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Influence of nutritional factors on xanthan production by *Xanthomonas malvacearum*

T. Selva Mohan, R.Babitha

P.G Department of Micro Biology, Sivanthi Aditanar College, Moniketti Pottal(P.O) Kanyakumari, India

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ABSTRACT

Xanthan gum is an extra cellular microbial hetero polysaccharide of great commercial importance, secreted by the yellow pigmented bacterium Xanthomonas malvacearum. Polysaccharides are macromolecules with diverse applications in the food, chemical, energy production and pharmaceutical Industries. The nutrient factors influencing the production of Xanthan polymer by Xanthomonas malvacearum isolated from infected cotton leaves were investigated. Xanthomonas malvacearum showed the highest yield of polymer 0.621 kg of sucrose at 48 hours. Xanthan production and biomass with varying carbon source, nitrogen source, different pH and different temperature were investigated. Physical characterization of Xanthan is solubility. Chemical characterization of Xanthan is Osazone test were investigated crystal shaped structure are viewed on viewing under microscope .To degrading the Xanthan gum using bacterial strains.

Keywords: *Xanthan*, Polysaccharide, Exopolysaccharide.

INTRODUCTION

Exopolysaccharides are important constituents of the surface of bacterial cells and play a critical role in the interaction of bacteria with the environment. Many bacteria produce complex Exopolysaccharide (EPS) which can remain attached to the cell surface in a capsular form or be released as slime. Exopolysaccharides producing microorganisms occur widely in nature in different types of habitat. Among the large number of isolated type of the genus *Xanthomonas*, many are mucoid that is capable of producing the polysaccharide called xanthan [9]. Xanthan gum is an extra cellular microbial hetero polysaccharide of great commercial importance, produced by the yellow pigmented bacterium Xanthomonas *malvacearum.* Xanthan gum has unusual rheological properties and is extensively applied in various industries as a stabilizing, emulsifying, suspending and thickening agent Xanthan gum is used as a food additive and rheology modified is produced by a process involving fermentation of glucose or sucrose by the xanthomonas species [7].).Bacteria of the genus xanthomonas are yellow pigmented, motile,

aerobic, gram negative rods. *Xanthomonas malvacearum* causes seedling blight, leaf spot, boll rot symptoms are severe. It infects Gossypium hirsutum a tetraploid species [3]. .

Xanthan gum was discovered an extensive research effort by Allene Rosaline Jeanes and her research team at the united states Department of agriculture which involved the screening of a large number of biopolymers for their potential uses. It was brought in to commercial production by the kelco company under the trade name Kelzan in the early 1960's. It was approved for used in foods after extensive animal testing for toxicity in 1968. It is accepted as a safe additive in the USA, Canada and Europe [1]. .

Polymers are recalcitrant to microbial degradation, they would remain in land fill sites semi permanently, polymer waste is recognized as one of the most troubles some categories of waste, and disposal of polymer waste has been blamed for shortening the life of landfill sites. In response to this problem some microorganisms and their enzymes have been reported to participate in the depolymerization of the polysaccharide, enzymes responsible for the complete depolymerization of Xanthan have not been identified.

Unlike other gums, it is very stable under a wide range of temperatures and pH. It helps to prevent oil separation by stabilizing the emulsion although it is not emulsifier.Xanthan gum also helps suspend solid particles, such as spices and also used in frozen food and beverages. Toothpaste often contains Xanthan gum where it serves as a binder to keep the product uniform. Xanthan gum also helps thicken commercial egg substitutes made from egg whites to replace the fat and emulsifier found in yolkes. In the oil industry, Xanthan gum is used in large quantities, usually to thicken drilling mud. In cosmetics Xanthan gum is used to prepare watergels usually in conjunction with benzoate days. Xanthan gum is a common ingredient in fake blood recipes. In the present study *Xanthomonas malvacearum* was isolated from the cotton leaf. In order to determine the yield of Xanthan production and also this paper describes the influence of pH, temperature, different carbon source and nitrogen sources on the production of xanthan polymer and degraded the polymer by using microbes [11].

MATERIALS AND METHODS

Bacterial Strain

Xanthomonas malvacearum was isolated from infected cotton leaves (*Gossypium hirsutum*) collected from a cotton field.

Isolation of *Xanthomonas Malvacearum* from Infected Leaves

The blight infected cotton leaves were collected from the cotton field. The diseased spots were dissected from the leaf and surface sterilized with mercuric chloride (0.1%w/v). The diseased leaf discs were then thoroughly washed with sterile distilled water and transferred aseptically to Hoitink and Sinden's (HS) medium plates. The plates were incubated at 30 degree Celsius for three days. A yellow bacterium was found to emerge from the diseases spots kept on the HS medium plates. [2].

Biochemical Identification Of *Xanthomonas Malvacearum*

The organism was confirmed by the routine bacteriological tests following **Berge's manual** to establish that the organism isolated from the cotton leaves was *Xanthomonas malvacearum.*

Inoculums Development

Xanthomonas malvacearum and slants were first transferred to freshly prepare each 50 ml of growth medium in 250 ml Erlenmeyer flask. After 48 hours incubation 10ml of this culture were used to inoculate in each 90 ml of the production medium, with carbon sources and nitrogen sources.

Nitrogen Source

2% of commercially available nitrogen sources such as peptone, beef extract, meat extract, tryptone and black gram husk were tested to determine the yield of Xanthan at 30^0C for 96 hours. Samples were drawn at every 24 hour interval for the analysis of Biomass production and Xanthan production. Optimization of Xanthan Production of *Xanthomonas Malvacearum* at Different pH, Temperature in the Production Medium

To determine the optimal temperature and optimal pH for Xanthan production the culture was incubated at different temperatures (27^0C, 37^0C, 47^0C) and different pH (5, 7, and 9) maintained in Xanthan production broth. The sample was collected after at 24 hour incubation. The production of Xanthan and Biomass were determined. [6].

Analytical Determination

Estimation of Biomass

Growth of the organism was determined by measuring the dry weight of the washed cell mass, cell containing broth (10 ml) was centrifuged at 5000 rpm for 15 minutes. The pellet was washed twice with demonized water and dried to a constant weight at 80-100^0C [12].

Estimation of Xanthan

10 ml of sample was centrifuged at 10,000 rpm for 15 minutes. The 10 ml of the supernatant was taken and add 2 to 3 volumes of (95% v/v) isopropyl alcohol were added with shaking to precipitate out the polysaccharide. The precipitate was separated by centrifugation at 4000 rpm for 20 minutes. The residue was transferred to preweighted aluminum foil cups and dried for 18 hours in hot air oven at 60 degree Celsius. The cups were cooled at 30 degree Celsius for 1 hour and their dry weight gave the Xanthan production of the fermented broth [12]. .

Isolation of Plasmid

Plasmid isolated by lyzozyme method

Mutation

A loopful of culture from the growth medium was inoculated into the Czapek dox broth containing 2% of gelatin. Then the tubes with 5 ml of cultures in 5 tubes were to the UV light for 5, 10, 15, 20 and 25 minutes respectively czapek dox plates were prepared and UV exposed cultures were spreaded in respective plates.

Enumeration of Eps Degrading Microbes

To enumerate the polymer degrading microbes 1 gm of polymer was dissolved in 20 ml of methylene chloride. 1000 ml of basal medium containing 100 mg/liter of triton X100 was added to the medium. Enumeration of polymer degrading microorganism was performed by the plate count technique. One gram of the soil sample was suspended in 99ml of distilled water. After 30 minutes of agitation the suspense was serially diluted and plated on to the media and the microorganism were cultivated at 28^0C for 10 days. [10]...

Physical Characterization of the Eps Solubility

In order to find out the solubility of the exopolysaccharide in different solvents, 100 mg of EPS was taken in different eppendroffs to which 2 ml of solvents such as water, acetone, chloroform, ethanol, butanol and benzene were added, mixed thoroughly using a mixture and observed for pellet formation.

Chemical Composition of Eps Osazone Test

To 0.5ml of phenyl hydrazine hydrochloride, 0.1 g of sodium acetate, 10 drops of glacial acetic acid and 5 ml of the hydrolyzed sample were added. The mixture was heated on a boiling water bath for about half an hour and cooled slowly which was then examined under a microscope for crystal formation. [8].

Estimation of Protein

A standard graph was prepared at different dilution (0.1 to 0.5 ml) of BSA (10 mg/100 ml) in test tubes. To this 4 ml of biurette reagent was added and incubated for 30 minutes. The optical density was measured at 520 nm by spectrophotometer. A standard graph was plotted by the protein concentration on x axis and optical density at Y axis.

A known amount of the sample was taken and ground it well with 80% of ethanol and centrifuged for 5 minutes at 5000 rpm. One ml of supernatant makeup to 5 ml within NaOH. Then from this 0.5 ml was taken and 5 ml of solution C was added finally. After incubation 0.5 ml of foline phenol reagent was added. The intensity of colour developed was measured using spectrophotometer at 640 nm.

RESULTS

Xanthomonas malvacearum was isolated from the infected cotton leaves. Fig. 1 showed the xanthan production obtained by the *Xanthomonas malvacearum*, under the production process with various carbon sources (sucrose, fructose, rice bran, wheat bran, molasses and grape juice) are tested. The most suitable for xanthan production is sucrose and the maximum is found to be 0.621 mg/10 ml at 48 hours. The xanthan production obtained by the *Xanthamonas malvacearum* under the production process with various nitrogen sources (Peptone, tryptone, meat extract, beef extract, soybean meal and black gram husk) are tested. The most suitable for xanthan production is peptone and the maximum is found to be peptone 0.615 mg/10 ml at 24 hours.

Fig.2 showed the biomass production obtained by the *Xanthomonas malvacearum,* under the production process with various carbon sources is tested. The maximum biomass production obtained by rice bran and the maximum is found to be 0.085 mg/10 ml at 48 hours. The effect of different nitrogen sources on the biomass production *Xanthomonas malvacearum*. The maximum biomass production is peptone found to be 0.225 mg/10 ml at 48 hours.

Fig.3 Showed The effect of optimal temperature and pH in the xanthan production of different carbon source by *Xanthomonas malvacearum* in Among different pH and temperature were tested, pH7 and the 37^0C appear to be most suitable for xanthan production of sucrose and it is found to 0.330 mg/ml and the minimum Xanthan production in pH5 and 47^0C and it is found to be 0.025 mg/10 ml. The effect of different pH, temperature and different nitrogen sources on the xanthan production of *Xanthomonas malvacearum* pH7 and temperature 37^0C appear to be most suitable for xanthan production of peptone and it is found to be 0.256 mg/10 ml.

Fig.4 showed that the optimal pH, temperature and different carbon sources on the biomass production of *Xanthomonas malvacearum* pH7 and temperature 37^0C appear to be most suitable for biomass production of sucrose and it is found to be 0.14 mg/10 ml. The effect of different pH, temperature and different nitrogen sources on the biomass production of *Xanthomonas malvacearum* pH7 and temperature 37^oC appear to be most suitable for biomass production of peptone and it is found to be 0.062 mg/10 ml.

Fig.5 showed that the estimation of protein in *Xanthomonas malvacearum*.
The resultant colony with halo, which is indicates the xanthan degradation. Such halo formation would suggest the depolymerization of the polymer in the agar plate.
Physical characterization of the EPS solubility, of the EPS was insoluble in other solvents such as chloroform, acetone, ethanol, butanol and benzene.
The hydrolyzed samples were dried for osazone test produced yellow osazone crystals.

Strain improvement by mutation
The mutation and screening industrially useful microorganisms are important for the successful development of the various strains required in the fermentation industry. The productivity of the parent strain under investigation was improved by UV mutation.

UV treatment for 25 minutes resulted in a strain designated as *Xanthomonas malvacearum*. It was observed that zone formation occurs in mutant strains by the addition of mercuric chloride. Zone formation is due to enzyme secretion by *Xanthomonas malvacearum.*

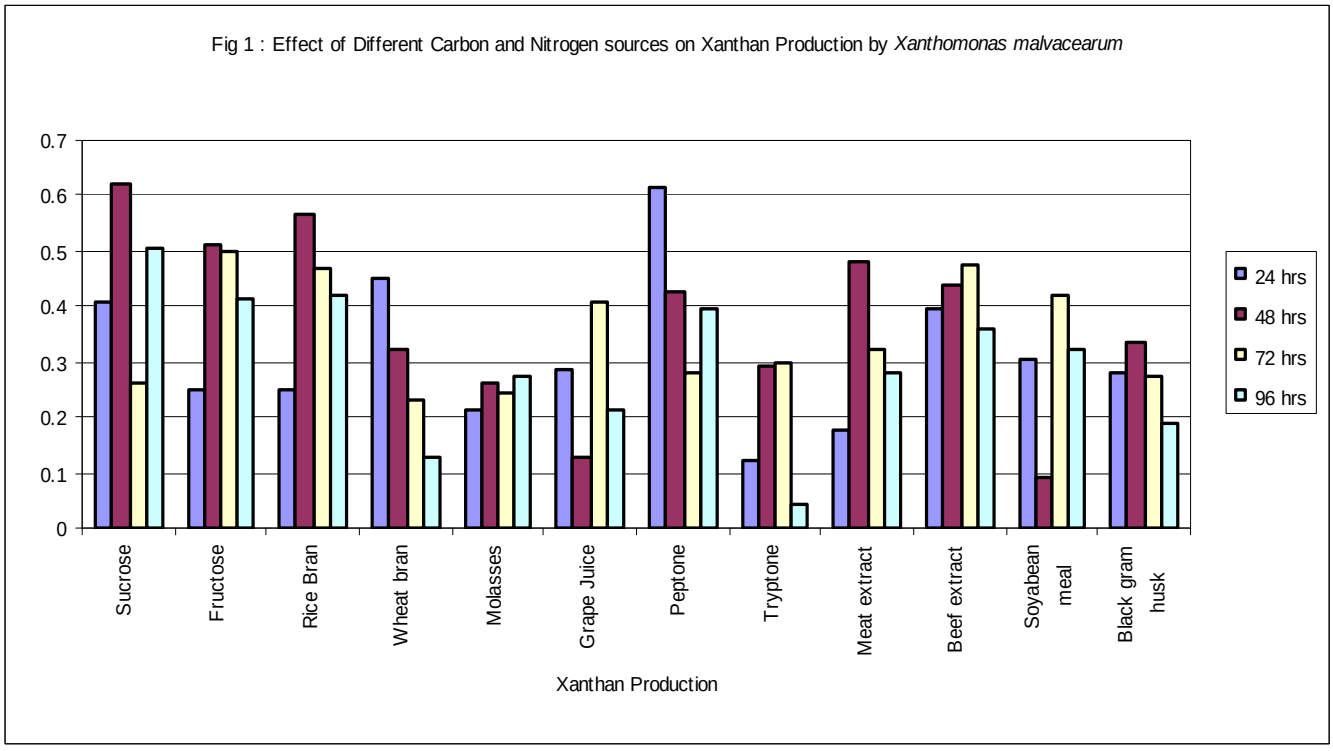


Fig 2 : Effect of Different Carbon and Nitrogen sources on Biomass Production by *Xanthomonas malvacearum*

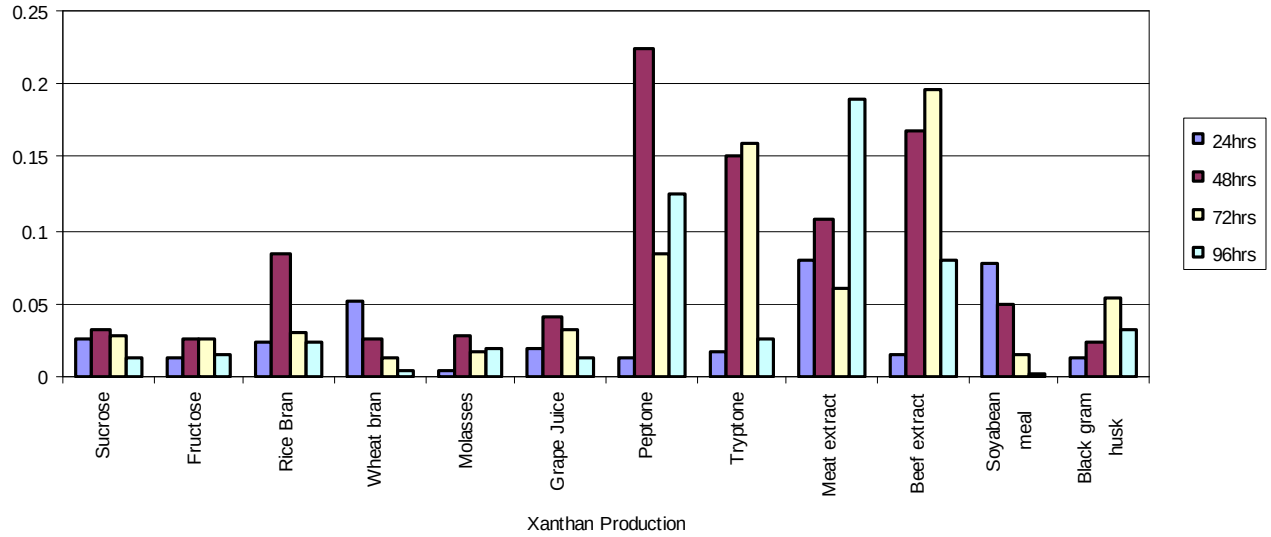
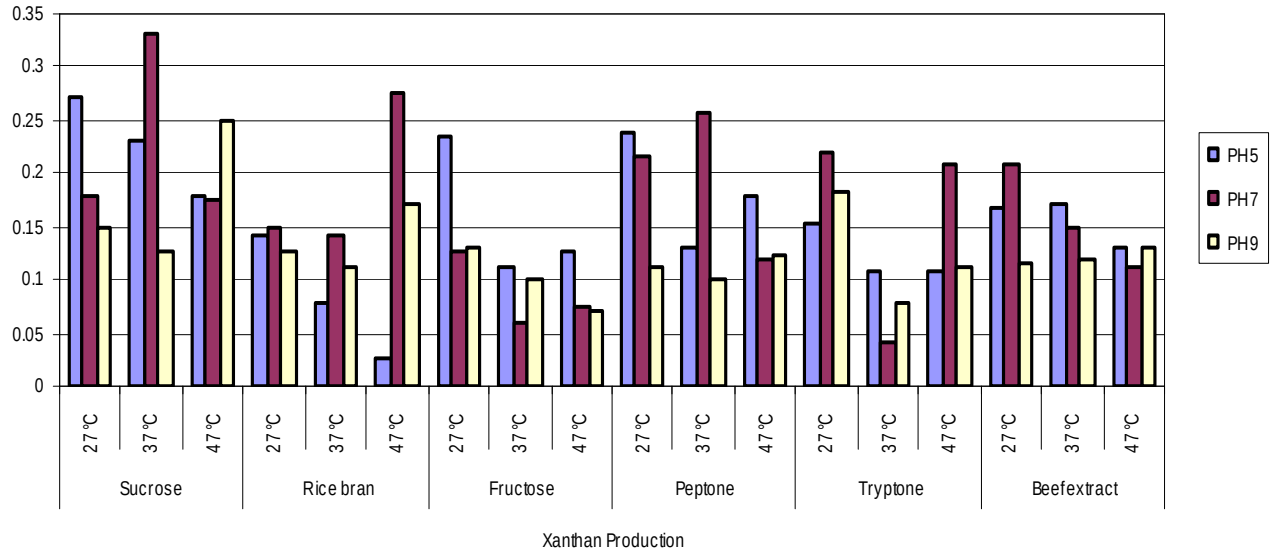


Fig 3 : Effect of Different pH and Temperature on Xanthan Production by *Xanthomonas malvacearum*



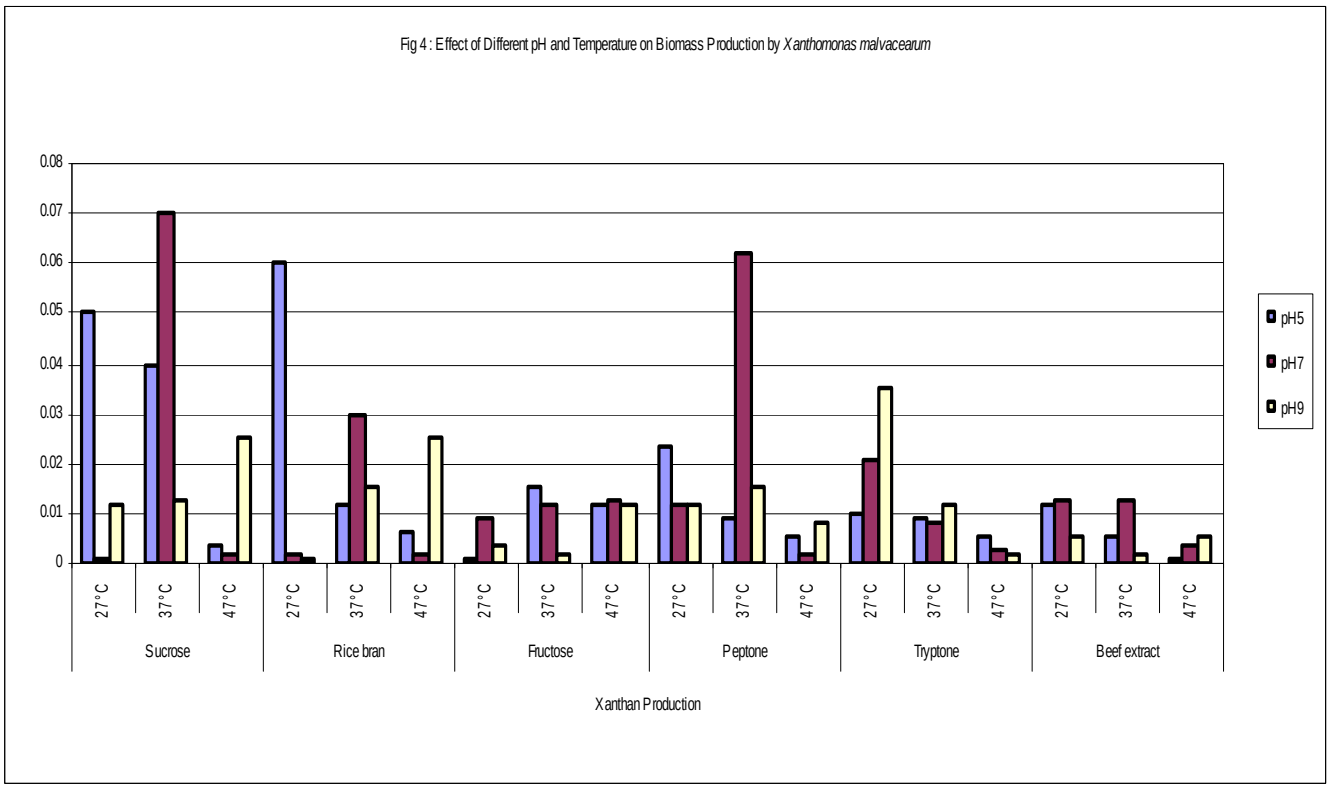
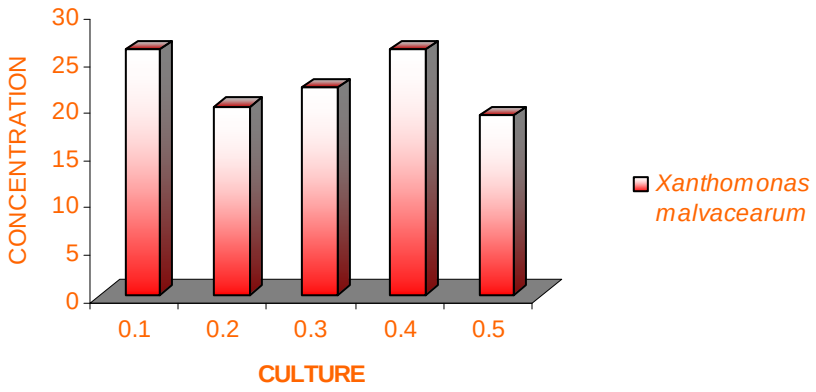


FIG. 5 PROTEIN ESTIMATION OF *XANTHOMONAS MALVACEARUM*



DISCUSSION

The production of xanthan gum, an industrially important microbial exopolysaccharide, was enhanced by using *Xanthomonas malvacearum.* Xanthan gum has unusual rheological properties and is extensively applied in various industries as a stabilizing, emulsifying, suspending and thickening agent.

In this present study the effect of different carbon sources and the nitrogen sources on the production of xanthan by *Xanthomonas malvacearum*. Among the six substrate (sucrose, fructose, rice bran, wheat bran, molasses and grape juice), tested the maximum, production polymer content was obtained with 0.621 mg/10 ml sucrose at 48 hours. During the active phase

of polymer synthesis, sucrose was rapidly utilized leading to its significant reduction in the medium.

[5]. suggested that sucrose gave the highest yield (dry weight) 1199 g/l and glucose was the second 10.8 g/l. Concerning the concentration of sucrose or glucose, the results confirmed that 4% gave the highest yield (dry weight) 14.3 g/l for glucose and 13.5 g/l for glucose.

In this present study the sucrose has gained interest in the production of xanthan. On the other hand the maximum biomass obtained at 48 hours of rice bran as carbon source. [17]. have noted that production of xanthan gum or may not be associated with biomass, Increased exopolysaccharide synthesis after the cessation of biomass.

In this present study the six nitrogen sources (peptone, tryptone, meat extract, beef extract, soyabean meal and black gram husk) tested, the maximum polymer content was obtained with 0.615 mg/10 ml.The Peptone showed the highest polymer production compared to other nitrogen sources. A maximum growth rate within the range $0.11\text{-}0.125^{-1}$ was obtained from the continuous culture data in defined medium, producing xanthan at rate up to $0.36g^{-1}h^{-1}$. Corresponding to a maximum 67% peptone conversion at a dilution rate (D) of 0.05 h^{-1} [13]..

In this present study the three various temperture (27^oC, 37^oC, 47^oC) and different pH (5, 7, 9) tested the maximum xanthan production a pH and the maximum temperature xanthan production is 37^oC. xanthan production of sucrose in pH7 and temperature 37^oC is found to 0.330 mg/10 ml. [16]. suggested that when the initial pH of the fermentation broth was increased from 5.0 upto 7.0, there was an increase in polysaccharide production. However higher values (pH8.0) caused a decrease in polysaccharide production with regard to temperature 30^oC – 40^oC was shown to be optimum, since higher or lower temperatures had a negative effect on polysaccharide production.

In this present study the increasing pH was inhibitory to biomass as well as polymer accumulation. Suggested the pH control of xanthan fermentations helps to maintain a constant sugar consumption rate. The pH did not affect bacterial growth but it affects sugar consumption.

Xanthomonas species showed similar pattern exhibiting an initial increase during the first 48 hours and then decreasing. The initial decreasing viable cell umber could reflect cell adaptation to differences in media composition. The inoculaton was produced in growth media which had high nutrient level, where as the production media was low in nutrient. It is known that low concentration of nitrogen in the culture medium generally stimular xanthan polymer synthesis [15]. .

In the present study resultant colony with halo, which is indicative of xanthan polymer degradation? Such halo formation would suggest the depolymerization of the polymer in the agar plates suggested colony with halo, which is indicative of polymer degradation the medium, was counted as polymer.

The *Xanthomonas malvacearum* strain produced considerably very higher yield with carbon source-sucrose, pH7 and temperature 37^oC. The characterization confirm that the xanthan is polymer.

Furthermore, using defined significant factors (high level of whey and pH of 6), gum production was carried out in a large-scale fermentor.

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