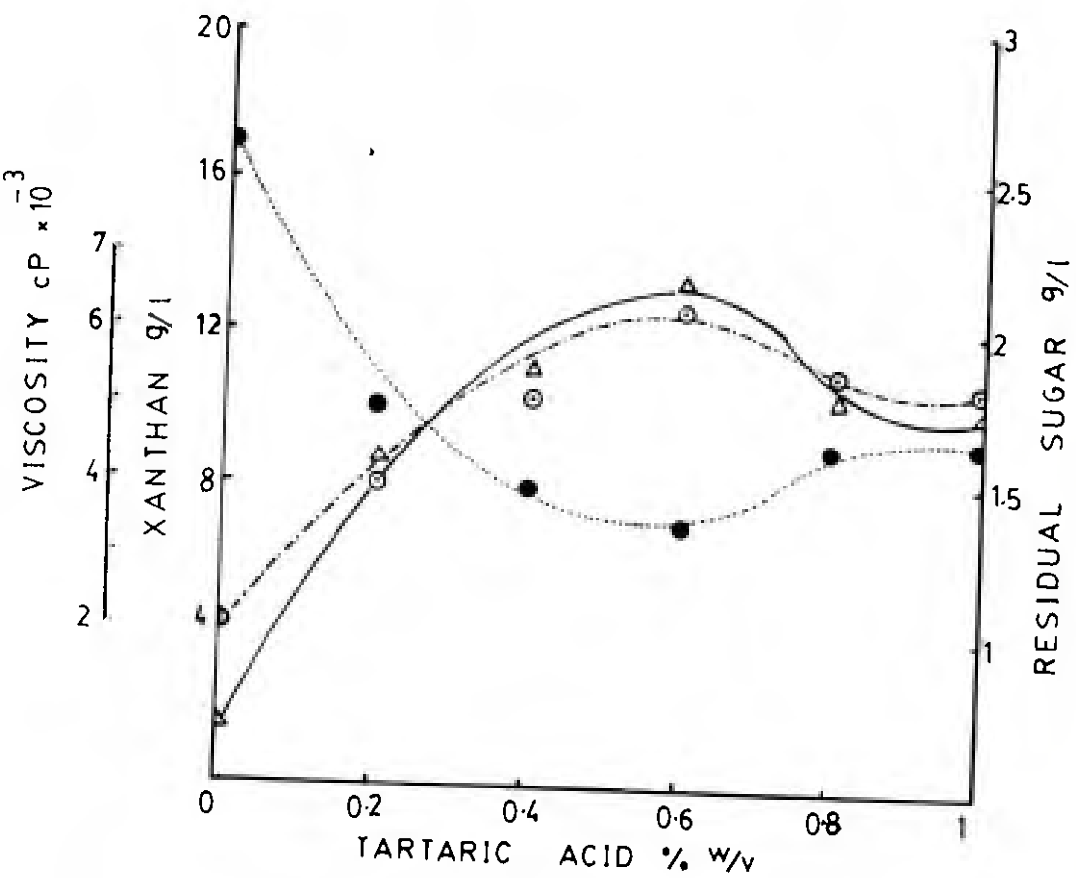


Fig.32 - Effect of Tartaric acid on the biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt medium in 1 liter conical shake flasks.

(Viscosity O---O, Xanthan Δ——Δ, Residual sugar ●—●)

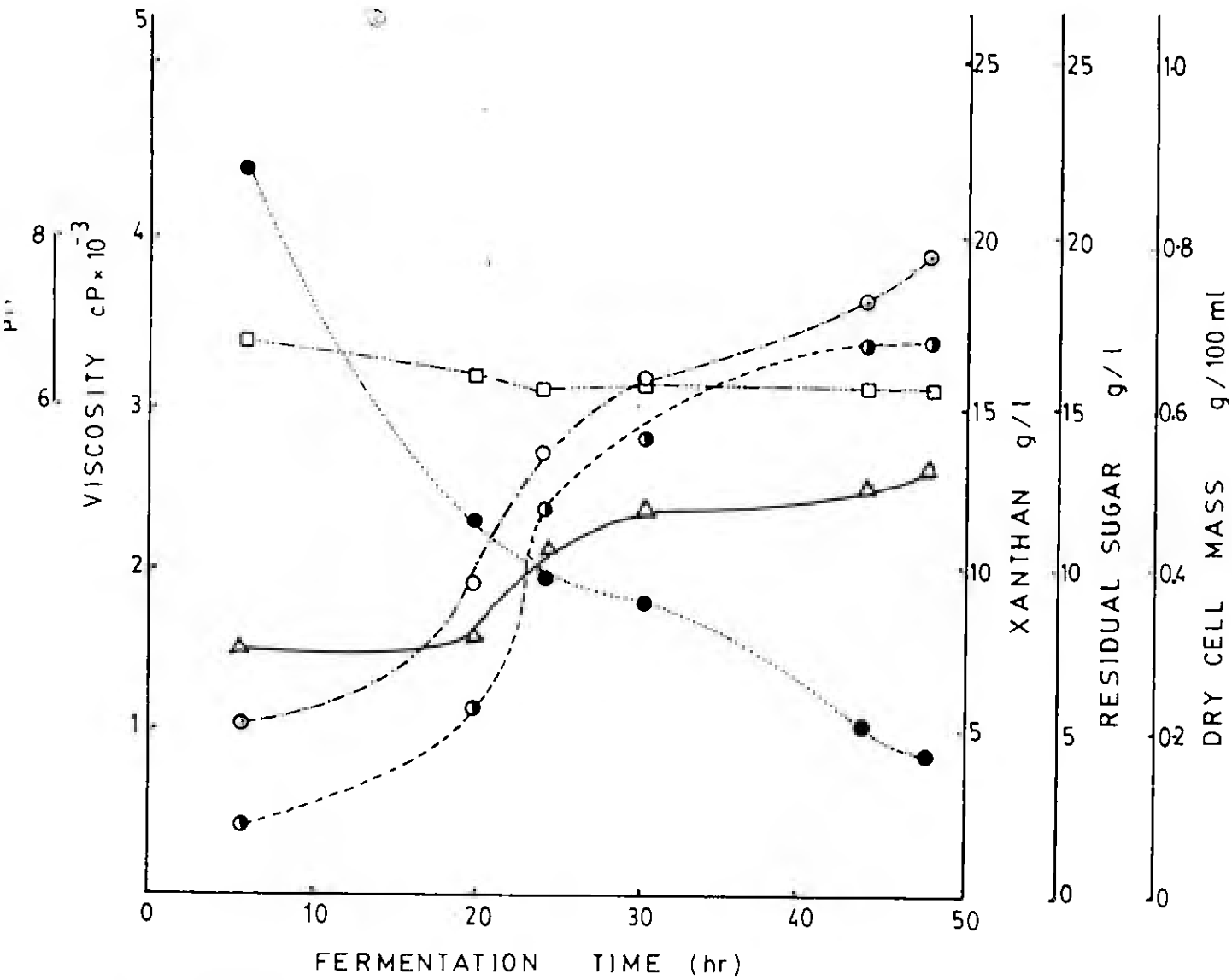
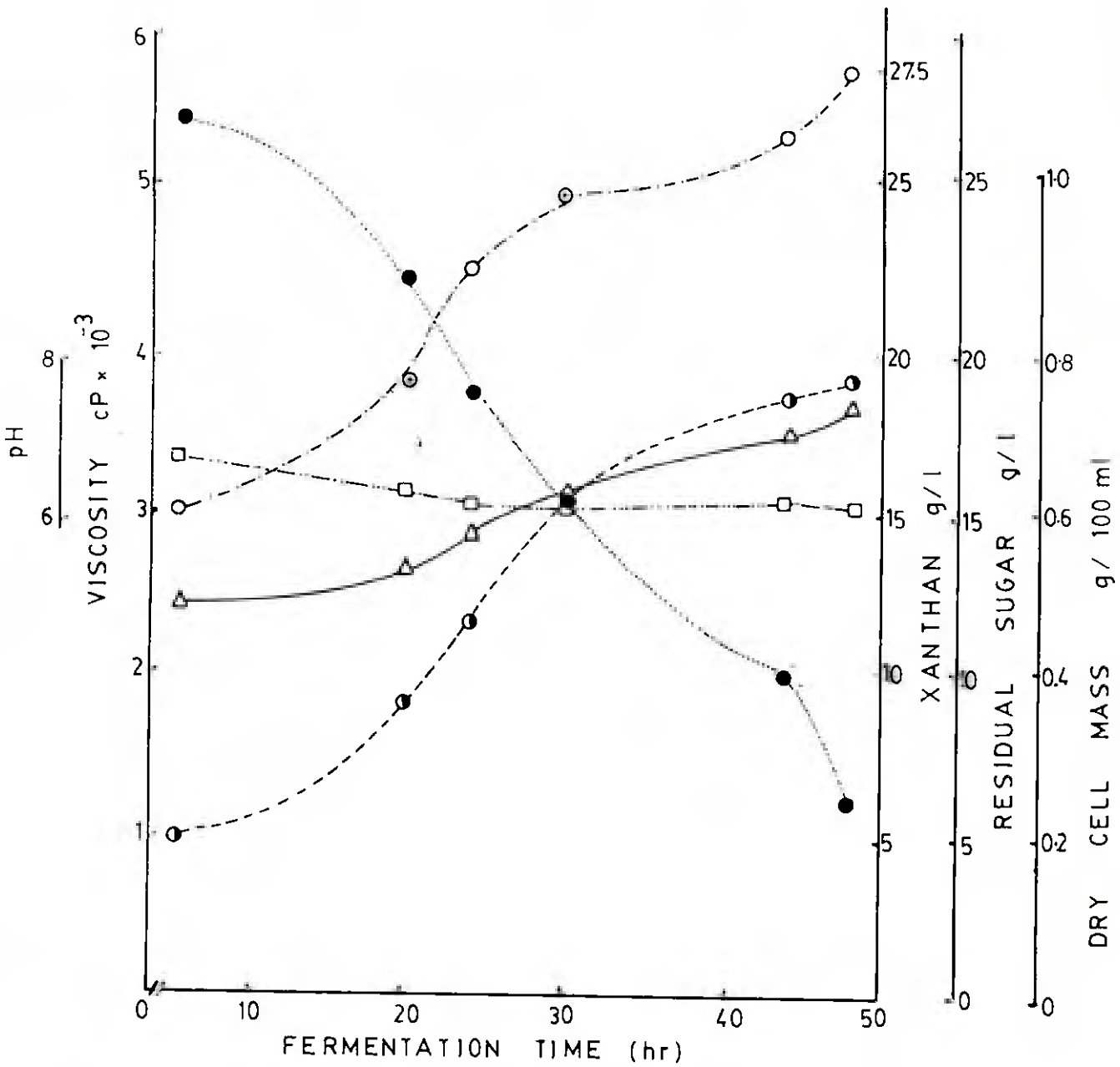


Fig.33 - Effect of Glucose on the biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in 10 liter fermenter.

(Dry cell mass ◑--◑ , Xanthan Δ—Δ , Viscosity ○--○ , pH □···□
Residual sugar ●—●)



Fig·34 - Effect of Sucrose on the production of Xanthan biopolymer by Xanthomonas cucurbitae PCSIR-52 in 10 liter fermenter.

(Residual sugar ●—● ; Xanthan Δ—Δ ; Viscosity ○—○ ; pH □—□
Dry cell mass ◑—◑)

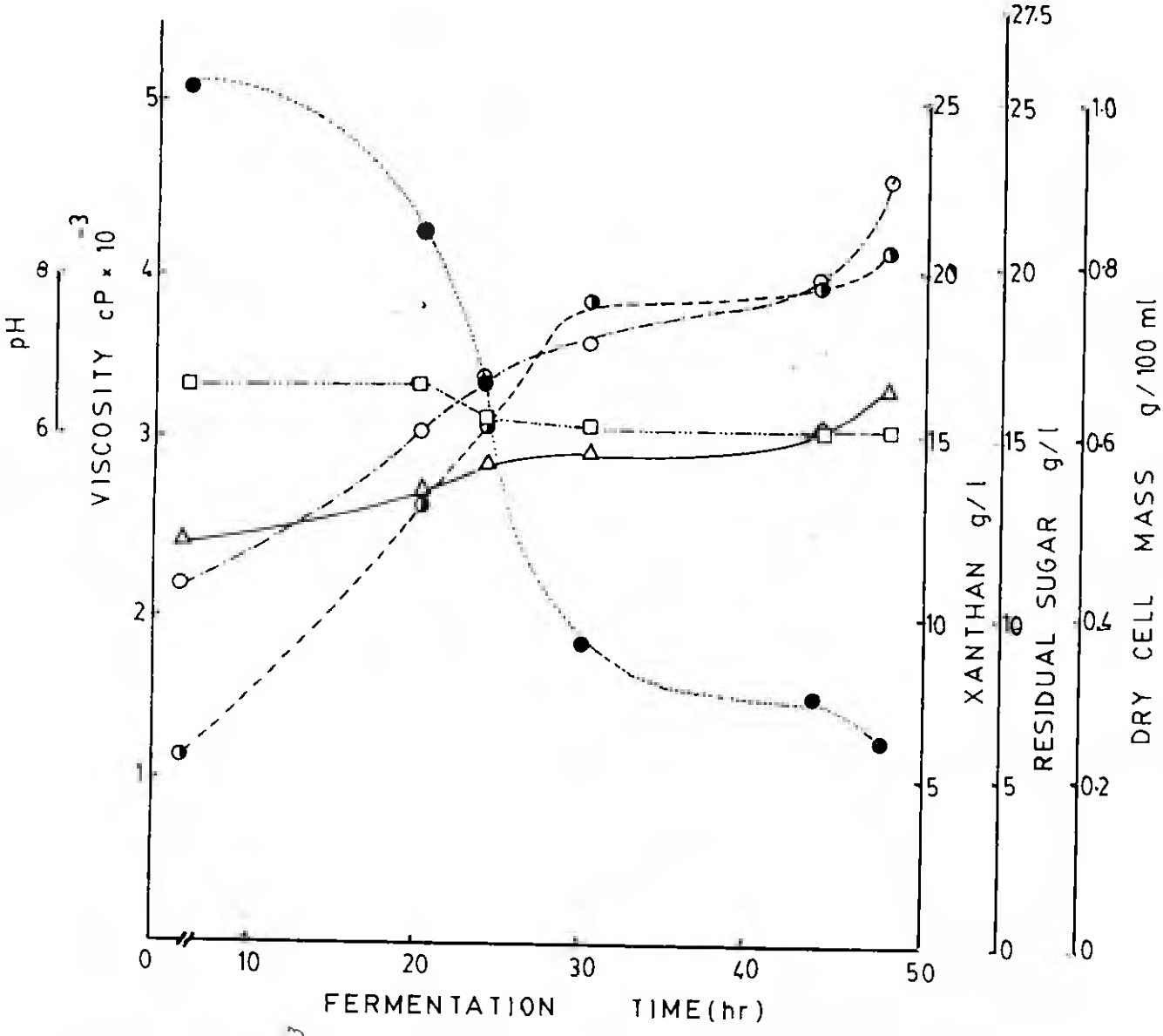


Fig.35 - Effect of Lactose on the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in 10 liter fermenter

(Viscosity o--o ; Dry cell mass ◑—◑ ; Residual sugar ●···● ,
pH □--□ ; Xanthan △—△)

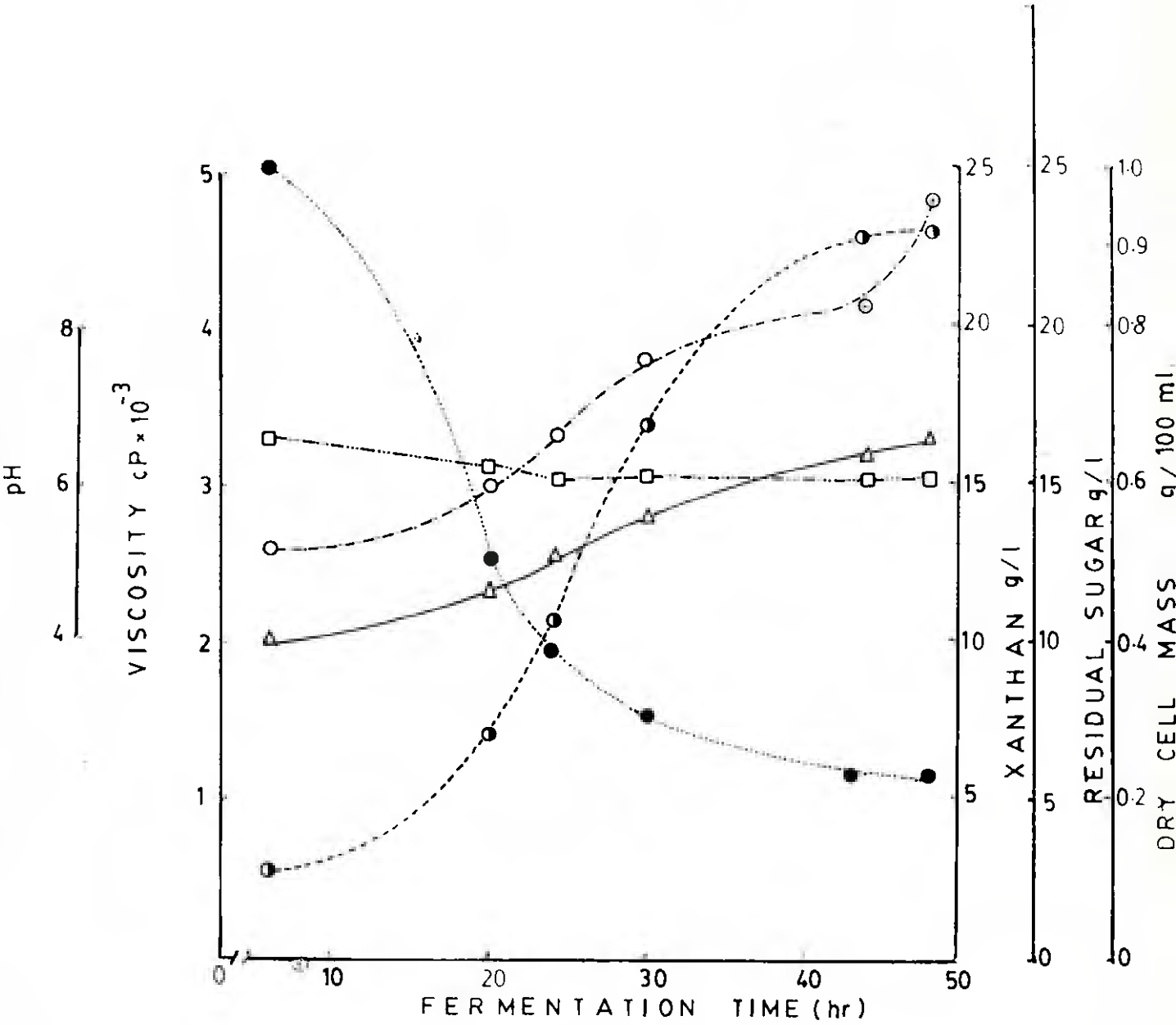


Fig.36 - Biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 using sucrose-salt-corn steep liquor in 10 liter fermenter

(Viscosity O--O , Xanthan Δ—Δ , Residual sugar ●—● , pH □--□
Dry cell mass ◑--◑)

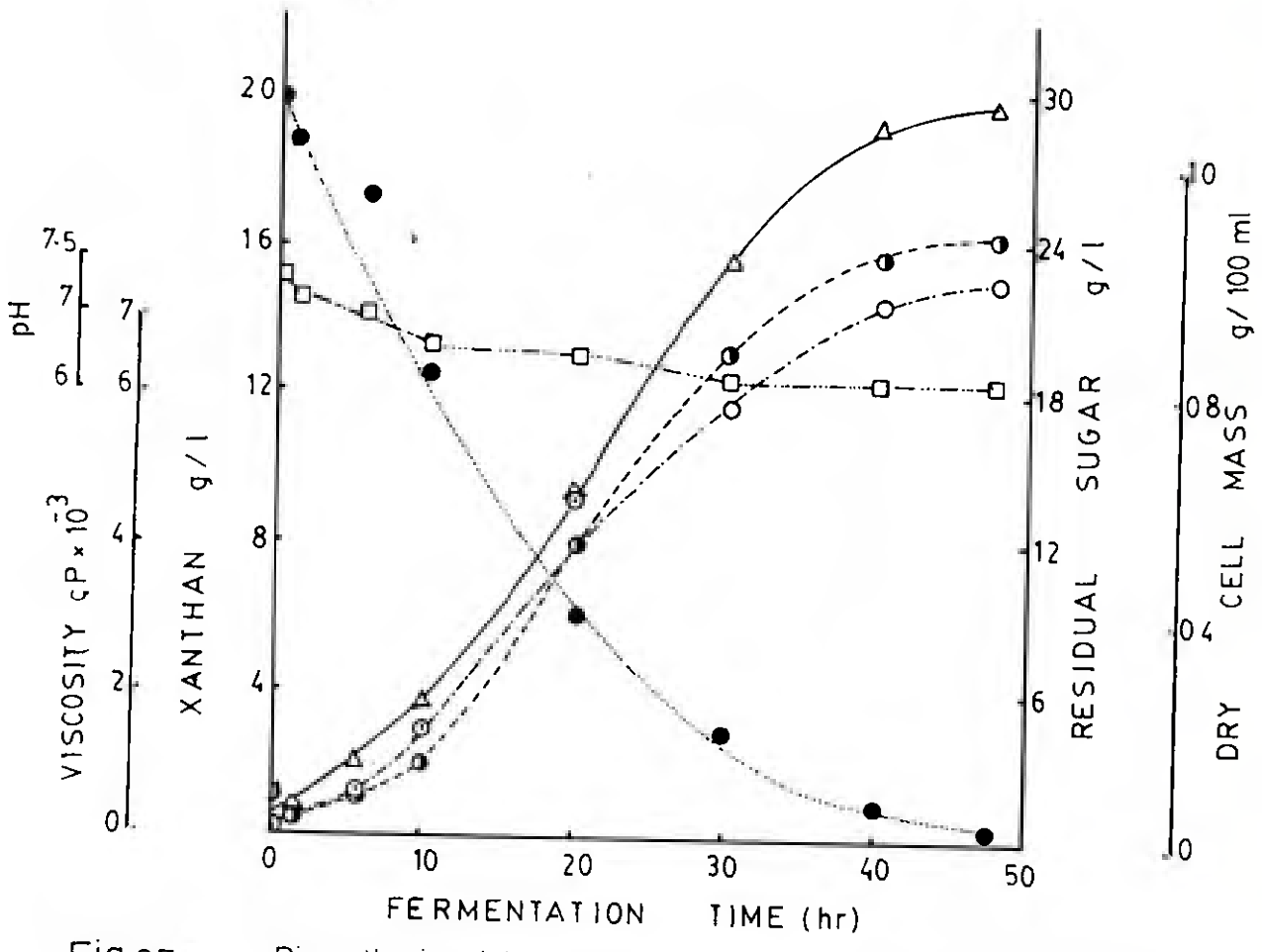


Fig.37 - Biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in 10 liter fermenter (sucrose-salt-proflo extract).
(Viscosity ○--○ ; Dry cell mass ◑--◑ ; Xanthan △—△ ,
pH □--□ , Residual sugar ●--●)

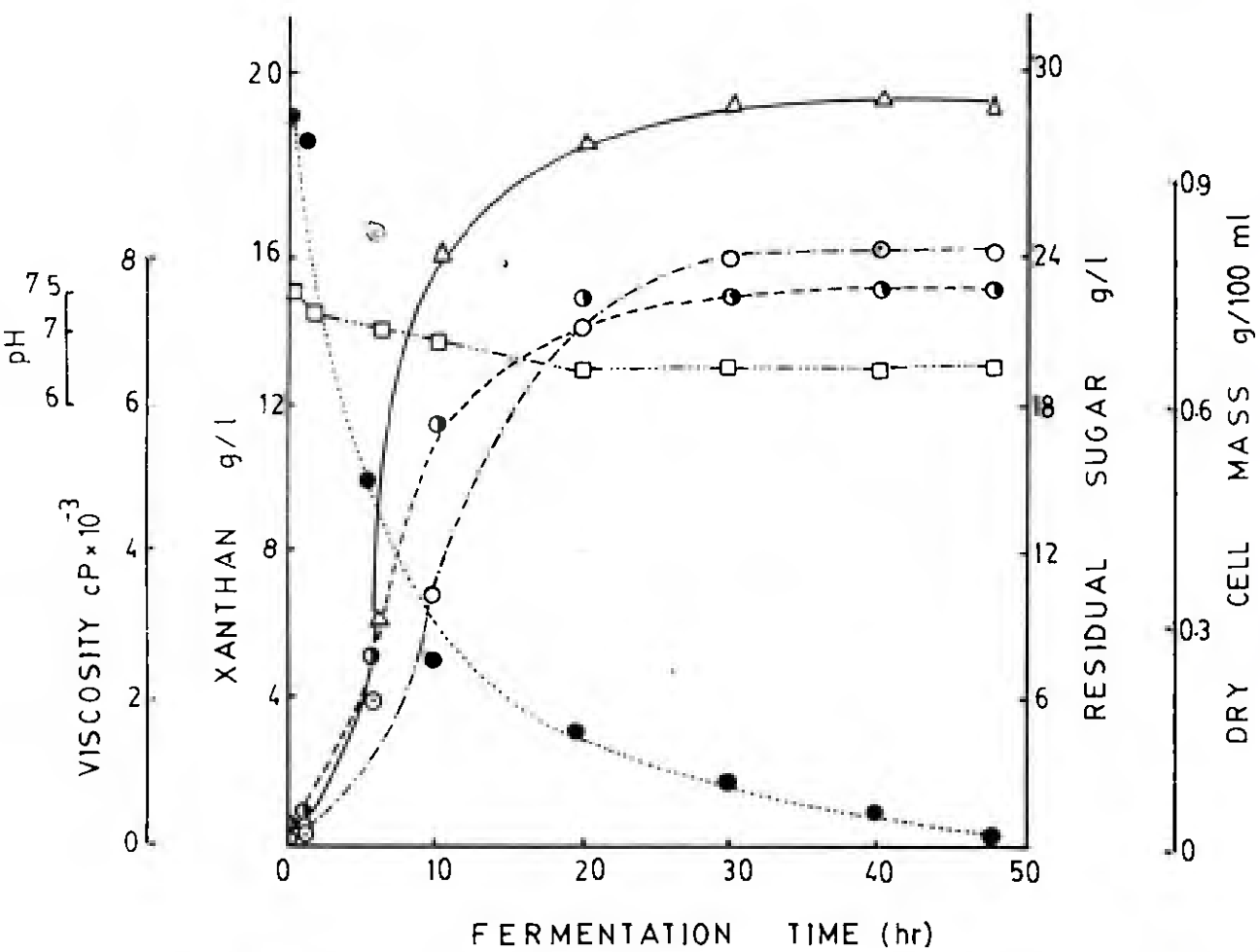


Fig.38 – Biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in 50 liter fermenter.

(Viscosity ○—○ , Xanthan △—△ , Dry cell mass ◑—◑ ,
pH □—□ , Residual sugar ●—●)

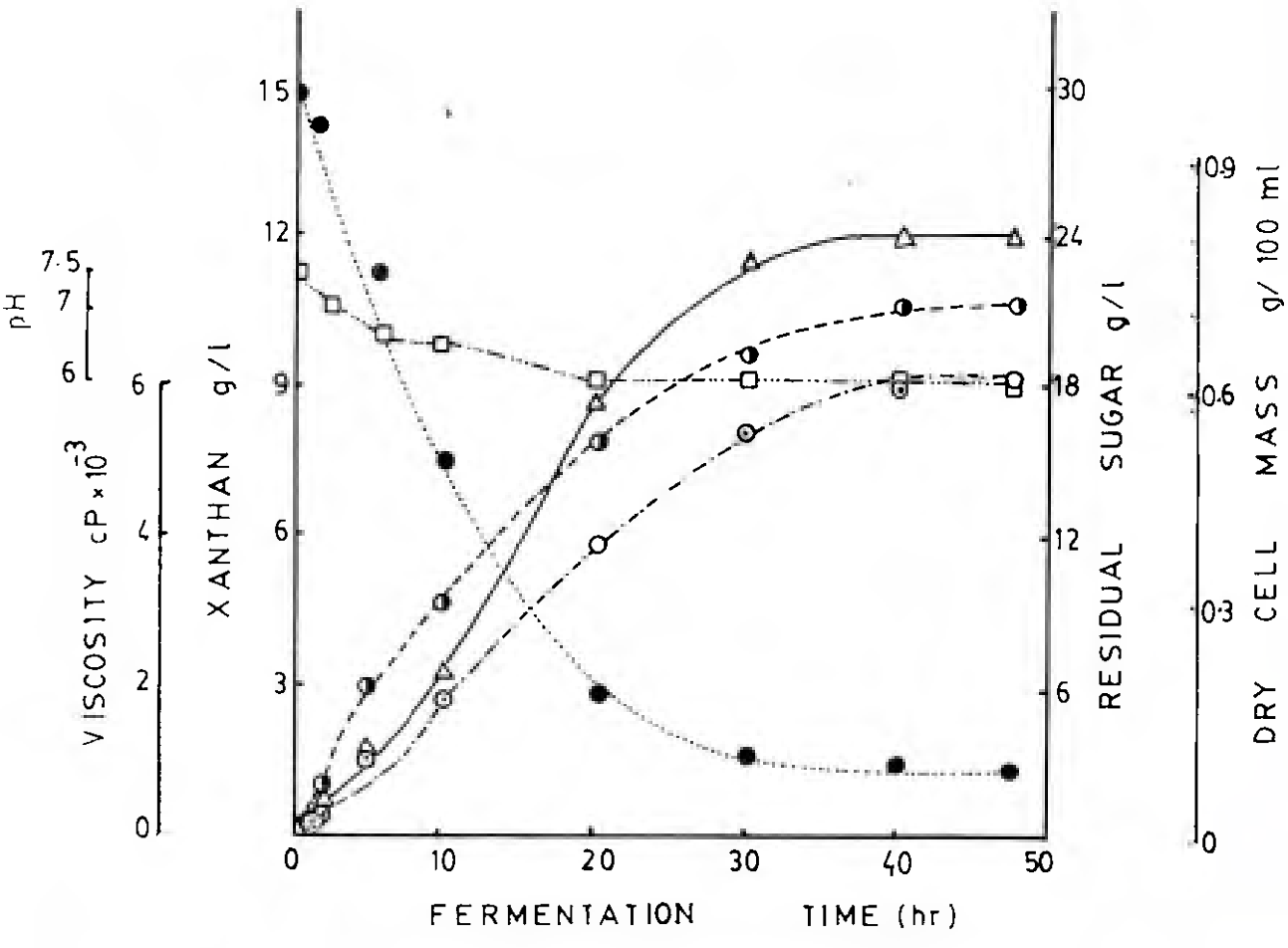


Fig.39 - Production of Xanthan biopolymer by Xanthomonas cucurbitae PCSIR-52, in 100 liter fermenter.

(Viscosity O--O ; Residual sugar ●---● ; pH □---□ ; Xanthan Δ—Δ ; Dry cell mass ◑---◑)

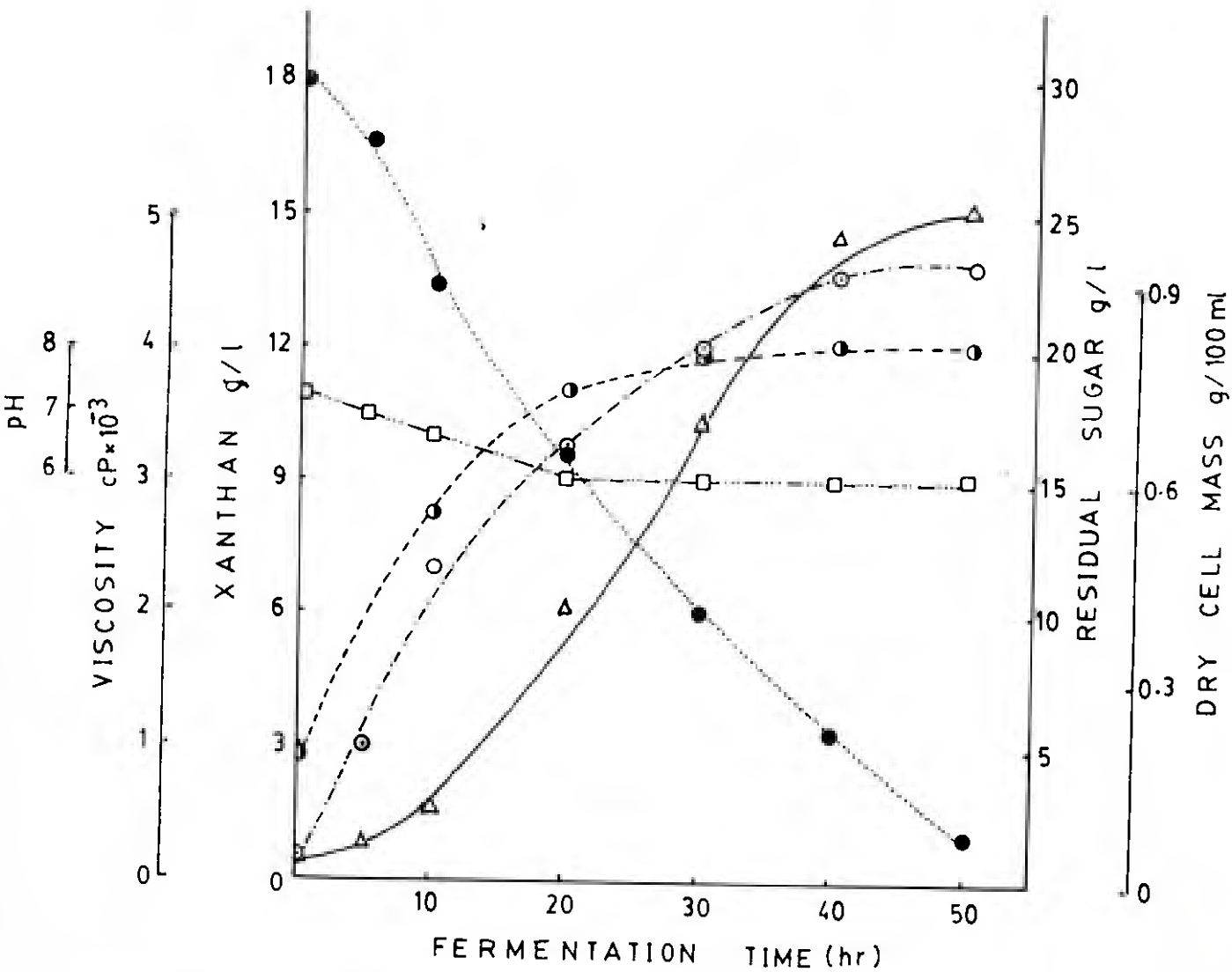


Fig.40—Production of Xanthan gum in 250 liter Stainless-steel fermenter.
(Xanthan △—△, Viscosity ○—○, Dry cell mass ◑---◑; Residual sugar ●····●;
pH □—□)

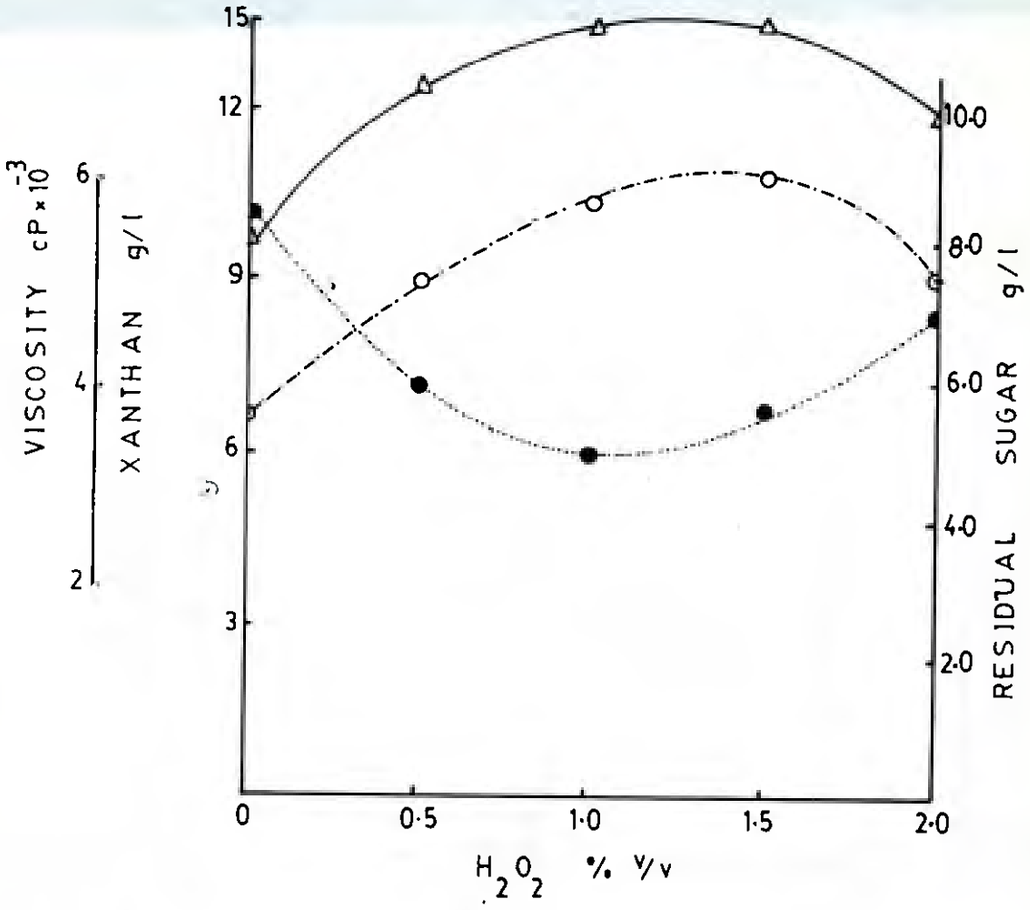


Fig.41 - Effect of hydrogen peroxide (added 24 hr after inoculation) on the biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in sucrose salt medium in 1 liter conical shake flasks.

(Viscosity o—o ; Xanthan Δ—Δ ; Residual sugar ●···●)

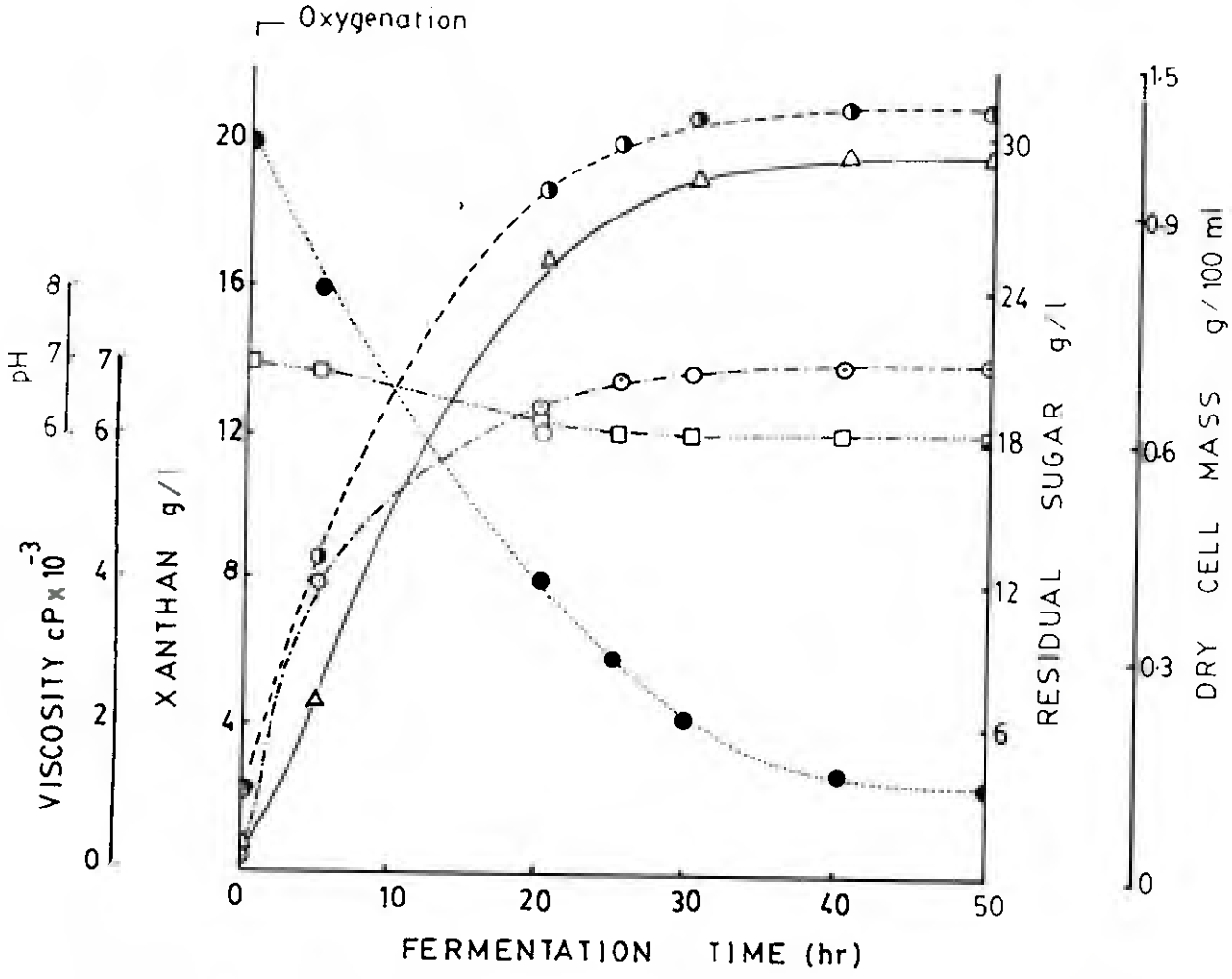


Fig.43 - Effect of partial replacement of air by Oxygen gas (5:1) on the biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52, in 10 liter fermenter, (0 hr after inoculation).

(Viscosity o--o, Residual sugar ●·····●, Dry cell mass ◑---◑, pH □·····□, Xanthan △——△)

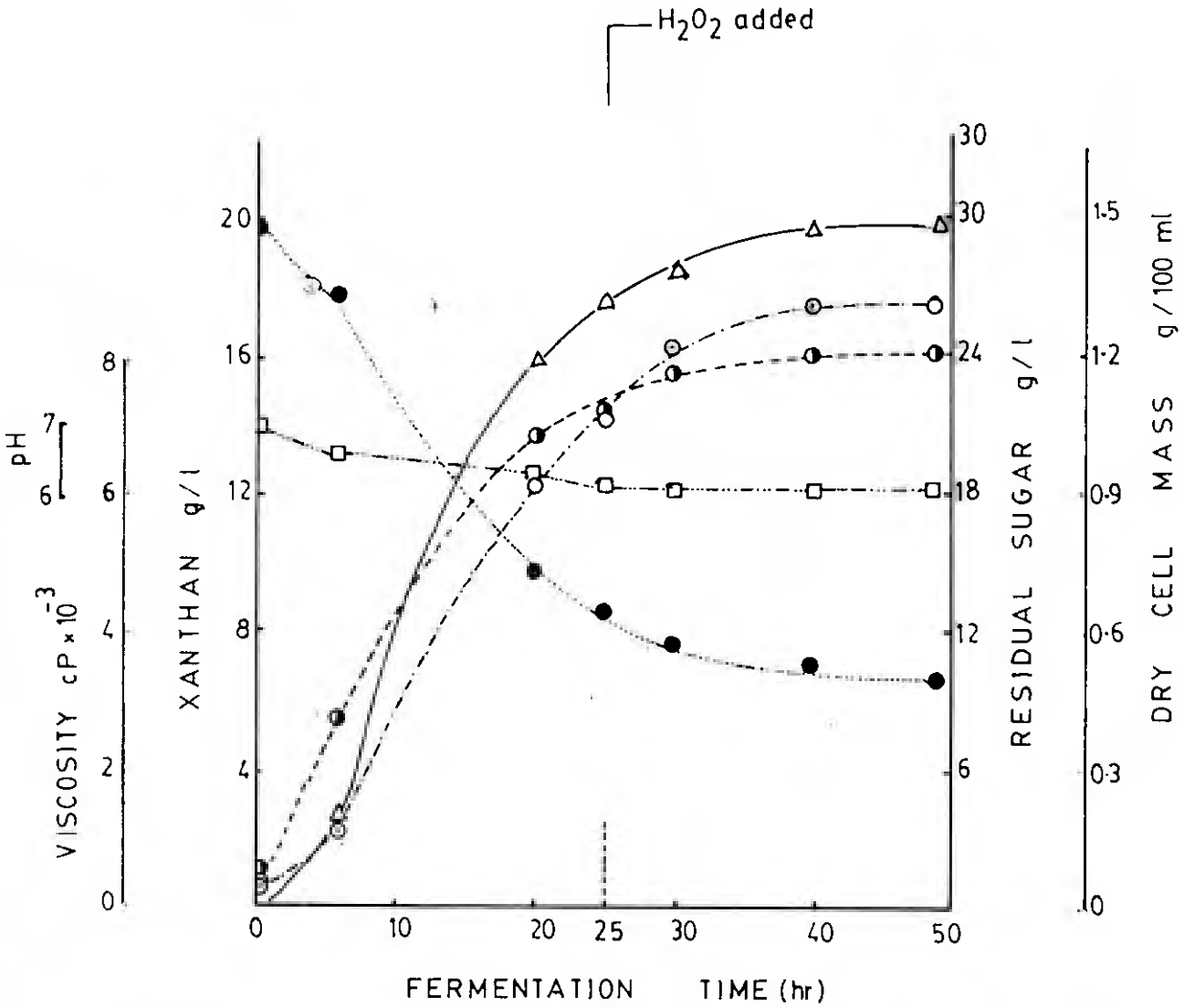
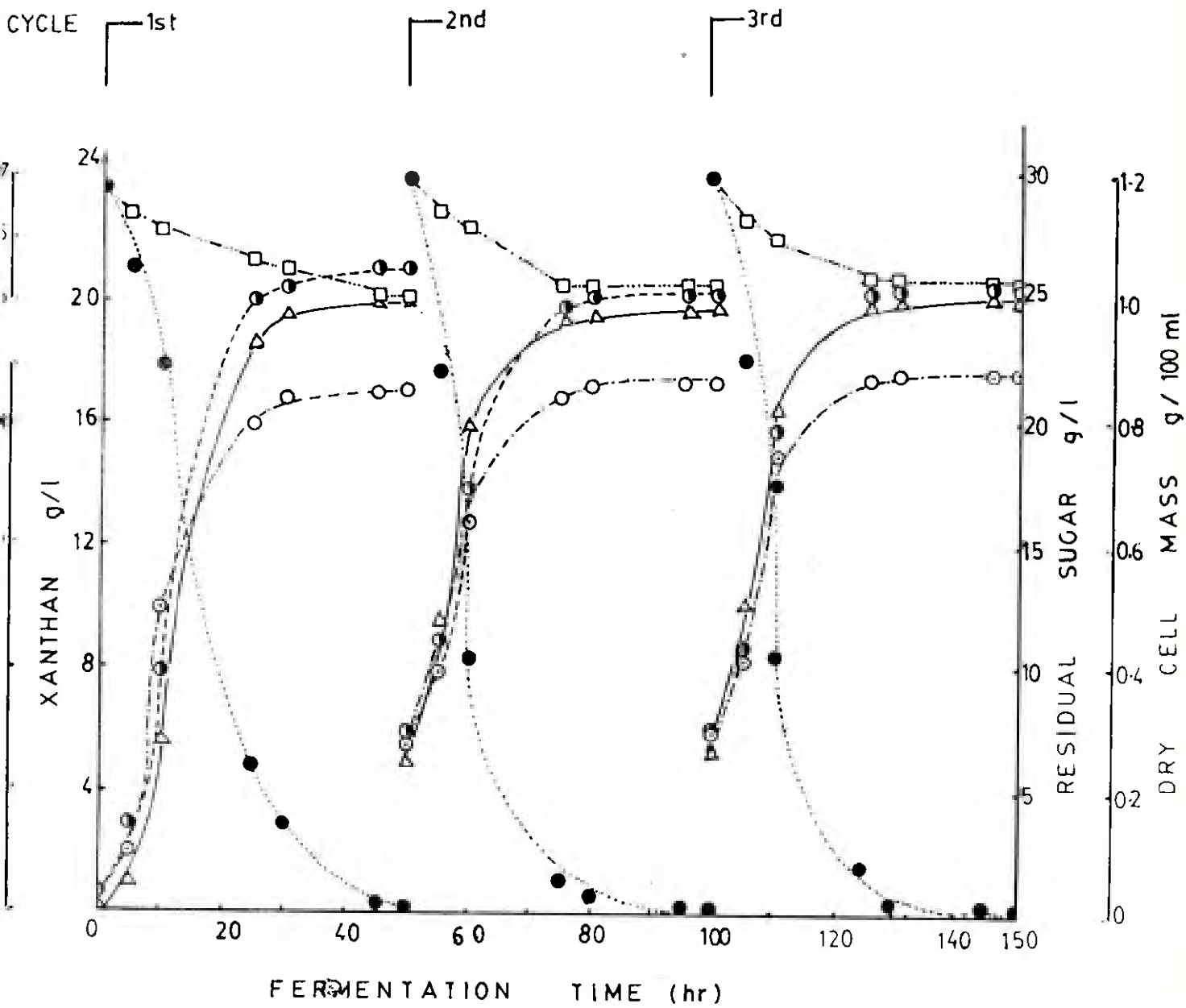


Fig.44 - Effect of feeding H_2O_2 on the biosynthesis of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in 10 liter fermenter

(Viscosity O--O Dry cell mass ◑---◑ pH □--□ Xanthan Δ—Δ Residual sugar ●—●)



3·45 – Repeated-batch fermentation; Sucrose-salt medium for the production of Xanthan gum by Xanthomonas cucurbitae PCSIR-52 in three cycles

(Viscosity o—o , Dry cell mass ◑----◑ , Residual sugar ●·····● , pH □—□ , Xanthan △—△)

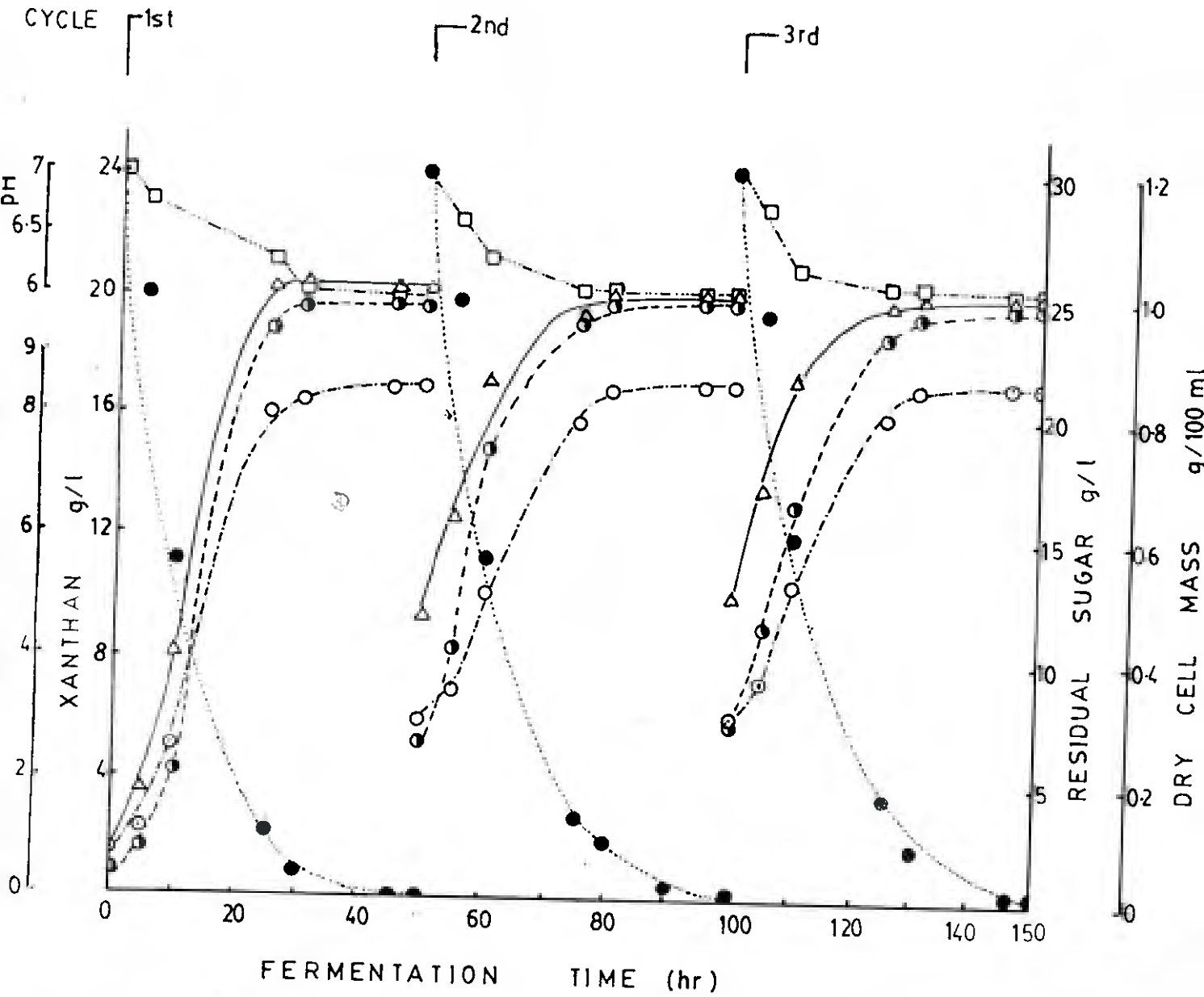


Fig.46 - Repeated-batch fermentation, Sucrose-salt-proflo medium for the production of Xanthan biopolymer by Xanthomonas cucurbitae PCSIR-52 in three cycles. (Viscosity ○---○ ; Dry cell mass ◑---◑ ; Xanthan gum △——△ ; pH □---□ Residual sugar ●---●)

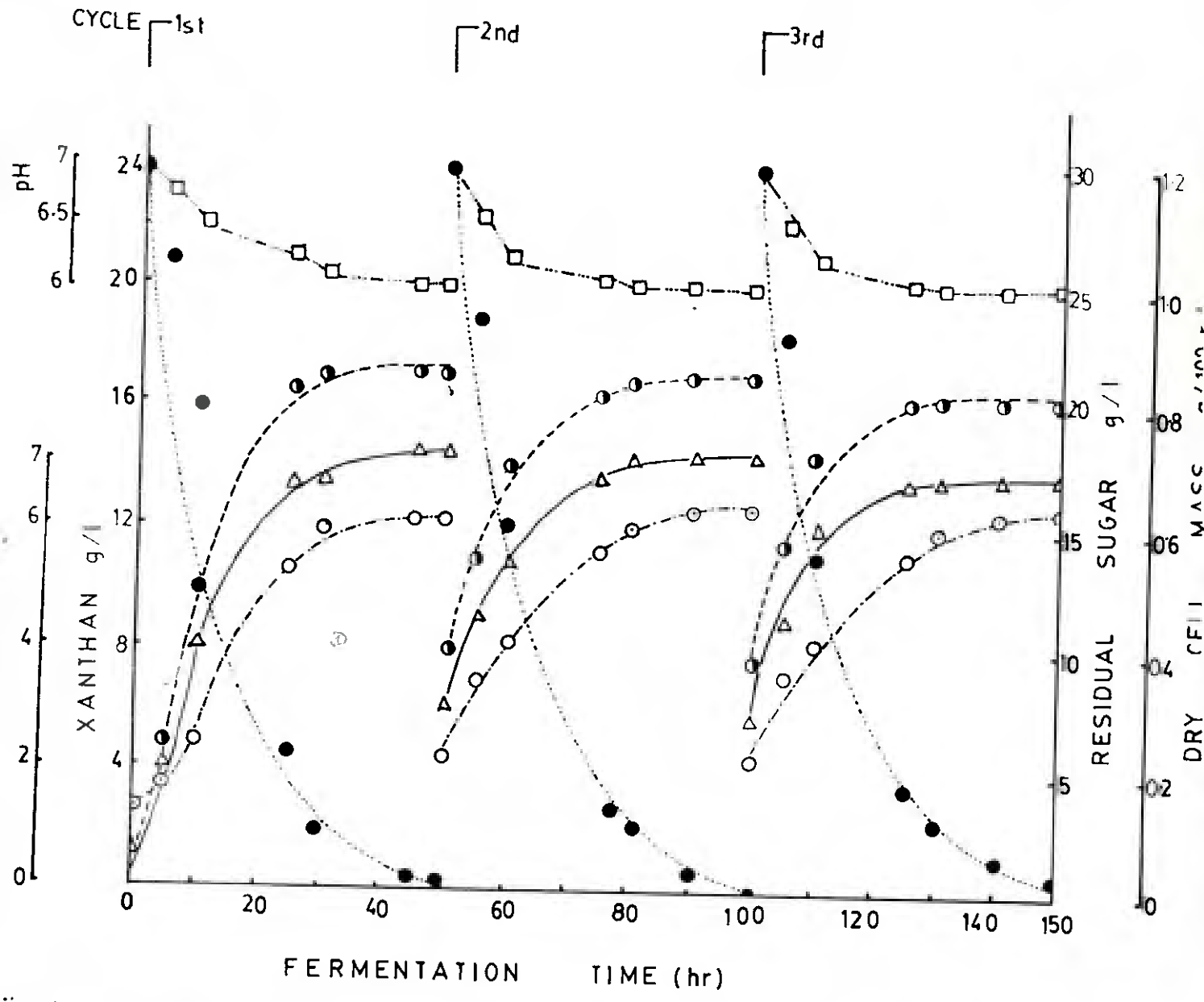


Fig.47 - Repeated-batch fermentation, Cane juice medium for the production of Xanthan gum in 10 liter fermenter, by Xanthomonas cucurbitae PCSIR-52.
(□-□ pH , ○-○ Viscosity, ◑-◑ Dry cell mass , Δ-Δ Xanthan gum ,
●-● Residual sugar)

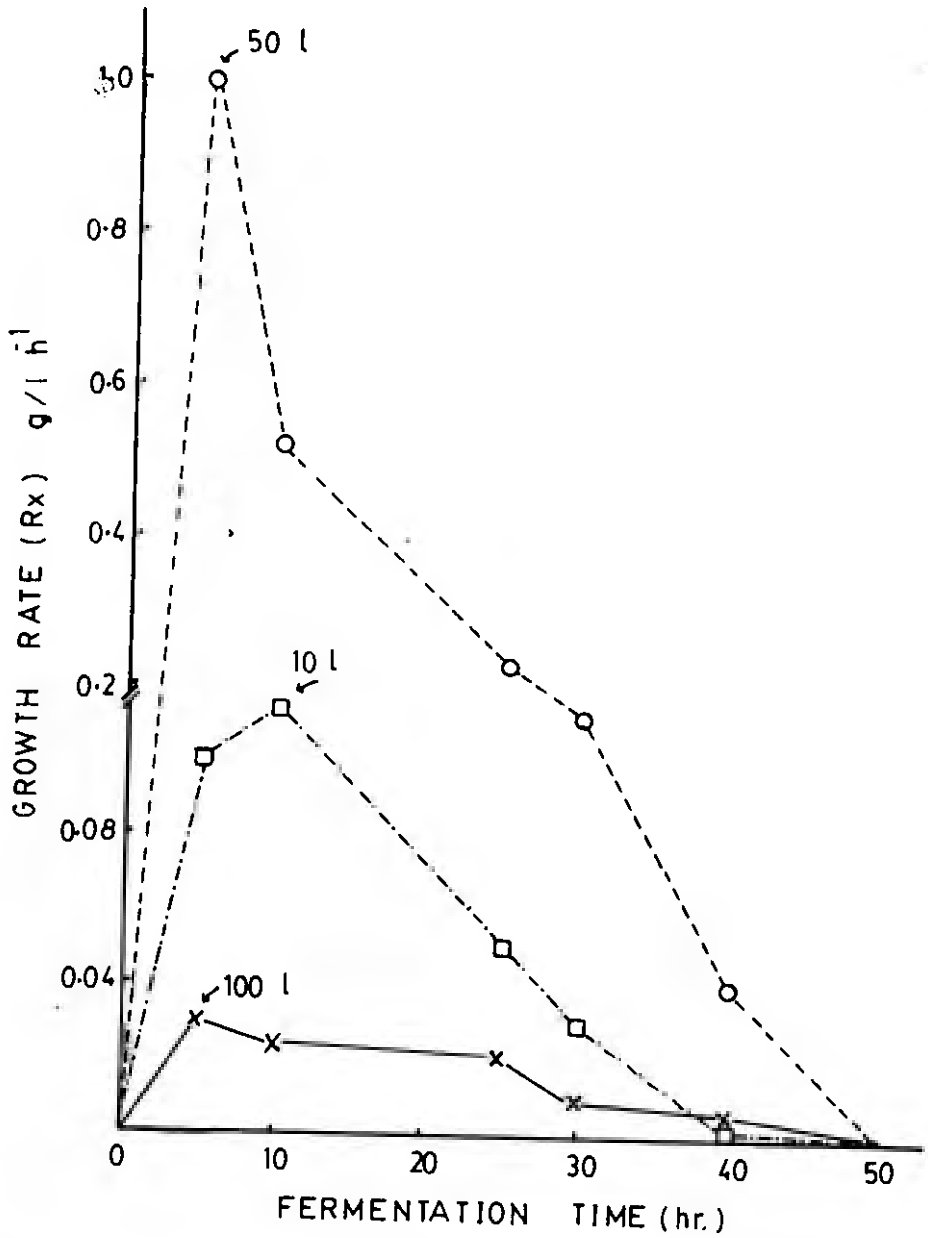


Fig.48 - Growth rate of Xanthomonas cucurbitae PCSIR-52 in Sucrose salt medium in 10, 50 and 100 liter fermenter.

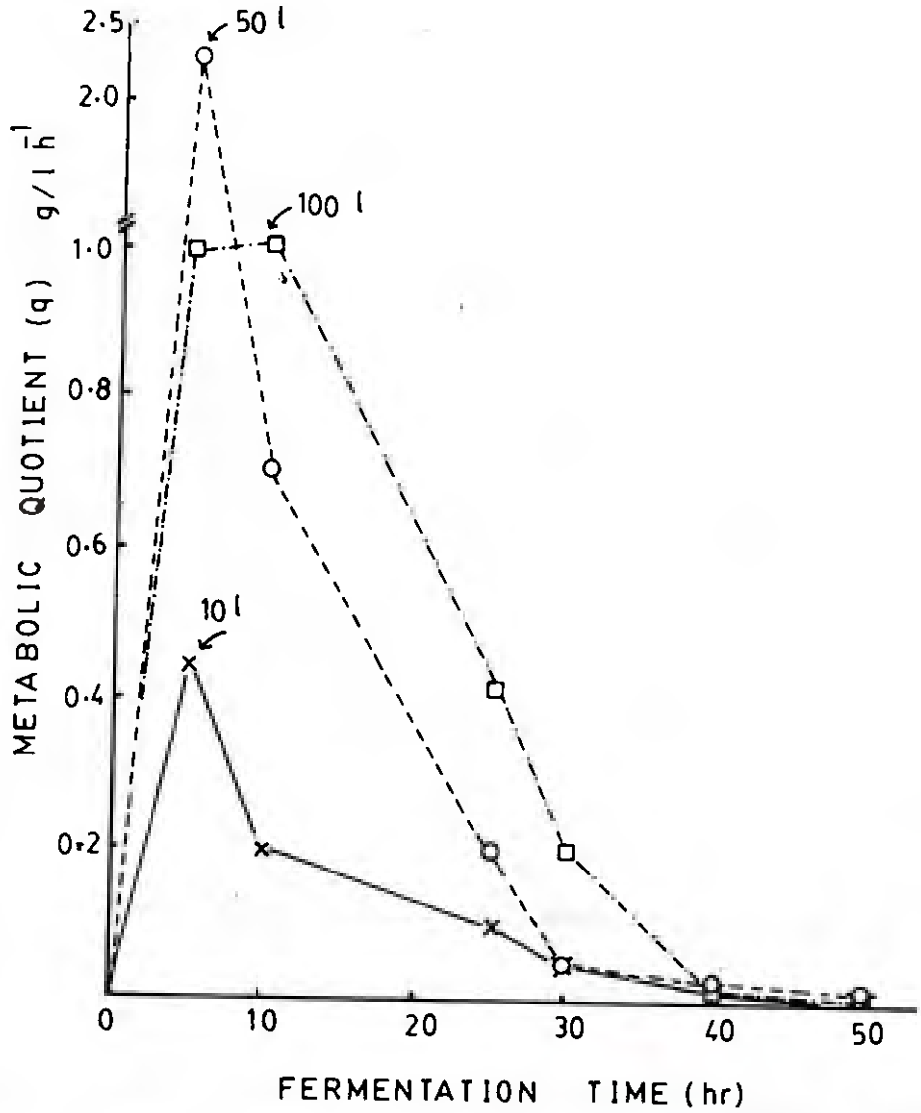


Fig.49 - Metabolic quotient of Xanthomonas cucurbitae PCSIR-52 using Sucrose-salt medium in 10, 50, and 100 liter fermenters.

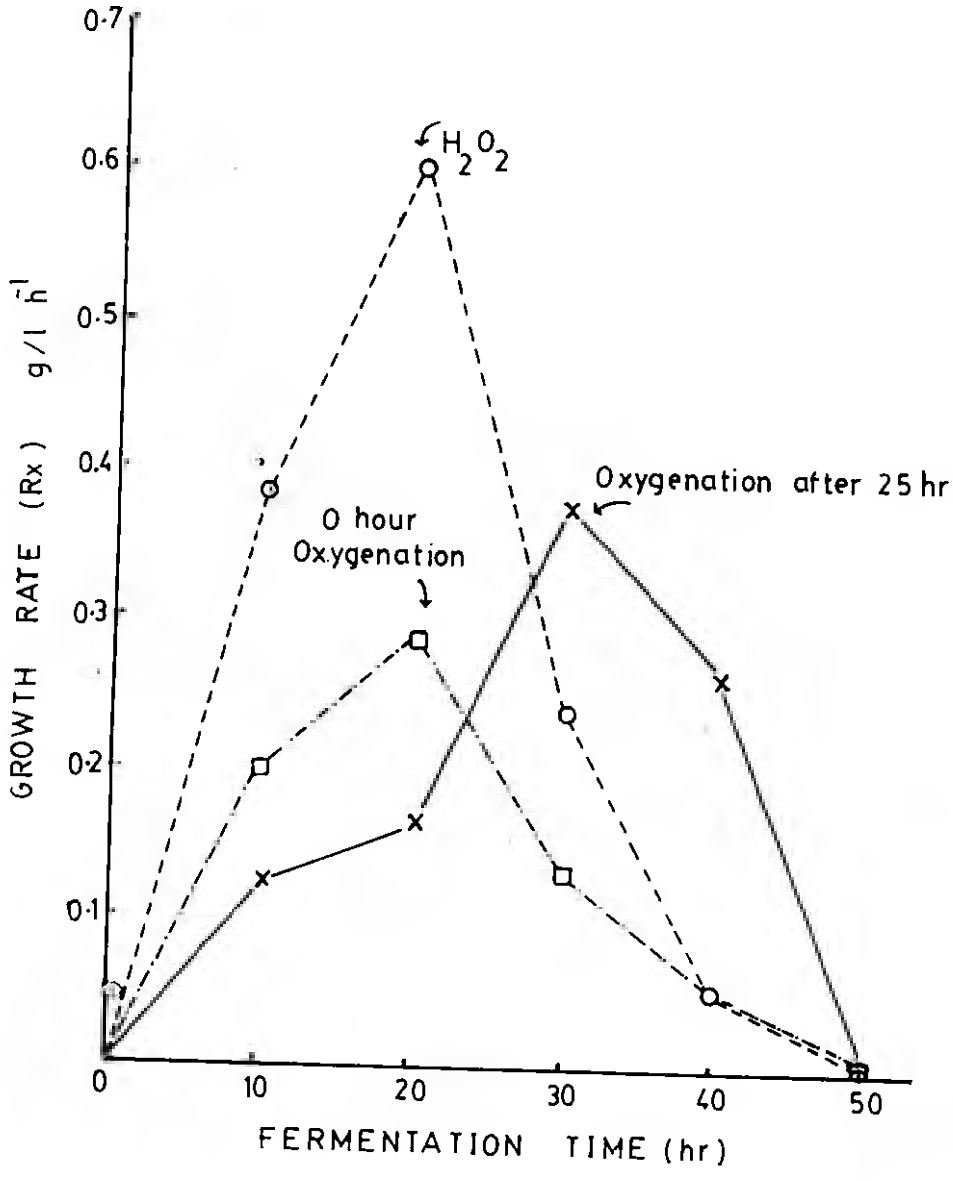


Fig.51 - Effect of Oxygenation on the growth rate of Xanthomonas cucurbitae PCSIR-52 in Sucrose - salt medium in 10 liter fermenter.

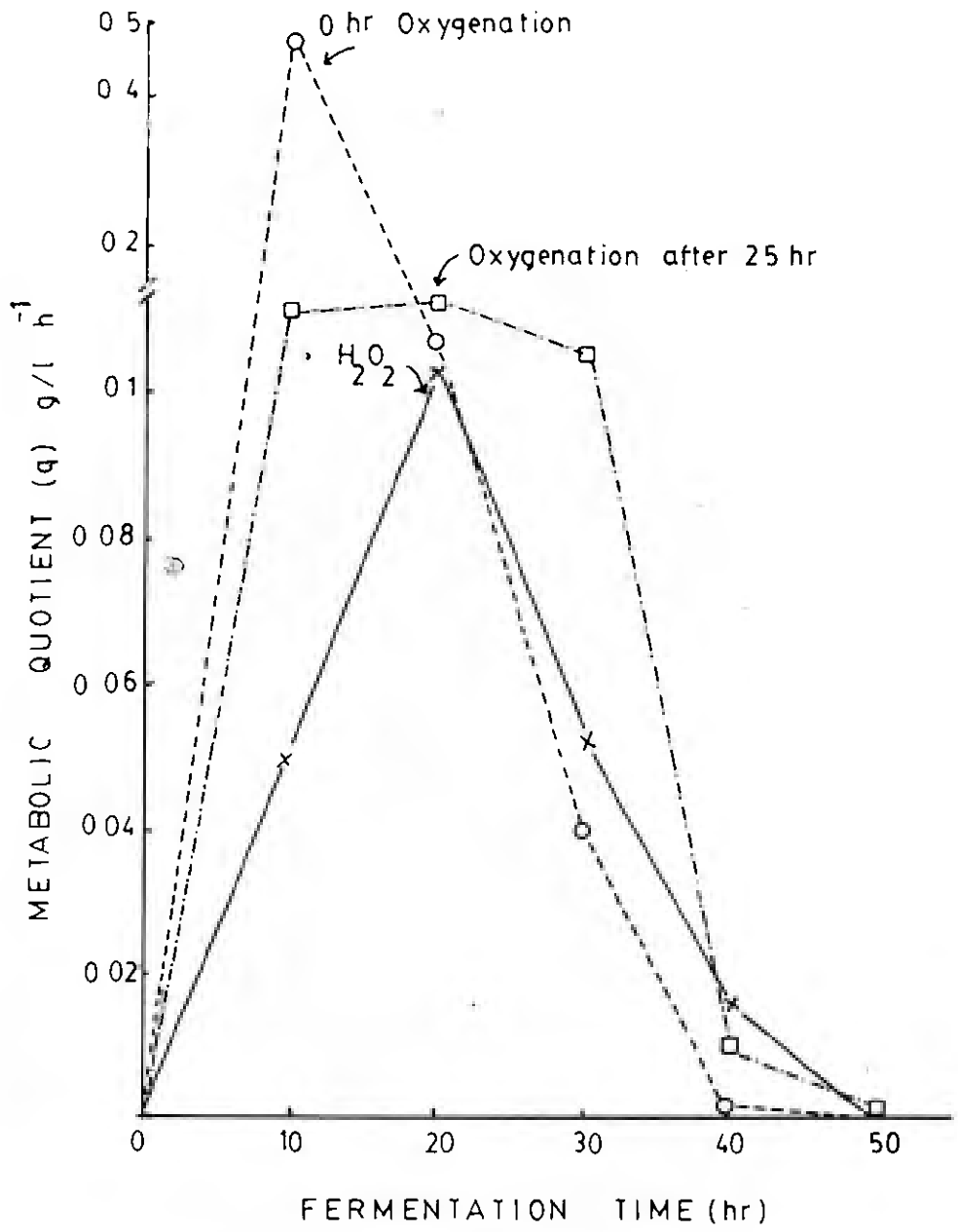


Fig. 52- Effect of Oxygenation on the Metabolic quotient of Xanthomonas cucurbitae PCSIR-52 in Sucrose – salt medium in 10 liter fermenter.

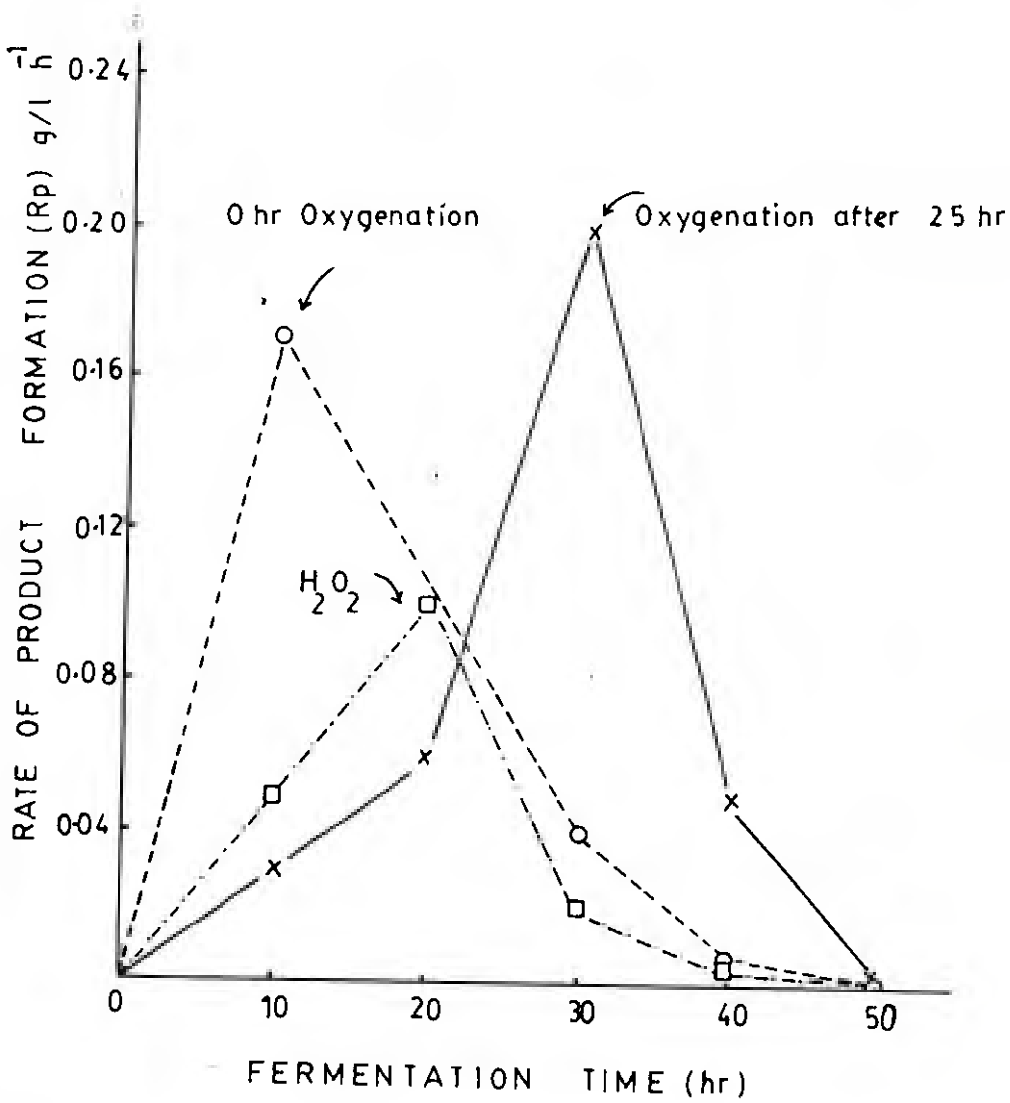


Fig.53 -Effect of Oxygenation on the rate of product formation by Xanthomonas cucurbitae PCSIR-52 using Sucrose-salt medium in 10 liter fermenter.

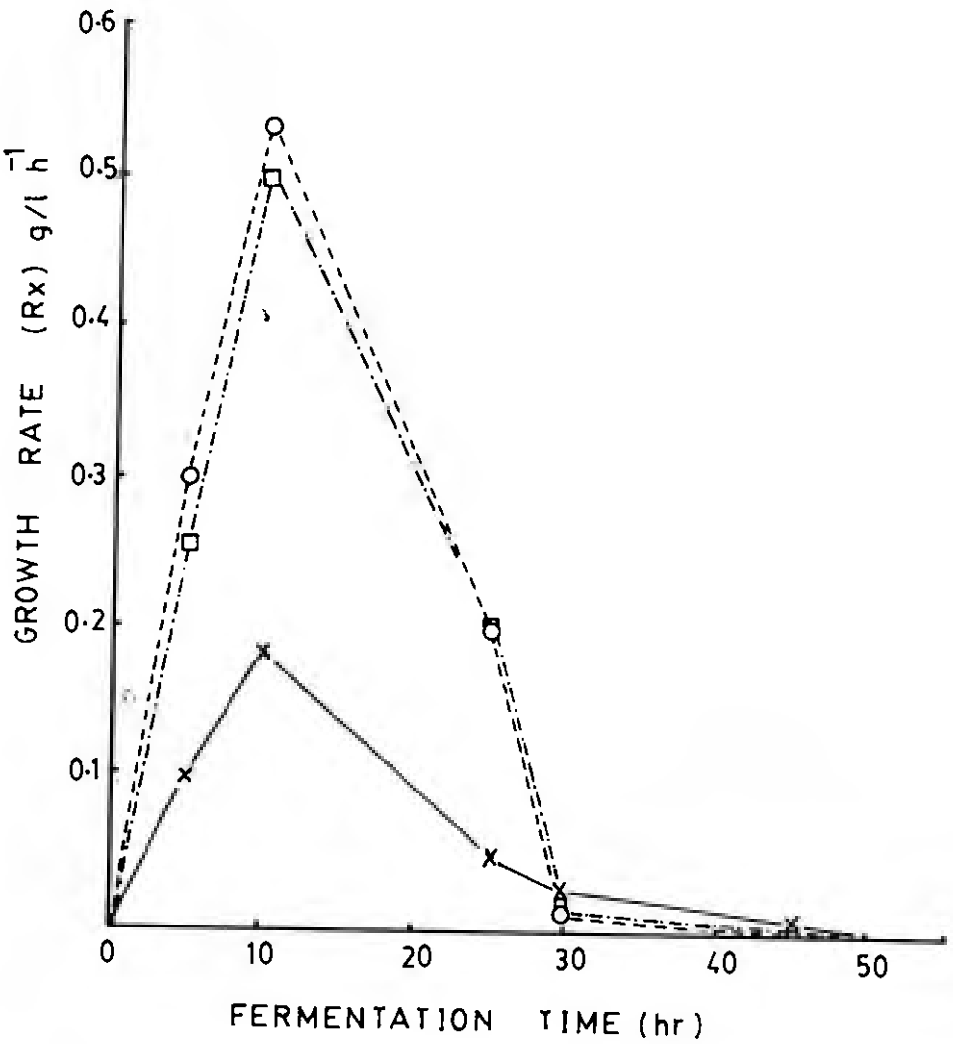


Fig. 54 - Growth rate of Xanthomonas cucurbitae PCSIR-52 in three cycles of Repeated-batch process using Sucrose-salt medium in 10 liter fermenter.

(1st cycle O--O ; 2nd cycle □—□ ; 3rd cycle x——x)

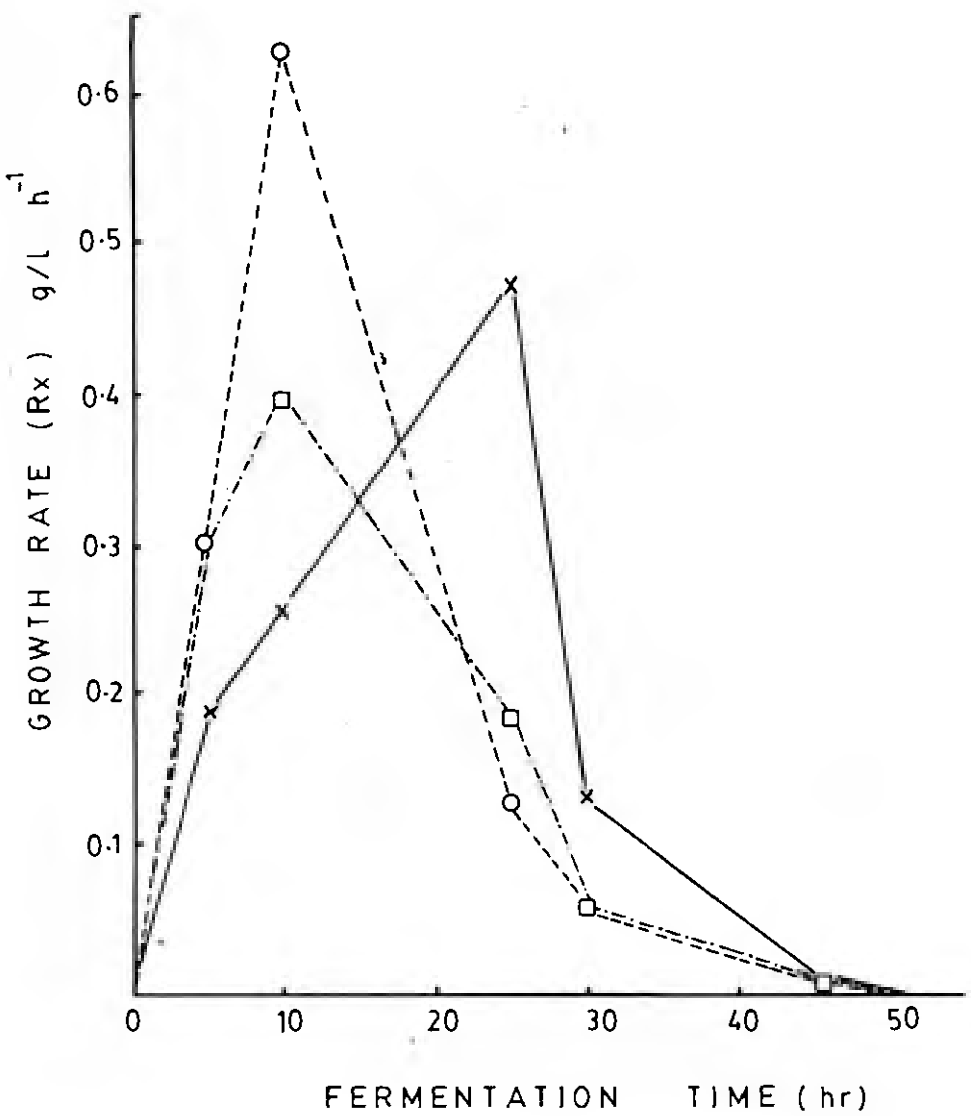


Fig.55- Growth rate of Xanthomonas cucurbitae PCSIR-52 in three cycles of Repeated batch process using Sucrose-salt medium (with Proflo) in 10 liter fermenter.

(1st cycle O--O , 2nd cycle □--□ , 3rd cycle ×—— ×)

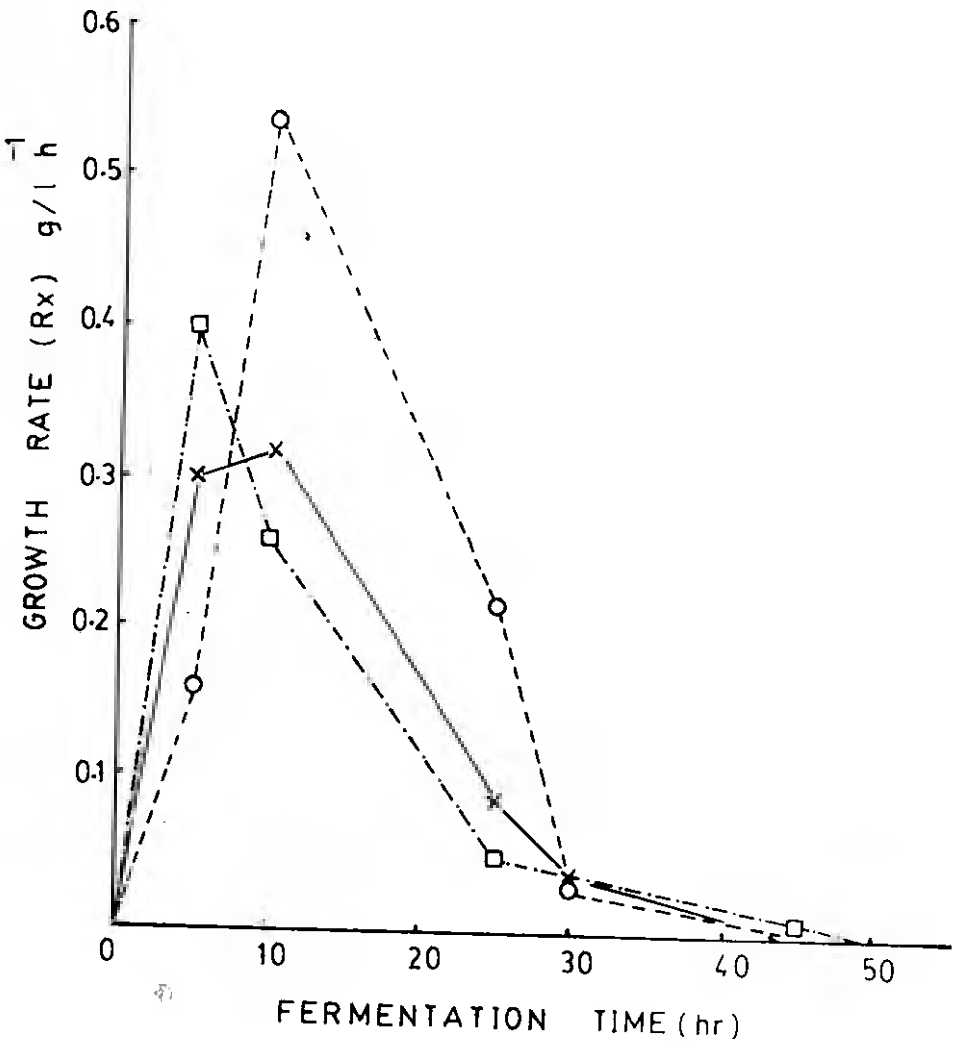


Fig. 56 – Growth rate of Xanthomonas cucurbitae PCSIR-52 in three cycles of Repeated-batch process using Cane juice in 10 liter fermenter.

(1st cycle o–o ; 2nd cycle □–□ ; 3rd cycle x——x)

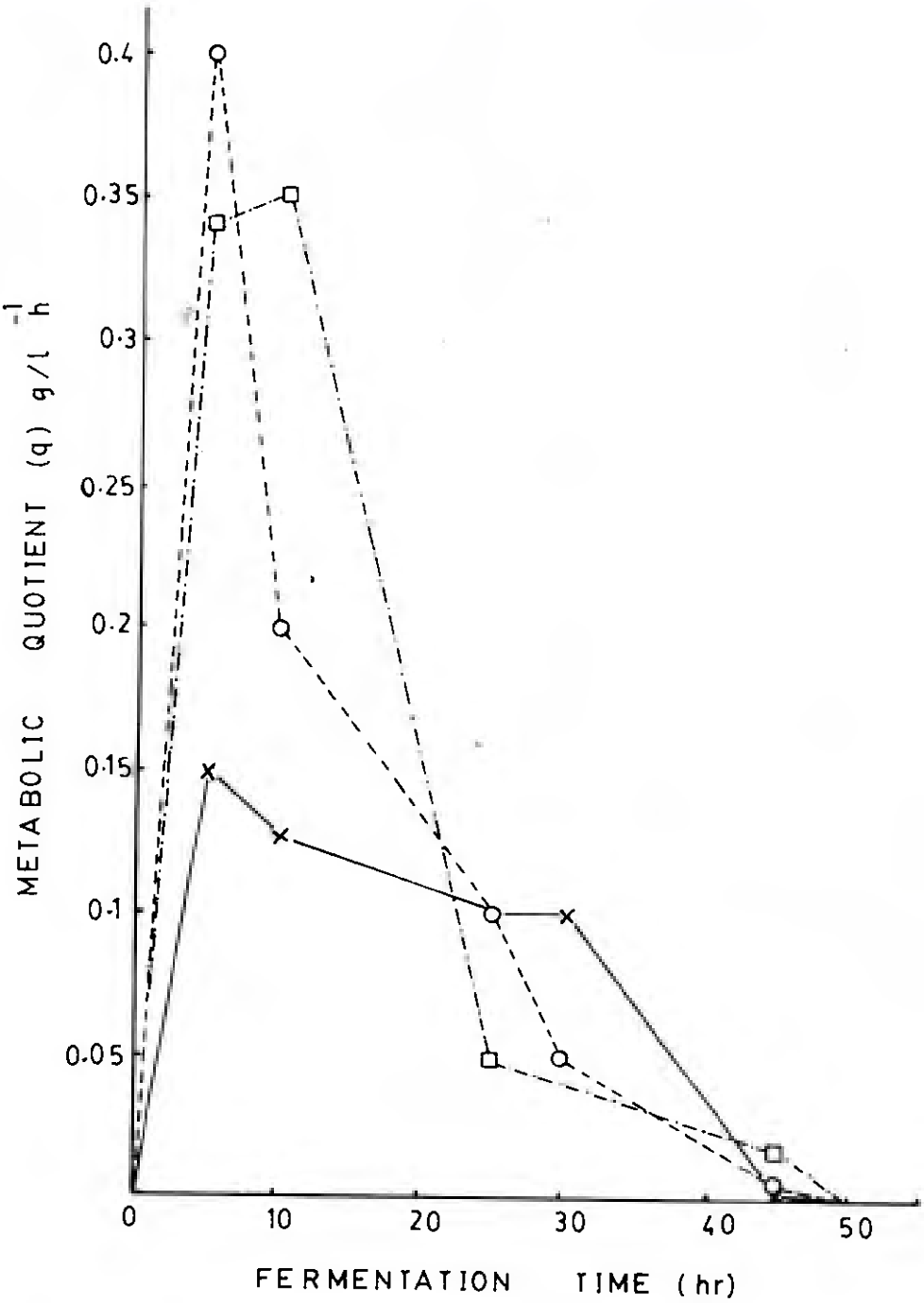
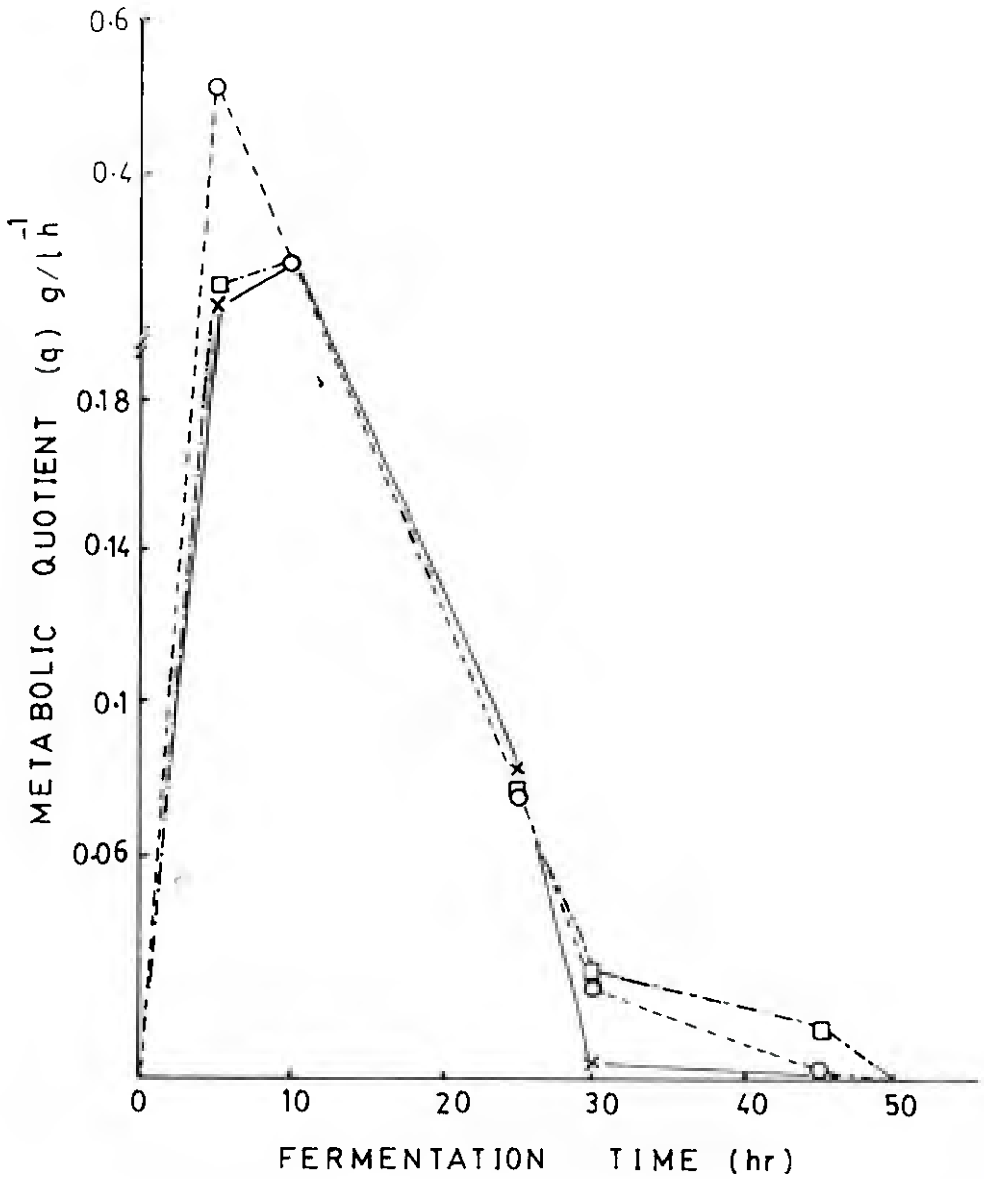


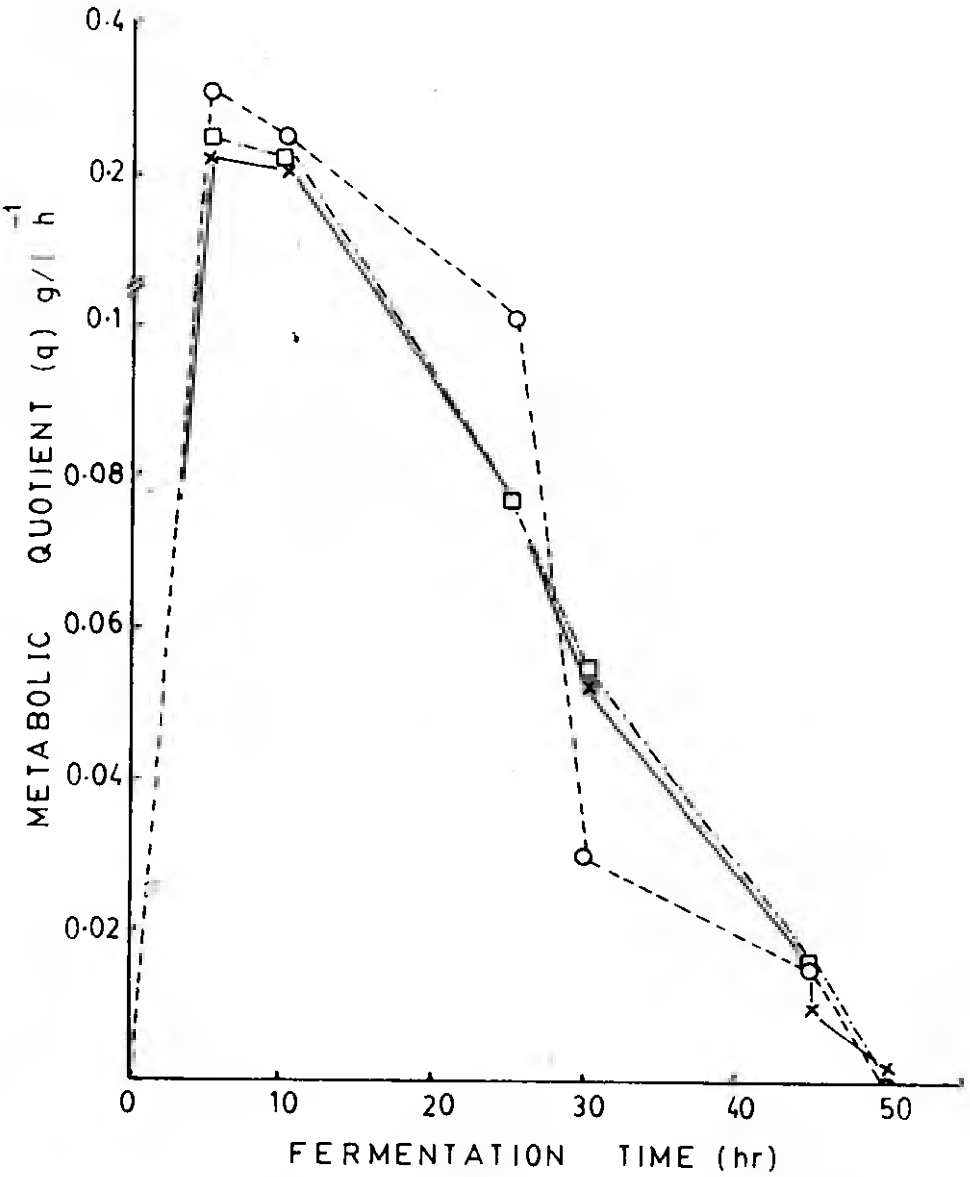
Fig.57 - Metabolic quotient in three cycles of Repeated-batch process using Sucrose - salt medium by Xanthomonas cucurbitae PCSIR-52, in 10 liter fermenter.

(1st cycle o--o ; 2nd cycle □-·-□ ; 3rd cycle x——x)



Fig·58- Metabolic quotient in three cycles of Repeated-batch process using Sucrose-salt-proflo by Xanthomonas cucurbitae PCSIR-52, in 10 liter fermenter.

(1st cycle O---O ; 2nd cycle □-·-□ ; 3rd cycle x——x)



Fig·59 – Metabolic quotient in three cycles of Repeated-batch process using Cane juice by Xanthomonas cucurbitae PCSIR 52 in 10 liter fermenter.

(1st cycle O---O , 2nd cycle □-·-□ , 3rd cycle x—x)

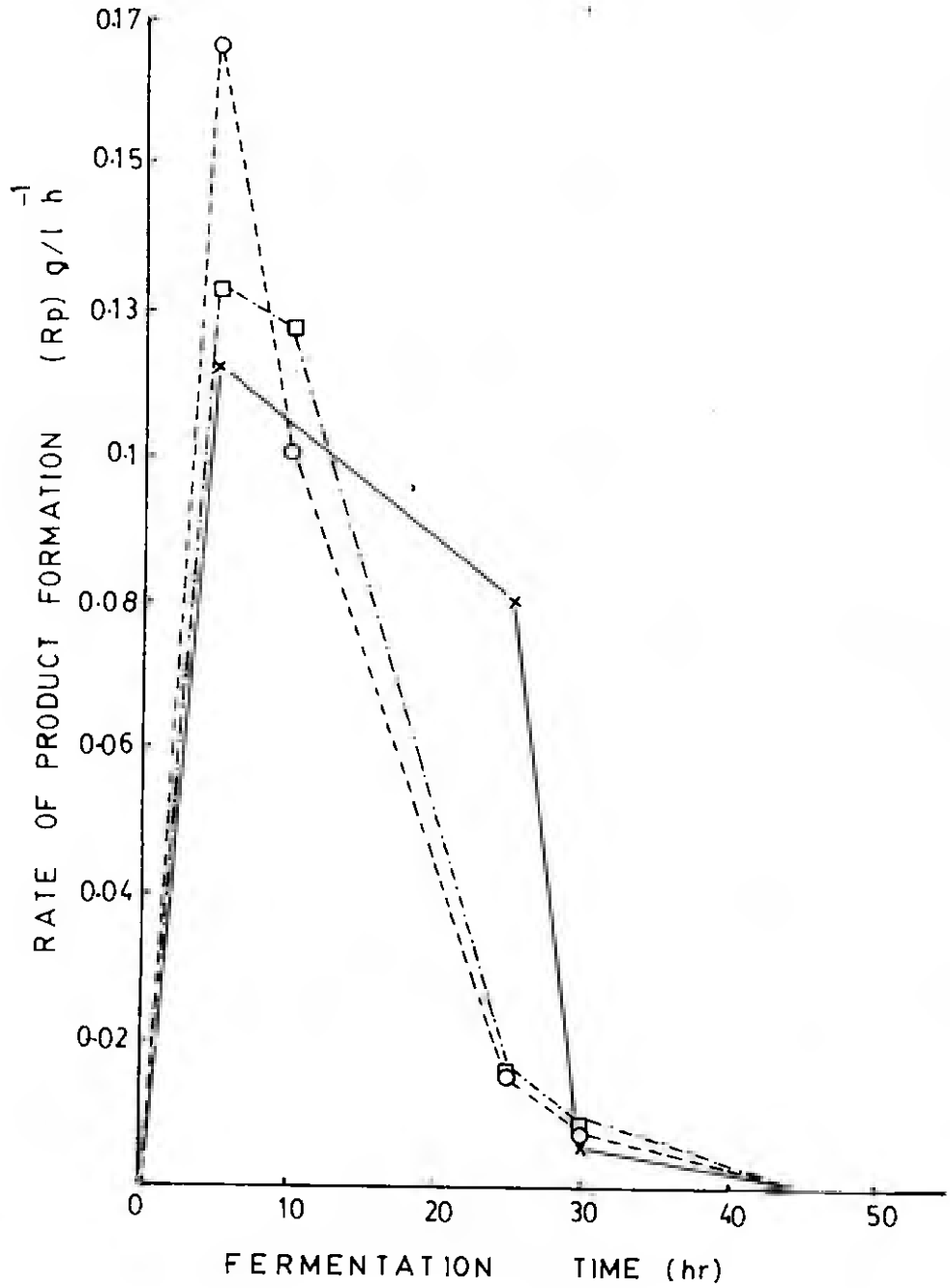


Fig. 60- Rate of product formation in three cycles of Repeated - batch process using Sucrose - salt medium by Xanthomonas cucurbitae PCSIR-52, in 10 liter fermenter.

(1st cycle O--O , 2nd cycle □-□ , 3rd cycle x——x)

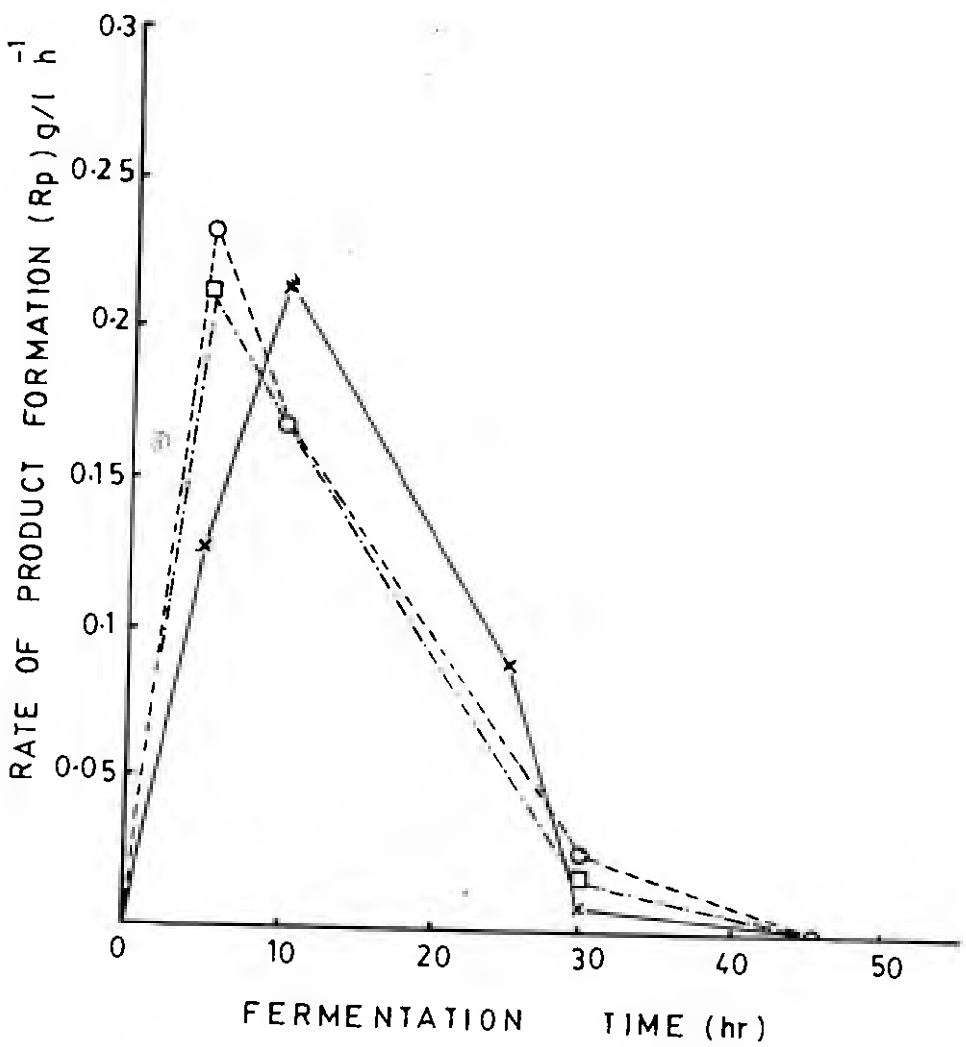


Fig.61 - Rate of product formation in three cycles of Repeated-batch process using Sucrose–salt–proflo medium by Xanthomonas cucurbitae PCSIR-52 in 10 ℓ fermenter.

(1st cycle O--O , 2nd cycle x—x , 3rd cycle □-□)

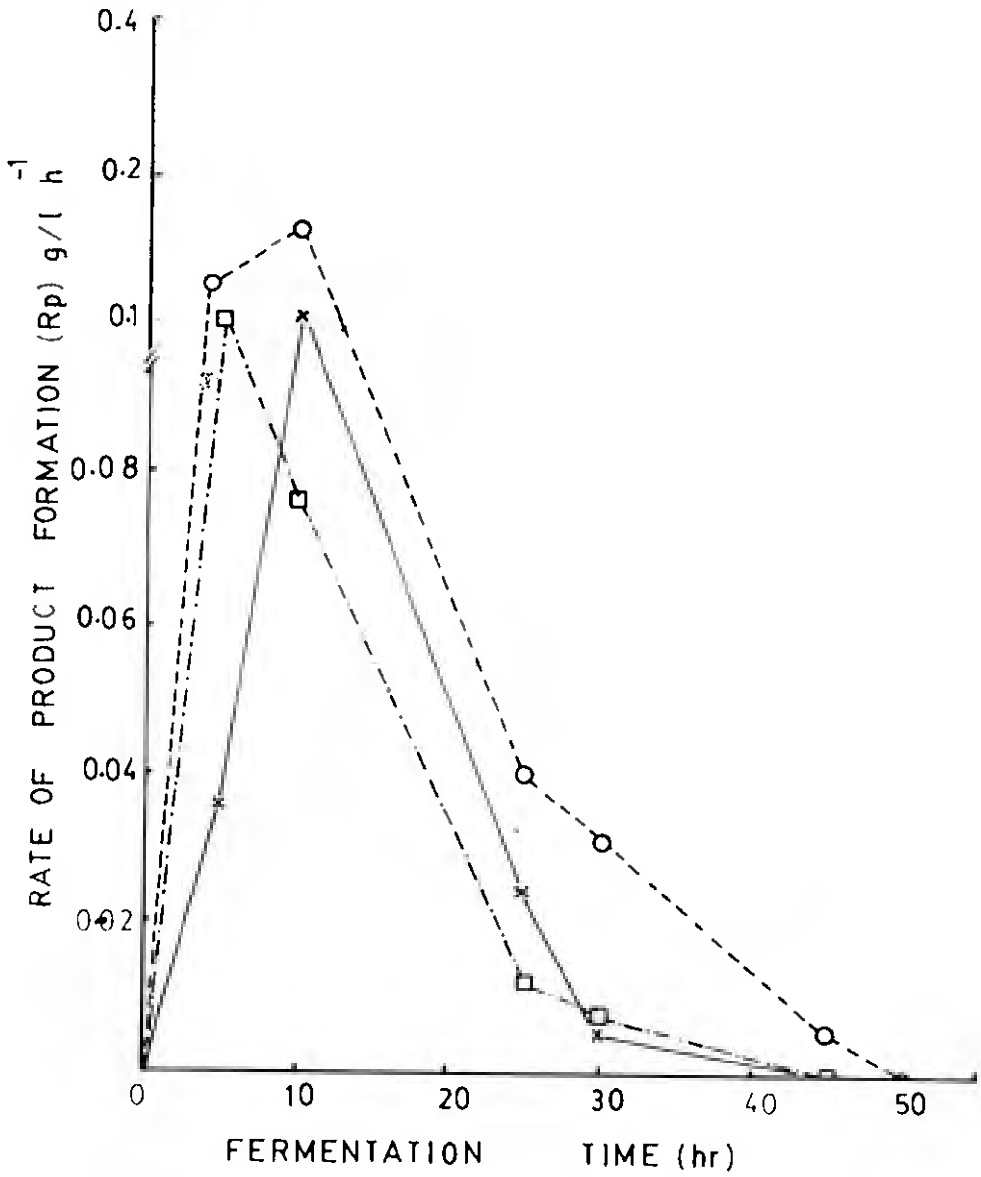


Fig.62 - Rate of product formation in three cycles of Repeated-batch process using Cane juice by Xanthomonas cucurbitae PCSIR-52 in 10 liter fermenter.

(1st cycle O-O , 2nd cycle ×—×, 3rd cycle □-□)

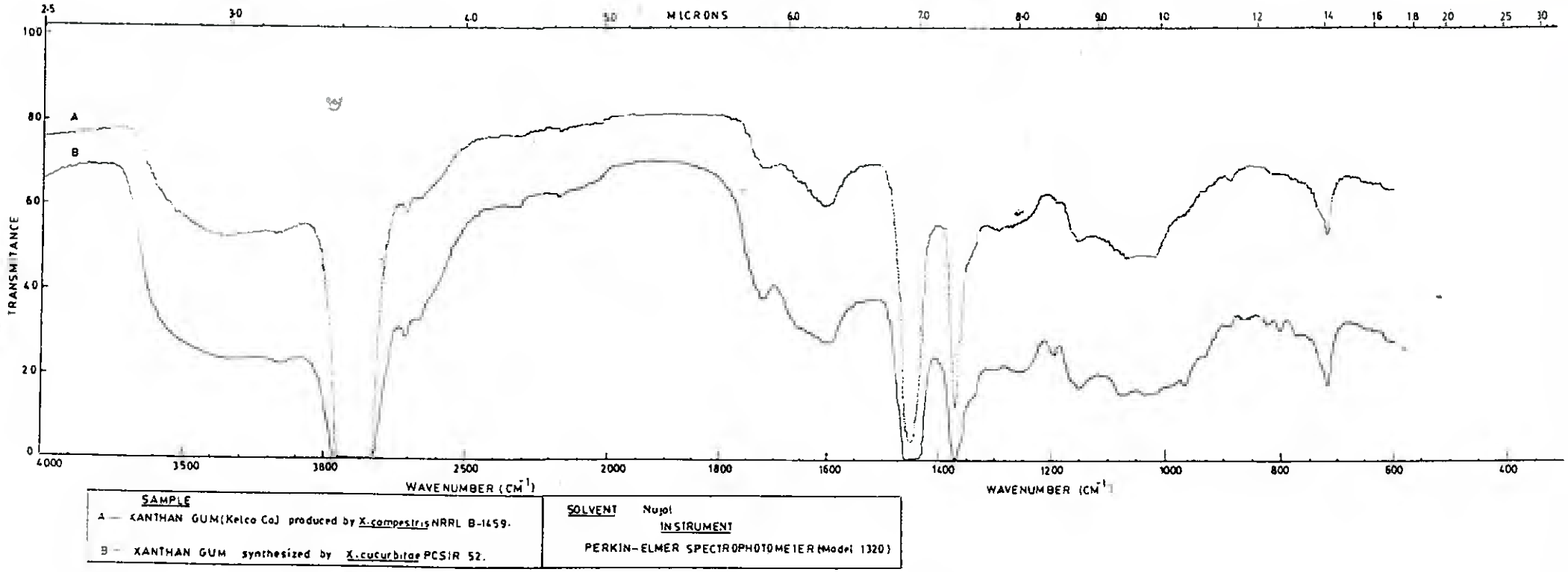


Fig. 63 — [redacted] — Infrared Spectra of Xanthan Gums

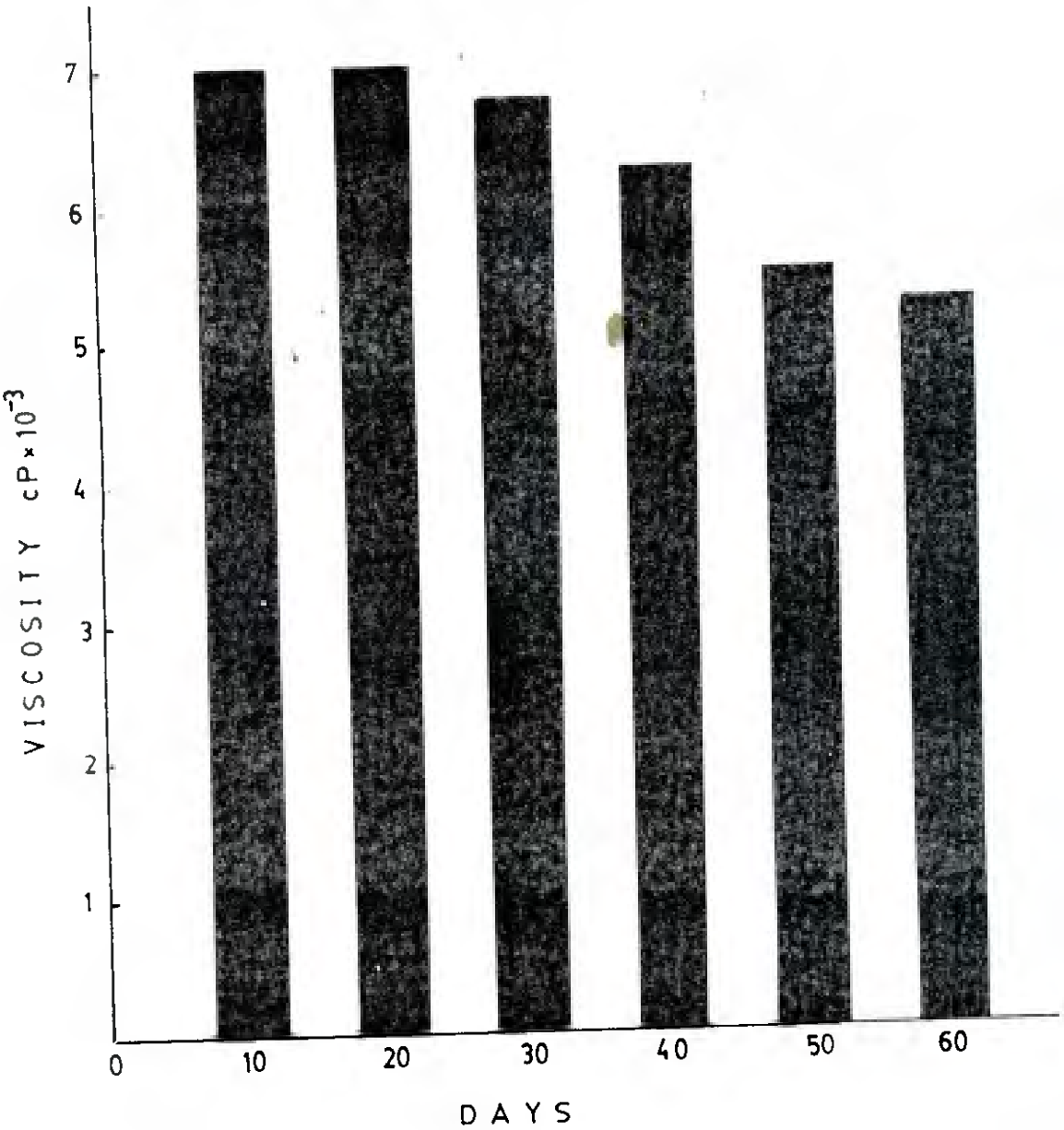


Fig. 64-Variations in the keeping quality of crude Xanthan solution after the addition of 0·3 % V/v formaldihyde.