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Variable nitrogen sources effect on *Xanthomonas campestris* ATCC 13915 ability for xanthan production in culture supplemented with pineapple waste

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Xanthan gum is widely known as the source for numerous applications, for example, in the food industry, pharmaceuticals, and more recently, in the improvement of oil production. It is used in many industries as this biopolymer's properties meet the industry's needs. However, due to economic constraints, xanthan gum is produced in many industries using continuous fermentation technology. Glucose is the carbon substrate in the commercial manufacture of xanthan, which raises the cost of xanthan synthesis. Using a less expensive substrate, like agricultural waste, is one technique to lower the price of xanthan. Besides, a suitable nitrogen source with optimal concentration is vital to obtain high xanthan production. Therefore, this study emphasises the effect of different nitrogen sources supplemented with liquid pineapple waste in the cultivation medium for high xanthan production. The result shows that the medium supplemented with 12 gL^{-1} ammonium dihydrogen phosphate successfully produced the highest xanthan production of about 12.5 gL^{-1}. This finding shows an increment of about 60% from the original medium used in xanthan production using *Xanthomonas campestris* ATCC 13951 in submerged cultivation.

Keywords: *Xanthomonas campestris*, xanthan, production, nitrogen source, pineapple waste

INTRODUCTION

In recent years, study on the synthesis of essential metabolites using agricultural waste as an alternative substrate in fermentation medium has been extensively explored (Vaishnav et al. 2022; de Souza et al. 2022; Awasthi et al. 2022; Dailin et al. 2019). The discharge of this agriculture waste effluent from different types of sources, such as food processing industries, including the canning of fruits, contains highly valuable bioactive compounds (Kandemir et al. 2022). Moreover, it supports the need for post-product that could be produced from this waste. For example, in Malaysia, canning industries specifically produce many types of waste, such as liquid pineapple waste and other industries in the downstream process (Yusof *et al.* 2020; Abdullah, 2017; Jusoh *et al.* 2014). Hence, this waste can be used in microbial products such as xanthan, pullulan, bacterial cellulose, alginate, curdlan, fructo-oligosaccharides and succinoglycan (Efremenko et al. 2022). Conclusively, agro-industrial waste is constantly increasing as the world population grows (Adejumo et al. 2020). The proper use of these leftovers to create bio-products by microorganisms offers numerous benefits, including value addition and waste management (Ayilara et al. 2020).

Exopolysaccharides produced by bacteria have great potential in industries and various types of exopolysaccharides such as kefiran, pullulan and xanthan have been studied extensively (El Enshasy et al. 2011; Nordin et al. 2020; Dailin et al. 2020; Dailin et al. 2022). A gram-negative bacteria called *Xanthomonas campestris* produces the exopolysaccharide known as xanthan for industrial use. It is a branching heteropolysaccharide

made up of pentasaccharide units with the molar ratios 2:2:1 of d-glucose, d-mannose, and d-glucuronic acid residues (Bhat et al. 2022). Previous researchers reported different types of Xanthomonas species, such as *Xanthomonas campestris* pv. *Manihotis* ISBF 1182 (Silva et al. 2020) and *Xanthomonas campestris* pv. *pelagornii* (Niknezhad et al. 2016). Extensive study has been done on the toxicological and safety characteristics of xanthan gum for use in pharmaceuticals and food (Tripathi et al. 2018). The exceptional characteristics of xanthan as a rheological control agent in aqueous systems and its stabilising properties in suspensions and emulsions could explain its great industrial importance (Steffens et al. 2022). The primary features of xanthan polymer are its rheological properties and molecular weight, where the variation in molecular weight of the as-produced product is accounted for by the different levels of association between the chains forming aggregates of individual xanthan units (Mohsin et al. 2021).

The environment condition, the composition of the culture medium, and the process operating parameters, such as temperature, pH, agitation speed, aeration rate, and fermentation duration, are just a few of the variables that might affect the production and quality of xanthan (El Sayed et al. 2016; Ozdal et al. 2019). Xanthan is successfully produced from a defined medium containing sucrose or glucose as carbon sources. Moreover, other approaches have been made in research using an alternative substrate, such as the volarization of industrial agriculture waste (Şen et al. 2022). One of the essential nutrients affecting cell growth and polysaccharide biosynthesis besides carbon source is nitrogen source. Nitrogen sources are important since these nutrients are connected to cell growth and metabolite production (Khani et al. 2016). Therefore, this study aimed to investigate the effect of using different nitrogen sources with culture supplemented with pineapple waste for xanthan production by *Xanthomonas campestris* ATCC 13951 in submerged cultivation.

MATERIALS AND METHODS

2.1 Producing microorganism

The reference strain was obtained from American Type Culture Collection (ATCC), *Xanthomonas campestris* ATCC 13951 and used as the producing microorganism in this study. The strain was cultured in a standard nutrient broth and stored at -70°C in a vial containing glycerol for further use.

2.2 Culture conditions

The inoculum was prepared with medium containing glucose (10 gL^{-1}), K_2HPO_4 (4 gL^{-1}), yeast extract (4 gL^{-1}), malt extract (5 gL^{-1}), and $MgS0_4.7H_2O$ (0.5 gL^{-1}). The pH was adjusted to 7 before autoclaving at 120°C for 20 minutes. The 250 ml shake flask containing 50 ml of the prepared medium was inoculated with *Xanthomonas campestris* ATCC 13951 and cultured in an orbital shaker at 30°C and 200 rpm for 24 hours. After 24 hours, the growth was measured using a spectrophotometer (Spectronic 200, PerkinElmer) to obtain the optical density at 600 nm.

2.3 Culture media for cell growth and Xanthan production

Xanthan production was performed simultaneously in 250 mL Erlenmeyer flasks containing 50 ml medium. 5% (v/v) of the previously prepared inoculum was inoculated into the shake flask after 24h cultivation process. The cultivation media contained different types of nitrogen sources including $(NH_4)_2SO_4$, $(NH_4)H_2PO_4$, urea, yeast extract, peptone and potassium nitrate. The best nitrogen source was further optimized for maximal xanthan production. Fermentation was carried out in an orbital shaker (model New Brunswick Innova 4000 Benchtop Incubator Shaker, USA) under aerobic conditions at 30 °C and 200 rpm for 96 hours.

2.4 Determination of biomass

Samples, in the form of two flasks containing 50 mL each, were taken at final cultivation time. Immediately after collection, the fermentation broth was centrifuged (Zentrifugen, Hettich, D-78532, Tuttlingen, Germany) in 50 mL falcon tubes at 7000 rpm for 15 minutes to precipitate the cells. The cells are dried in an oven for 24h at 60°C and weighed until constant weight.

2.5 Determination of xanthan

The xanthan produced during fermentation was obtained by a precipitation process with 96% (v/v) chilled ethanol. The supernatant was mixed with ethanol at a ratio of 1:3 (v/v) and 1% (v/v) KCl was added as an electrolyte using the standard xanthan precipitation method (Rončević et al. 2019). The mixture was then kept overnight at 4 °C in a refrigerator and later centrifuged at 7000 rpm and 4 °C for 15 min (Universal 320R, Hettich, Germany). The precipitate obtained was dried in an oven at 60 °C and weighed to a constant value. The precipitated product contained xanthan.

2.6 pH Determination

The pH of the media was measured during the sampling process for the shake flask study by using pH meter (Mettler-Toledo Delta 320, Greifensee, Switzerland).

2.7 Data analysis

Data were collected in triplicates and the average value was calculated using the standard deviation. The data presented include biomass, xanthan, pH of broth and specific production rates.

RESULTS

Effect of Different Types of Nitrogen Sources on Cell Growth and Xanthan Production

Different types of nitrogen sources ($(NH_4)_2SO_4$, $(NH_4)H_2PO_4$, urea, peptone, yeast extract and potassium nitrate) were added to the production medium to evaluate their suitability to support xanthan production. Figure 1 demonstrates the effect of different types of nitrogen sources having a strong influence on cell growth and xanthan production. The highest cell dry weight (CDW) was obtained at about 4.25 gL^{-1} in the medium containing a mixture of both ammonium dihydrogen phosphate and ammonium sulfate. This is followed by $(NH_4)_2SO_4$, $(NH_4)H_2PO_4$, urea, yeast extract and potassium nitrate. However, the use of a single type of nitrogen source in the medium containing LPW indicates that ammonium sulfate produced the highest cell amount compared to the rest of nitrogen sources. The negative control medium obtained the least number of cells, indicating insufficient or imbalance in nutrients of the medium led to the stunted proliferation of cell growth.

Further analysis of xanthan production shows that the LPW medium supplemented with only ammonium dihydrogen phosphate produced the highest xanthan of about 5.5 gL^{-1}. This is followed by ammonium sulphate, a combination of ammonium dihydrogen phosphate and ammonium sulfate, potassium nitrate, urea, peptone, and cultured supplemented with yeast extract. In the negative control medium in which medium without any supplementation with a nitrogen source, the xanthan production was highest compared to the medium containing yeast extract, urea and peptone. This study's results are similar to those obtained by Murad et al. (2017). They evaluated the impact of organic and inorganic sources on xanthan production using pre-treated whey lactose supplemented with 1% sucrose as a carbon source. It was found that xanthan production yield was promising by using ammonium dihydrogen phosphate as the inorganic nitrogen source in which 20 gL^{-1} xanthan was produced. To improve the yield of desired metabolites, an accurate selection of nitrogen sources as the most critical nutrients in the growing medium, and precise determination of their concentrations are required (Grahovac et al. 2014). The type of nitrogen source used as a growth-limiting nutrient is critical in optimising the production of several secondary metabolites (Davis et al. 1999). The pH values of the media having different types of carbon sources ranged between 5.2-8.2.

To better understand the relationship between cell growth and xanthan production, the specific xanthan production [$Y_{p/x}$] was calculated. The maximal value of 4.6 g/g was obtained in the negative control culture, which was almost 75 % higher than those obtained in cultures supplemented with double nitrogen sources (ammonium dihydrogen phosphate and ammonium sulfate). The cell performance for xanthan production was of the following manner: potassium nitrate > $(NH_4)H_2PO_4$ > $(NH_4)_2SO_4$ > peptone > urea > yeast extract.

Hence, the early screening of nitrogen sources revealed that ammonium dihydrogen phosphate is a highly suitable nitrogen source with culture supplemented with LPW for xanthan production. Thus, it was used for the following experiment to identify the optimal concentration needed to obtain maximal xanthan production.

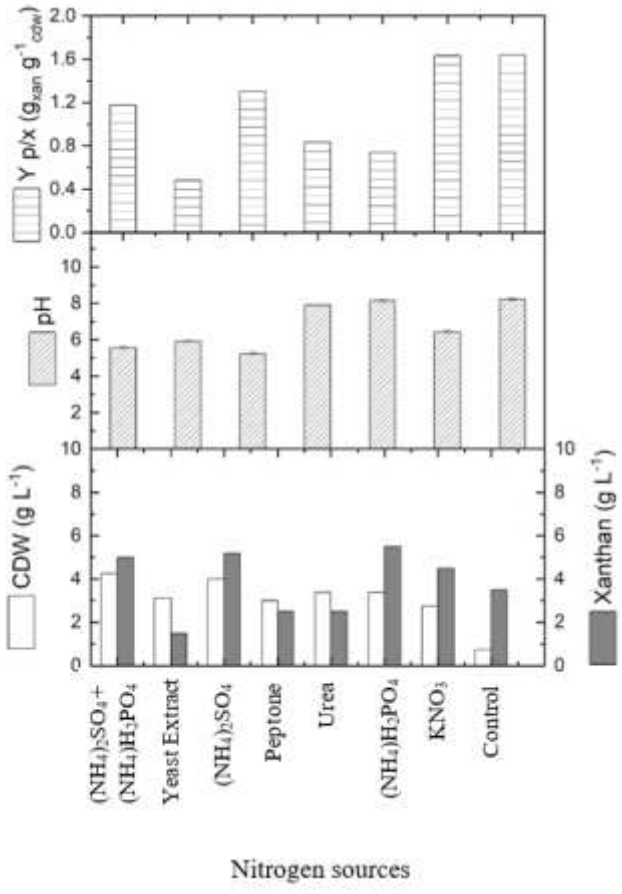

Figure 1: Effect of different nitrogen sources on xanthan production by *X. campestries* in shake flask cultures. Data were taken after submerged cultivations for 96 hours (Error bars represent standard error calculated)

Effect of different Ammonium Dihydrogen Phosphate Concentrations on Cell Growth and Xanthan Production

Figure 2 depicts the effect of different ammonium dihydrogen phosphate concentrations as nitrogen sources for biomass and xanthan production. The cultivation process was conducted for 96 hours. It is worth noting that the specific xanthan production was increased up to 9.09 gg^{-1} when 12 g L^{-1} of $(NH_4)H_2PO_4$ was used in the medium. Further increases in $(NH_4)H_2PO_4$ concentration decrease the specific xanthan production. To study the effect of nitrogen concentration on both biomass and xanthan production, the control medium excludes nitrogen sources. Medium without nitrogen sources produced the

lowest CDW compared to other media supplemented with nitrogen. There are no significant differences in cell growth between the control and different nitrogen concentrations up to 10 gL^{-1}. However, cell proliferation increases at nitrogen concentrations of 12 g L^{-1} and begins to decrease as nitrogen concentrations increase up to 16 gL^{-1}.

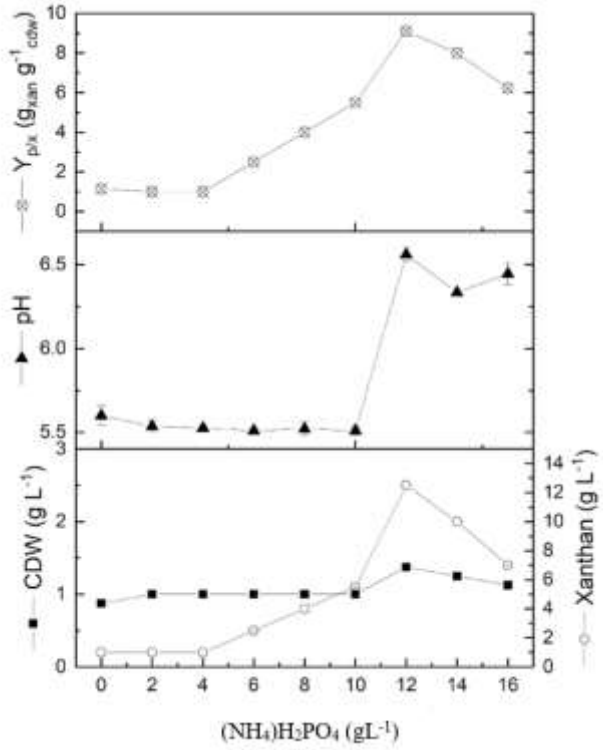

Figure 2: Effect of different $(NH_4)H_2PO_4$ concentrations on xanthan production by *X. campestries* in shake flask cultures. Data were taken after submerged cultivations for 96 hours (Error bars represent standard error calculated)

A similar trend was observed in a study by Moreno et al. (1998) using acid hydrolysate of waste from different sources such as melon, cucumber, and tomato. They revealed that media with supplementation of ammonium chloride showed an increased biomass concentration. In a study by Rosalam and England (2006), it was noted that increased biomass production yields could be obtained at high nitrogen sources concentration and temperature values lowered from 35 to 30 ˚C, with the maximum biomass obtained of about 3.74 gL^{-1}. In the study conducted by Dodic et al. (2011), the optimal pH for xanthan production was between 6.0 and 6.8. Nevertheless, the optimum pH conditions during the process were between 6.5 to 7.5 (Kalogiannis et.al. 2003). The investigations into xanthan productions were also carried out under similar conditions. The maximum xanthan yield is 12.5 g/L considering the optimum level of nitrogen concentrations 12 g/L. Further addition of nitrogen into the medium resulted in decreasing amount of xanthan produced. This could be probably due to the over-supply of this cell-growth element whereby it's hindering the cell to proliferate. Lower nitrogen source use may result in lower medium costs on a large scale and may encourage the commercialisation of xanthan production (Khosravi-Darani et al. 2011).

CONCLUSION

In this study, it can be concluded that the type of organic nitrogen sources highly influenced the xanthan production. The best nitrogen source to support high xanthan production is ammonium dihydrogen phosphate. The optimal concentration of ammonium dihydrogen phosphate was 12 gL^{-1} to produce maximal xanthan production of 12.5 gL^{-1}. The current study showed the possibility of using this type of nitrogen source with a fermentation medium supplemented with liquid pineapple waste as a low-cost alternative substrate for xanthan production. The findings from this study serve as the foundation for further research into xanthan synthesis from agricultural waste and the potential transfer of technology from the laboratory to the industrial level. To boost fermentation yield, bioreactor studies also can be conducted. Nevertheless, more research is needed to discover the ideal process parameters for xanthan production using agricultural waste as a fermentation substrate.

CONFLICT OF INTEREST

The authors declared that the present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

ARR and SS were involved in data collection and writing the manuscript. DJD, SZH, SFI and HAE reviewed the manuscript. All authors read and approved the final version.

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