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EFFECTS OF SALTS IN BIODIESEL DERIVED CRUDE GLYCEROL ON VANCOMYCIN PRODUCTION FROM *AMYCOLATOPSIS ORIENTALIS* ATCC® 19795™

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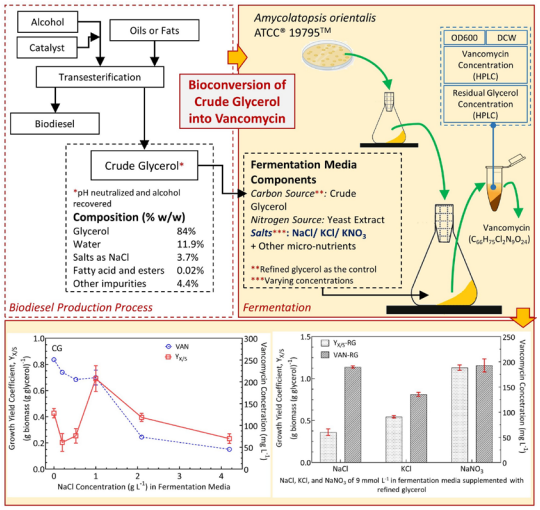
Graphical abstract



Abstract

Bioconversion of crude glycerol (CG), the primary byproduct in biodiesel generation, is currently of great interest due to its promising potential in producing chemicals of high commercial interest. The efficient use of CG is still limited primarily due to the presence of impurities that may vary in composition based on the parent feedstock, biodiesel generation process, and any recovery/ purification conducted. In this study, laboratory scale fermentation of vancomycin using *Amycolatopsis orientalis* ATCC® 19795™ was analyzed based on the presence of salts as an impurity in biodiesel derived CG. Results showed that CG as the sole carbon source inhibited the cell growth, but the vancomycin production was approximately the same (158±2 mg L^{-1}) as that of refined glycerol (RG)-based media at varying initial glycerol concentrations studied (5 g L^{-1} to 40 g L^{-1}). NaCl as an impurity in biodiesel derived CG reduced both the growth yield coefficient ($Y_{X/S}$) and vancomycin formation at high concentrations. Among other salts tested are $NaNO_3$ and KCl. $NaNO_3$ (0.8 g L^{-1}) supplemented media generated a higher $Y_{X/S}$ (1.1 g biomass (g glycerol consumed)$^{-1}$) and a similar vancomycin concentration (193 mg L^{-1}) compared to the media with added NaCl ($Y_{X/S}$ of 0.4 g biomass (g glycerol consumed)-1 and a vancomycin concentration of (190 mg L^{-1})). Relatively, both the $Y_{X/S}$ (0.55 g biomass (g glycerol consumed)$^{-1}$) and vancomycin concentration (135 mg L^{-1}) was reduced in KCl (0.7 g L^{-1}) supplemented media. The results suggest the potential of using CG (with salts i.e., NaCl as impurities) as an excellent low-cost alternative for RG in fermentation of vancomycin using *Amycolatopsis orientalis* ATCC® 19795™.

Keywords: *Amycolatopsis orientalis*, Biodiesel, Crude glycerol, Impurities, Vancomycin

1.0 INTRODUCTION

In terms of economic feasibility, production scalability, and reliability, biodiesel is a popular energy substitute in replacing fossil fuel needs in transportation [1, 3]. In spite of the numerous environmental benefits over fossil fuel derivatives, biodiesel production process is far from being a sustainable commercial operation and one such persistent challenge has been the voluminous generation of a by-product, crude glycerol (CG). The most common method of biodiesel production is homogeneous base-catalysed transesterification of triglycerides contained in fats (vegetable oils or animal fats) and generates a mixture of CG and other by-products/wastes alongside [4, 5, 6]. The composition (type and concentration of impurities) of CG varies significantly with the parent feedstock, transesterification method, and post purification methods used in biodiesel production process [7]. In general, biodiesel derived CG comprises of glycerol, alkalis (alkali soaps and hydroxides), methyl esters, alcohols, inorganic salts, and water [8, 9]. CG contains a variety of elements from the primary oil and the catalyst [K (0-217 ppm), P (12-37 ppm), S (14-128 ppm), Na (1.06-1.40%), C (24-37%), N (0.04-0.12%), and protein (0.05-0.44%)] [9]. In addition, CG yield is stoichiometrically equivalent to 10% w/w of total biodiesel produced and the rapid upsurge in biodiesel production has therefore inadvertently resulted in a CG surplus during last two decades [10]. Currently, over two

third of global glycerine supply comprises of biodiesel derived CG [11, 12, 13]. However, majority of industrial applications in pharmaceuticals, food, personal care products, polymer, and chemical industries demands refined glycerol (RG) of higher purity (>96%) [11, 14, 15]. High levels of contamination and high volume that is being produced have thereby limited the commercial use of biodiesel derived CG and instead yielded a readily available, attractive carbon source virtually at no cost [15, 16, 17, 18].

Various chemical and biological processes have been implemented to convert CG into green commodity chemicals of high value with little or no purification required [11, 19]. Bioconversion of biodiesel derived CG is currently a prevalent value-added technique where CG is used as a carbon and energy source in biological processes such as anaerobic digestion and fermentation, to produce a variety of high-end products or precursors [10, 15, 19, 20, 21]. A large group of microorganisms can assimilate glycerol however, impurities in CG may affect their metabolic pathways [8]. Therefore, when biodiesel derived CG is used as a substrate in fermentation, it is essential to use microorganisms that can tolerate impurities present in CG, particularly salts and alcohols [22, 23]. Actinomycetes are among the microorganisms and mixed microbial cultures that exhibit considerable levels of xerotolerance and halotolerance [23]. Soil actinomycete, *Amycolatopsis orientalis* is used in commercial production of vancomycin, a clinically important, first generation glycopeptide antibiotic used in the treatment of gram-positive bacterial infections [24, 25]. When Zeng et al. (2013) used biodiesel derived CG as the carbon source in batch cultivation of *A. orientalis* XMU-VS01, an approximate 3-fold increase in vancomycin yield was reported compared to other carbon sources (sucrose, d-glucose, d-fructose, maltose, dextrin, and soluble starch).

It is essential that the effect of salts in vancomycin production being investigated since salts are inclusive in biodiesel derived CG. Effect of salts on vancomycin biosynthesis has not yet been reported. Nonetheless, many studies including Abdelwahed & El-Naggar (2011), Dunstan et al. (2000), Jung et al. (2007), McIntyre et al. (1996), Nagy et al. (1993), and Zeng et al. (2013) used defined, semi-defined or minimal media supplemented with NaCl, KCl, or other salts at varying amounts. A study by McIntyre et al. (1996) presented the significance of media components of a semi-defined media in vancomycin production, in which the effects of NaCl and KCl were recorded as not significant. In addition, Zeng et al. (2013) reported that using KNO_3 as an additive in the production medium with an organic nitrogen source enhanced the vancomycin yield. In this study, the effect of salts in biodiesel derived CG on vancomycin fermentation using *Amycolatopsis orientalis* ATCC® 19795™ was analysed based on changes in growth yield coefficient ($Y_{X/S}$) and vancomycin formation.

2.0 METHODOLOGY

Microorganism and Materials

Amycolatopsis orientalis (ATCC® 19795™, American Type Culture Collection) was used in the study. Vancomycin hydrochloride (≥ 80%) from *Streptomyces orientalis* (Sigma-Aldrich, Germany) was used for analysing crude vancomycin in fermentation broth. Composition of the pH-neutralized, biodiesel derived CG (Patum Vegetable Oil Co., Ltd, Thailand) was 84% w/w of glycerol, 11.9% w/w of water, 3.7% w/w of salts as NaCl, 0.018% w/w of fatty acid and esters, and 4.4% w/w of other impurities. All other chemicals used were of analytical or chromatographic grades.

Shake Flask Fermentation

Amycolatopsis orientalis ATCC® 19795™ was grown in 250 mL shake flasks containing 50 mL of seed medium (17 g L^{-1} of glucose, 11 g L^{-1} of peptone, 3 g L^{-1} of malt extract, and 3 g L^{-1} of yeast extract) and were kept in a shaking incubator at 250 rpm for 48 hours at 30°C. Primary medium contained 2 g L^{-1} of yeast extract, 1 g L^{-1} of NaCl, 0.1 g L^{-1} of KH_2PO_4, 0.5 g L^{-1} of $MgSO_4 \cdot 7H_2O$, and 1 mL L^{-1} of a trace solution (1 g L^{-1} of $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$). Primary medium was supplemented with a carbon source, either RG or biodiesel derived CG and inorganic salts (NaCl, KCl, and $NaNO_3$) at their desired concentrations. Initial concentration of RG, unless otherwise specified, was 12 g L^{-1} and the initial concentration of CG was taken equivalent to that of RG samples with the purity of CG (84% w/w) in consideration. Initial concentration of NaCl in media was changed from 0 – 4.2 g L^{-1} and KCl and $NaNO_3$ were added at 0.7 g L^{-1} and 0.8 g L^{-1}, respectively.

Three replications of each medium were prepared with 50 mL of the defined media in 250 mL shake flasks. Initial pH of each medium was changed to pH 7 using either 1 N of NaOH or HCl. The suspension was inoculated with 2% v/v of the seed culture of $OD_{600} \approx 0.2$, and was incubated in a shaking incubator at 250 rpm for 5 days at 30°C. Each flask containing medium was sterilized at 121°C for 15 minutes prior to inoculation with the bacteria.

Determination of Cell Biomass Concentration

Growth of *Amycolatopsis orientalis* ATCC® 19795™ in fermentation media was gravimetrically determined by dry cell weight (DCW). Aliquots of the culture samples (1 mL) were centrifuged at 10,000 rpm for 20 minutes at room temperature. After the supernatant is removed, resulting pellets were washed with distilled water and were pelleted again by centrifugation. The washed cells were dried in an oven at 60°C for 48 hours or until constant weight. Presence of any insoluble components in the medium were eliminated by subtracting weight of sterile, cell free culture media from the sample dry cell weight.

Determination of Residual Glycerol Concentration

HPLC analysis of glycerol was performed using an Agilent 1260 Infinity II HPLC equipped with a VertiSep™ NH2 GES (4.6 × 250 mm, 5 µm), refractive index detector (RID), and a UV detector. The supernatant of the centrifuged fermented broth (10,000 rpm for 20 minutes at room temperature) was mixed with an internal standard (sucrose), diluted with the mobile phase (acetonitrile 75% in deionized water), and was filtered through a 0.2 µm syringe filter. Mobile phase flow rate was 1 mL min^{-1} and the column was maintained at room temperature. Injection volume was 20 µL.

Determination of Vancomycin Concentration using HPLC

Quantification of crude vancomycin in the fermented broth was conducted in the Agilent 1260 Infinity II HPLC equipped with a Luna® C18 column (4.6 × 250 mm, 5 µm, 100 Å) and a UV detector at 275 nm. The column was maintained at the room temperature. Supernatant of the centrifuged fermented broth (10,000 rpm for 20 minutes at room temperature) was mixed with an internal standard (caffein), diluted with the mobile phase (acetonitrile 30% in HPLC water with 0.1% formic acid (v/v)), and was filtered through a 0.2 µm syringe filter. Mobile phase flow rate was 0.5 mL min^{-1}. Injection volume was 10 µL. A calibration curve of known concentrations of vancomycin standard versus area under the HPLC peak was plotted and the unknown concentration of crude vancomycin in the fermented broth was calculated using the linear equation obtained from the calibration curve.

Calculation of Growth Yield Coefficient ($Y_{X/S}$)

In batch fermentation, limiting substrate is utilized in cell material growth, metabolic products formation, and cell maintenance [31, 32]:

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\frac{dX}{dt} - \frac{1}{Y_{P/S}}\frac{dP}{dt} - mX \qquad (1)$$

where X, S, and P are the cell mass, limiting substrate, and product concentration at a given time, t, respectively. The cell maintenance coefficient is denoted by m. The stoichiometric parameters, $Y_{X/S}$ and $Y_{P/S}$ are biomass growth yield based on substrate consumption and product yield based on substrate consumption, respectively. Assuming the amount of limiting-substrate used in product formation and cell maintenance is negligible, Equation (1) becomes:

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\frac{dX}{dt} \qquad (2)$$

Hence, $Y_{X/S}$ is used in assessing the biomass yield based on the limiting substrate utilization in batch growth [31, 32, 33]:

$$Y_{X/S} = \frac{dX}{dS} = \frac{X_e - X_0}{S_0 - S_e} \qquad (3)$$

where X_0 and X_e are the initial and maximum biomass (*Amycolatopsis orientalis* ATCC® 19795™) concentration (g L^{-1}) during batch growth, S_0 and S_e are the initial and final limiting substrate (glycerol—RG or CG) concentrations during batch growth (g L^{-1}), and $Y_{X/S}$ is the growth yield coefficient (g biomass (g glycerol consumed)$^{-1}$).

3.0 RESULTS AND DISCUSSION

Impact of Crude Glycerol Composition on $Y_{X/S}$ and Vancomycin Production

Composition of the pH-neutralized, biodiesel derived CG used is 84% w/w of glycerol, 11.9% w/w of water, 3.7% w/w of salts as NaCl, 0.018% w/w of fatty acid and esters, and 4.4% w/w of other impurities. It was presumed that in CG supplemented media, the concentration of impurities (NaCl, fatty acids and esters, and other) is directly proportional to the initial glycerol concentration in fermentation media. $Y_{X/S}$ and vancomycin produced during the exponential growth phase (< 72 hours) were determined in batch fermentation of *Amycolatopsis orientalis* ATCC® 19795™ in RG and CG-based media, separately at different initial glycerol concentrations. Initial concentration of CG was taken equivalent to that of RG samples of 5 g L^{-1}, 12 g L^{-1}, 20 g L^{-1}, and 40 g L^{-1} (with the purity of CG (84% w/w) in consideration). During the exponential growth phase, the amount of glycerol spent on cell maintenance and product formation were presumed negligible. The results are summarized in Figure 1.

Higher initial glycerol concentrations naturally suggest an abundance of nutrition and energy available for growth of microorganism. Evidently, $Y_{X/S}$ gradually increased at higher initial substrate concentrations of RG (Figure 1). In RG-based media, the lowest and highest $Y_{X/S}$ of 0.39±0.06 g biomass (g glycerol consumed)$^{-1}$ and 0.91±0.13 g biomass (g glycerol consumed)$^{-1}$ were observed in media supplemented with initial RG of 5 g L^{-1} and 40 g L^{-1}, respectively. At high concentrations of glycerol, cell growth can be inhibited due to increased osmotic pressure in the fermentation medium [34, 35]. Zeng et al. (2013) reported that higher concentrations (>80 g L^{-1}) of CG negatively impact both vancomycin yield and cell growth due to reduced mass transfer in culture. Comparably in the present study, the highest $Y_{X/S}$ of 0.58±0.02 g biomass (g glycerol consumed)$^{-1}$ was recorded in CG-based media supplemented with the lowest initial glycerol concentration of 5 g L^{-1}. Samples with higher initial CG concentrations (12 g L^{-1}, 20 g L^{-1}, 40 g L^{-1}) generated approximately similar $Y_{X/S}$ (0.45±0.02, 0.43±0.03, and 0.45±0.05 g biomass (g glycerol consumed)$^{-1}$, respectively).

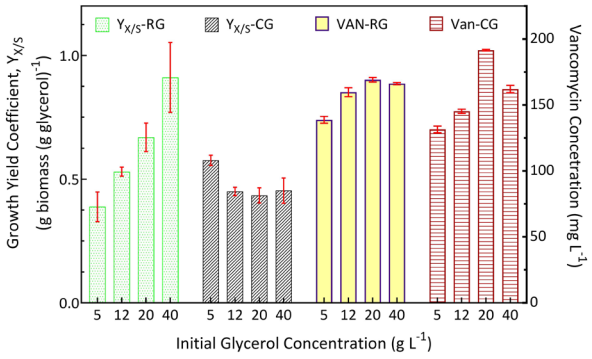


Figure 1 Growth yield coefficient ($Y_{X/S}$) and vancomycin produced (VAN) at 72 hours at four different initial concentrations (5 g L^{-1}, 12 g L^{-1}, 20 g L^{-1}, and 40 g L^{-1}) in refined glycerol (RG) and crude glycerol (CG) supplemented media

Vancomycin is a growth associated, secondary metabolite that initiates product synthesis during the exponential growth phase and continues to the stationary phase [29, 36]. This was further confirmed by a Luedeking–Piret model developed for quantifying vancomycin formation kinetics in this study (unpublished). In the Luedeking–Piret model, both growth and nongrowth associated coefficients (α and β) were nonzero, however the growth associated-terms were approximately 120-290 times larger than the non-growth associated terms (α >> β) in both RG and CG-based media. Therefore, the growth associated phase is rather important in production of

vancomycin and is significantly controlled by factors linked to cell growth [37]. However, the limiting-substrate effect on the product formation was neglected in the model. As illustrated in Figure 1, a gradual increase of vancomycin formation (138.7±2.6 mg L^{-1}, 159.7±3.4 mg L^{-1}, and 169.2±1.8 mg L^{-1} in RG supplemented media; 131.5±2.5 mg L^{-1}, 145.2±1.6 mg L^{-1}, and 191.6±0.5 mg L^{-1} in CG supplemented media) with increasing initial substrate concentrations (5 g L^{-1}, 12 g L^{-1}, and 20 g L^{-1}, respectively) were observed. In both RG and CG supplemented media, the highest vancomycin concentrations were reported in media with 20 g L^{-1} of initial glycerol concentrations (166.2±0.8 mg L^{-1} and 191.6±0.5 mg L^{-1}, respectively) and further increase of initial glycerol concentration (40 g L^{-1}) recorded a reduced vancomycin formation (166.2±0.8 mg L^{-1} and 162.1±2.8 mg L^{-1}, respectively). In this study, samples of higher initial glycerol concentrations recorded lower $Y_{X/S}$ (as mentioned in previous section) suggesting higher unused carrying capacities of substrates [32]. As confirmed by a modified logistic model developed to analyse the substrate consumption in CG-based media at different initial glycerol concentrations (unpublished), the carrying capacity coefficient (k) was lessened at higher initial glycerol concentrations (> 20 g L^{-1}). A reduced carrying capacity coefficient (k) signifies a slower depletion of substrate and the substrate availability over an extended period of time suggests an elongated growth phase with a possible delay in reaching the maximum carrying capacity of the medium. Since vancomycin is prominently growth associated, an elongated growth phase suggests a reduced product accumulation over the fermentation time considered in the study, hence the reduced vancomycin production. Concurrently, Figure 1 displays similar vancomycin concentrations in both RG and CG-based media at their corresponding initial glycerol concentration levels. Nonetheless, the vancomycin concentrations portrayed in Figure 1 were accumulated only during the exponential growth phase (< 72 hours). As a secondary metabolite that is synthesized during both the exponential growth phase and stationary phase, the reported vancomycin concentrations are likely gross understatements. As described in next section, CG-based media could instead generate a comparatively higher vancomycin concentration at 120 hours of fermentation (stationary phase) to that of RG-based media.

No specific trends were identified between both $Y_{X/S}$ and vancomycin produced and the amounts of salts, fatty acid and esters, and other impurities in CG. Nonetheless, within the range of initial glycerol concentrations studied (5 g L^{-1} to 40 g L^{-1}), the amount of biomass produced per CG consumed were comparatively less than those of RG-based media; vancomycin produced were approximately similar in both media with a potential increase of vancomycin concentration in CG-based media at extended fermentation times. Thereby, the presence of salts and impurities presumably affected the $Y_{X/S}$ of CG-based media. And the approximately constant $Y_{X/S}$ at increasing initial CG concentrations suggested an inhibitory effect at higher initial glycerol concentrations (> 5 g L^{-1}).

Impact of NaCl concentration on $Y_{X/S}$ and Vancomycin Production

$Y_{X/S}$ and vancomycin concentration at 120 hours of fermentation when the primary media (without 1 g L^{-1} of NaCl) was supplemented with 12 g L^{-1} of RG and NaCl at different concentrations (0 – 4.2 g L^{-1}) are illustrated in Figure 2(a). Figure 2(b) illustrates $Y_{X/S}$ and vancomycin concentration when the aforementioned experiment was replicated by replacing RG with an equivalent amount of CG of 84% w/w purity. In Figure 2(b), x-axis denotes the total amount of NaCl concentration in fermentation media: the sum of NaCl available as an impurity in CG (0.524 g L^{-1}) and the amount of NaCl added in media (0 – 4.2 g L^{-1}).

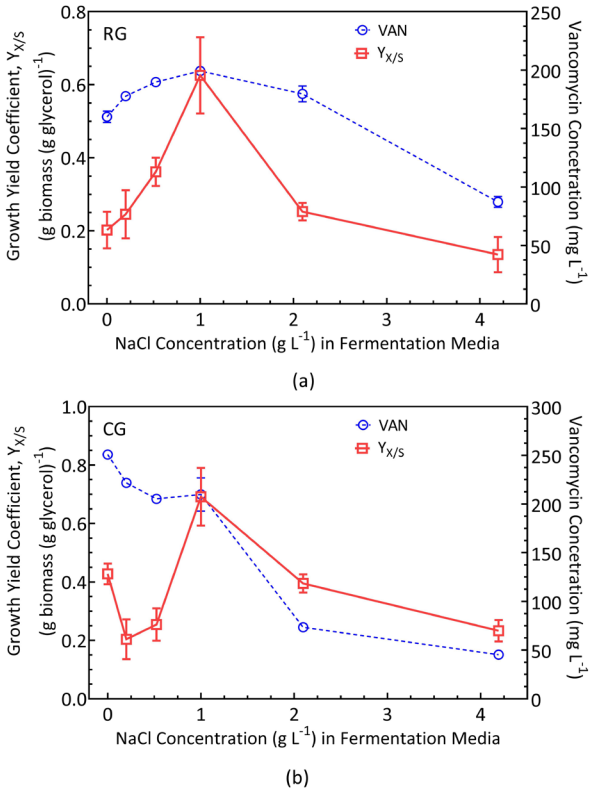


Figure 2 Effect of NaCl concentration on vancomycin concentration (VAN) (⊖) and growth yield coefficient ($Y_{X/S}$) (⊟) at 120 hours of fermentation in (a) refined glycerol (RG) and (b) crude glycerol (CG) supplemented media

Figure 2(a) suggests that the concentration of NaCl in fermentation media exhibits significant effect on both $Y_{X/S}$ and vancomycin production. In addition, at all NaCl concentrations used in the study (< 4.2 g L^{-1}), vancomycin generation assumed a significant likeness to the trend of $Y_{X/S}$. In RG-based media, samples with NaCl of 1 g L^{-1} recorded the highest $Y_{X/S}$ of 0.63±0.10 g biomass (g glycerol consumed)$^{-1}$ and vancomycin concentration of 199.26±0.71 mg L^{-1} (Figure 2(a)). At lower concentrations of NaCl (< 1 g L^{-1}), $Y_{X/S}$ was increased by 0.43 g biomass (g glycerol consumed)$^{-1}$ per 1 g L^{-1} of NaCl added. At higher concentrations of NaCl (> 1 g L^{-1}), $Y_{X/S}$ was reduced by 0.14 g biomass (g glycerol consumed)$^{-1}$ per 1 g L^{-1} of NaCl added. Fermentation media-without NaCl produced approximately 160 mg L^{-1} of vancomycin. Vancomycin production further declined below 160 mg L^{-1} at higher concentrations of NaCl (> 2.5 g L^{-1}). Thus, limited presence of NaCl in the RG-based fermentation media (< 2.5 g L^{-1}) was favourable for a higher production of vancomycin (160 mg L^{-1} < [Vancomycin] < 200 mg L^{-1}). Assuming that CG comprises only

of RG and NaCl, a RG-based fermentation medium with NaCl of 2.5 g L^{-1} is identical to a CG sample with a NaCl composition of 17 w/w%. Accordingly, a CG sample with NaCl as an impurity with a composition as high as 17 w/w% would nevertheless generate a higher vancomycin concentration (> 160 mg L^{-1}).

CG-based fermentation media recorded the highest $Y_{X/S}$ of 0.69±0.10 g biomass (g glycerol consumed)$^{-1}$ in samples with 1.524 g L^{-1} of NaCl and the highest vancomycin concentration of 250.76±4.36 mg L^{-1} is recorded in samples with 0.524 g L^{-1} of NaCl (Figure 2(b)). Here, CG-based media with 1.524 g L^{-1} and 0.524 g L^{-1} of NaCl denote the CG-based primary media supplemented with 1 g L^{-1} and 0 g L^{-1} of NaCl, respectively, since biodiesel derived CG used in the study consisted of 3.7% w/w of salts as NaCl corresponding to an inherent NaCl concentration of 0.524 g L^{-1} in CG-based fermentation media. Both equivalent samples in RG-based media (samples with 1 g L^{-1} and 0 g L^{-1} of NaCl) recorded lesser $Y_{X/S}$ (0.63±0.10 g biomass (g glycerol consumed)$^{-1}$) and vancomycin concentration (189.77±2.16 mg L^{-1}), respectively. Therefore, the maximum values of both $Y_{X/S}$ and vancomycin produced in CG-based media were higher than those of RG-based media. Nevertheless, $Y_{X/S}$ of CG-based media varied approximately similar to that of RG-based media. At lower concentrations of NaCl (< 1 g L^{-1}), $Y_{X/S}$ was increased by 0.33 g biomass (g glycerol consumed)$^{-1}$ per 1 g L^{-1} of NaCl added. At higher concentrations of NaCl (> 1 g L^{-1}), $Y_{X/S}$ was reduced by 0.13 g biomass (g glycerol consumed)$^{-1}$ per 1 g L^{-1} of NaCl added. CG-based media without-added NaCl—of an inherent NaCl concentration of 0.524 g L^{-1} as an impurity in biodiesel derived CG—produced the highest amount of vancomycin (250.76±4.36 mg L^{-1}) which was then declined as the NaCl concentration was increased. Hence, CG-based media without-added NaCl was favourable for a higher production of vancomycin. Therefore, both RG and CG-based media with lower NaCl concentrations (<1 g L^{-1} of NaCl added) recorded higher vancomycin concentrations; both media with 1 g L^{-1} of NaCl added recorded higher $Y_{X/S}$.

In high concentrations of salts media, bacteria may immediately undergo hyperosmotic shock or the lipid layer of the bacterial cell membrane may negatively be affected by the swelling due to lessened van der Waals forces which ultimately results in altered biochemical pathways, metabolite production rates, and bacteria growth rates [8, 38, 39]. Thereby, it is suggestive that NaCl as an impurity at low concentrations in CG or a simulated CG (a RG-based medium prepared by adding a low concentration of NaCl) are favourable for higher vancomycin production and higher $Y_{X/S}$; higher concentrations of NaCl may be detrimental to both vancomycin production and higher $Y_{X/S}$.

Impact of KCl and $NaNO_3$ concentrations on $Y_{X/S}$ and Vancomycin Production

To study the effect of other types of inorganic salts on vancomycin production and $Y_{X/S}$, primary media (without 1 g L^{-1} of NaCl) with 12 g L^{-1} of RG was supplemented with inorganic salts, KCl and $NaNO_3$ of 0.7 g L^{-1} and 0.8 g L^{-1}, respectively such that the initial compositions of the salts in media were equivalent to 9 mmol L^{-1} of Na^+ or Cl^- (inherent NaCl of 0.524 g L^{-1} in biodiesel derived CG used in the study). Both $Y_{X/S}$ and vancomycin concentration at 120 hours of fermentation are illustrated in Figure 3.

Both NaCl and $NaNO_3$ supplemented media generated approximately similar and higher vancomycin concentrations of 190±2 mg L^{-1} and 193±13 mg L^{-1}, respectively; KCl supplemented media recorded the lowest at 135±4 mg L^{-1}. Nonetheless, the highest $Y_{X/S}$ of 1.13±0.03 g L^{-1} was recorded with the $NaNO_3$ supplemented media while both NaCl and KCl recorded significantly lower $Y_{X/S}$ (0.36±0.04 and 0.55±0.01 g biomass (g glycerol consumed)$^{-1}$, respectively). Both Na^+ and K^+ are essential cations in substrate uptake systems in cells and are used as primary osmotic solutes at low medium osmolarities [40, 41]. In general, anions in growth media (Cl^- and NO_3^-) strongly influence the cell growth rates and their relative effects on cell growth and/or inhibition can be further explored based on the specific ion (Hofmeister) effects [38]. However, when $NaNO_3$ is used, a significant increase of vancomycin formation and cell growth occurred since $NaNO_3$, the inorganic salt of nitrogen acts also as a nitrogen source providing nutrients required for cell growth and product synthesis [42]. Thereby the amount of cell growth per glycerol consumed, as denoted by $Y_{X/S}$ in $NaNO_3$ supplemented media is higher compared to non-nitrogen salts, NaCl and KCl.

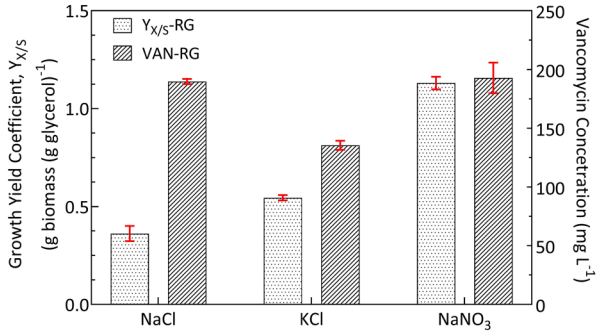


Figure 3 Effect of salts (NaCl, KCl, and $NaNO_3$ of 9 mmol L^{-1}) on growth yield coefficient ($Y_{X/S}$) and vancomycin concentration (VAN) and at 120 hours of fermentation in refined glycerol (RG) supplemented media

4.0 CONCLUSION

Optimal fermentation media formulation is essential in economical operation of secondary metabolite production in laboratory and industry alike. When biodiesel derived CG is used as an abundant low-cost carbon substrate in fermentation, impurities in CG may have a detrimental effect on the biochemical synthesis of products with the selected microorganism. In this study, the effect of salts as an impurity in biodiesel derived CG on vancomycin production is studied using *Amycolatopsis orientalis* ATCC® 19795™.

Within the range of initial glycerol concentrations studied (5 g L^{-1} to 40 g L^{-1}), the amount of biomass produced per glycerol consumed was comparatively less in CG-based media than those of RG-based media. At high initial glycerol concentrations (> 5 g L^{-1}), $Y_{X/S}$ in CG-based media were nearly identical. Higher initial CG concentrations are indicative of added impurities in media which further confirms the inhibitory effect on biomass growth. The amount of vancomycin produced were approximately similar in both RG and CG-based media of which the latter may record higher vancomycin concentrations at extended fermentation times. Therefore, biodiesel derived CG

portrays a promising low-cost substrate alternative to RG in the production of vancomycin using *Amycolatopsis orientalis* ATCC® 19795™.

Impurities in biodiesel derived CG may interact with each other and have a synergistic effect. This study explored the effect of NaCl, as an inherent impurity in biodiesel derived CG, on vancomycin production presuming the effects are of individual nature. It was observed that low concentrations of NaCl in media (<1 g L^{-1}) are favourable for higher vancomycin production and higher $Y_{X/S}$. A higher vancomycin formation was reported in both media comprised of CG with no NaCl added and RG with NaCl of 1 g L^{-1}. Consequently, the need for cumbersome substrate purification processes and relative expenses can be dismissed, given that a low concentration of NaCl as salt impurities are present in biodiesel derived CG, thereby granting direct use of CG in fermentation medium. Effect of other salts (KCl and $NaNO_3$) on biomass growth and vancomycin formation were also analysed. Comparatively, $NaNO_3$ supplemented media generated high $Y_{X/S}$ and similar vancomycin concentrations to those of NaCl supplemented media; KCl as a salt was less favourable to both $Y_{X/S}$ and vancomycin concentrations. In addition, as an inorganic salt of nitrogen, $NaNO_3$ is cell growth promoting and significantly influence the vancomycin synthesis, thereby is a complementary nitrogen additive in the fermentation media. Nonetheless, it is essential that other impurities and their synergistic effects on vancomycin formation are examined along with the optimization strategies based on fermentation media, microorganisms used, process conditions, and downstream operations to facilitate future scaling-up needs.

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