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EFFECTS OF ROSE-WATER ON THE TOTAL PHENOL AND FLAVONOID CONTENT AND RADICAL SCAVENGER ACTIVITY IN FERMENTED BROTH WITH *KOMAGATAEIBACTER XYLINUS*

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Abstract: *Komagataeibacter xylinus* is a cellulose-producing bacteria that is symbiotic with yeast-associated and kombucha production. Several herbs and spices are commonly added during the fermentation process to enhance the flavour of kombucha. Nevertheless, the efficiency of rose water on the quality of kombucha remains limited. Therefore, the current study aims to determine the effect of rose water derived from petals of Bishop's Castle species on the total phenolic, flavonoid, and radical scavenging activity of the broth rose. The *K. xylinus* was inoculated to various concentrations of rose water (0 to 100% v/v) and analysed for the chemical compound and scavenging activity after fermentation for 15 days. The results revealed that bacterial growth, total phenolic and flavonoid content, and radical scavenging activity decreased with the concentrations of rose water applied in the culture medium. The highest total phenol (0.07 mg/mL) and flavonoid (0.07 mg/mL) content was obtained from broth containing 20% (v/v) rose water. The radical scavenging activity was comparable to the control medium in treatment containing lower rose water concentrations (20% to 60% v/v). In conclusion, biologically active chemicals in the rose water might inhibit the growth of bacteria and contribute to the total content of analysed secondary metabolites in the broth. Thus, further study should be conducted to determine the effects of lower concentrations and a variety of rose water applied after the bacteria attained the growth phase.

Keywords: Bacteria, broth, fermentation, rose.

Introduction

Komagataeibacter xylinus (*K. xylinus*), formerly known as *Acetobacter xylinum*, is a gram-negative bacterium belonging to the Acetobacteraceae family, is among the bacteria cellulose producer (Lahiri *et al.,* 2021). According to Seddiqi *et al.* (2021), *K. xylinus* is extensively employed in biomedical applications due to its purity, biocompatibility, and remarkable properties of bacterial cellulose. In addition, the tea drink used during fermentation is used as a fermented beverage known as kombucha (Abaci *et al.,* 2022). This species can grow and associate with other species and yeast, referred to as a Symbiotic Culture of Bacteria and Yeast (SCOBY) (Coelho *et al.,* 2020). Typically, SCOBY is used as an inoculum for kombucha production (Abaci *et al.,* 2022). Kombucha has been increasing due to the belief as a natural remedy and has health benefits such as probiotics (Lahiri *et al.,* 2021; Antolak *et al.,* 2021), positive effects on digestion and gut health (Batista *et al.,* 2022) and anti-inflammatory (Kochman *et al.*, 2020).

To enhance the flavour and quality of kombucha, several plant extracts that serve as natural nutrient supplements were added to the fermentation media for microbial growth and metabolic activity (Zhao *et al.,* 2021). The infusion of spices, herbs, juices, and plant extract into the fermentation medium promotes the production of desired metabolites and provides the microorganisms with a well-balanced nutritional environment (Salem *et al.,* 2020). Moreover, plant extracts are natural sources

rich in bioactive substances like polyphenols, flavonoids, and essential oils that frequently enhance kombucha quality (Abaci *et al.*, 2022). As a result, choosing the right plant extracts is one of the elements in improving kombucha quality.

Roses are known for their medicinal value. However, the active ingredient in the rose differs from one variety to another (Ren *et al.,* 2016). Different varieties of kombucha beverages, as well as the use of rose extract as a starting substrate for fermentation, may have antioxidant activity that is beneficial to human health (Lahiri *et al.,* 2021; Agarwal *et al.*, 2022). Therefore, the utilisation of appropriate rose variety can contribute to the quality of the product. According to Sadraei *et al.* (2013), the *Rosa damascene* Mill flower is rich in essential oil and used for perfume production. In addition, the Bishop's Castle Rose is frequently employed in traditional medicine. However, the effect of this rose species on the quality of the fermented beverages with *K. xylinus* remains limited. Therefore, this study's objective was to investigate the effects of the water extract concentrations from the petal flowers of Bishop's Castle Rose on the growth of *K. xylinus* and the content of total phenolic and flavonoid as well as 2,2-diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging in the fermented solution.

Material and Methods

Source of Plant Material and Preparation of Water Extract

The delicate petals of the Bishop's Castle rose (Figure 1) were obtained from the Zety Roses garden located in Kuala Berang, Terengganu, Malaysia. The samples were brought to a laboratory at the Universiti Malaysia Terengganu and dried in an oven at 60°C for 12 hours. Rose water was prepared by boiling the 6 g dried petal in 1 L of distilled water for 15 minutes. Subsequently, a varying ratio of rose water and Hestrin Schramm (HS) medium (Sagen, 2023) was prepared and kept in a freezer for further use.

Figure 1: The morphological characteristic of Bishop's Castle rose flowers obtained from the Zety Roses garden

Preparation of Treatment Medium and Culture Conditions

The treatment media used HS medium mixed with rose water at concentrations of 0, 20, 40, 60, 80 and 100% (v/v), respectively. All media were adjusted to pH 5 (HI2002-02 pH-meter; Hanna Instruments, Romania) using 1.0 N HCl or 1.0 M NaOH solution. The mixture was mixed by stirring on a magnetic stirrer to ensure uniform distribution of the rose water in the medium. The inoculum was prepared by inoculating a single colony of *K. xylinus* (into a liquid HS medium and incubated for three days at 30°C. The absorbance of broth (bacteria

cell) was determined using a spectrophotometer (UV-1800 PC Shimadzu spectrophotometer), where 1.0 at OD600 nm, equivalent to 8 x 10^8 *K. xylinus* cells per millilitre was used as an inoculum. Accordingly, 10 mL bacteria broth was inoculated into a 90 mL treatment medium that was placed in a 250 mL Erlenmeyer flask. The flask was then covered with aluminium foil and incubated at 30°C. Cells were measured every three-day interval for 15 days of fermentation. Subsequently, the total phenol, total flavonoid and radical scavenging activity were determined. Three replicates were prepared for each concentration of rose water treatment.

Total Phenolic Content

At harvest (15 days of fermentation), the total phenolic content was determined using a spectrophotometer (UV-1800 PC Shimadzu) based on the method previously described by Singleton and Rossi (1965). The reaction mixture consisted of 0.5 mL culture broth (kombucha solution), 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of 20% (w/w) sodium carbonate. The solution was made up to 10 mL with distilled water and incubated at room temperature, 28°C, for 120 minutes. Correspondingly, absorbance was measured at a wavelength of 750 nm with the blank using an HS medium. Gallic acid was employed as a calibration reference, and the findings were represented in milligrams of gallic acid equivalent per millilitre of sample (mg GAE/mL).

Total Flavonoid Content

The total flavonoid content was determined using the spectrophotometric method published by Yamin *et al.* (2021), with slight modifications. A 3 mL bacteria broth sample was combined with 5.4 mL of distilled water, 0.5 mL of sodium nitrite solution (5%), 0.3 mL of aluminium chloride hexahydrate (10%), and 1 mL of sodium hexahydrate (1 mol/L). Absorbance was measured at 510 nm with the blank on the HS medium. Rutin was employed as a calibration reference, and the findings were represented in milligrams as rutin equivalents per millilitre of sample (mg RE/mL).

DPPH Radical Scavenging Activity

The radical scavenging activity based on DPPH was assayed according to the method of Morales and Jimenez-Perez (2001). The reaction mixture consisted of 4.8 mL of DPPH solution and 1.0 mL of sample broth. The mixture was left at room temperature for 60 minutes. The absorbance was then measured at 515 nm with the blank on the HS medium. The DPPH Radical Scavenging Ability (RSA) was represented in percentages (RSA_{DPPH}) and computed using the following equation. Where A blank was the absorbance of the blank and A sample was the absorbance of the sample.

$$RSA_{DPPH}\,(\%) = (\text{A blank-A sample})/(\text{A blank}) \times 100.$$

Experimental Design and Statistical Analysis

The data were analysed using one-way ANOVA. The significant difference in the treatment was assessed using a post hoc test at $p < 0.05$, which was significantly different among the treatments. Each treatment was repeated three times, and the findings were reported as the mean value ± standard deviation using Microsoft Excel 2019.

Results

Bacterial Cell Counts and Cellulose Density

Results in Figure 2 illustrate the estimated cell counts of *K. xylinus* bacteria for various concentration treatments throughout 15 days of fermentation. For all treatments, the cell counts were significantly increased from the first day to the third day of fermentation, with no significant difference until nine days ($p < 0.05$) and declined after that (Figure 2). Among the treatments, the lower bacteria cell counts were from the rose water, followed by 80%, 60%, 40%, and 20% rose water (Figure 2). The control medium exhibits the highest cell count among the treatments until the end of the experiment. Figure 3 displays the density of cellulose in different concentration treatments after 15 days of fermentation. The highest cellulose density was observed in the treatment medium containing 20% rose water and was almost similar to the control. On the other hand, a treatment containing the highest concentration of rose water produced the lowest cellulose density by the *K. xylinus* after 15 days of fermentation period (Figure 3).

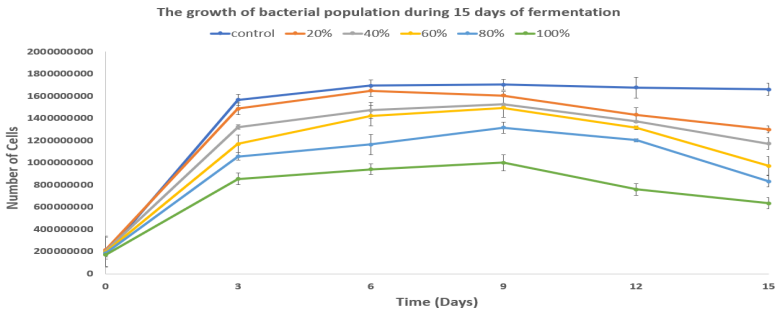

Figure 2: The bacteria population from the broth with different concentrations of rose water, sampled at 3-day intervals during fermentation

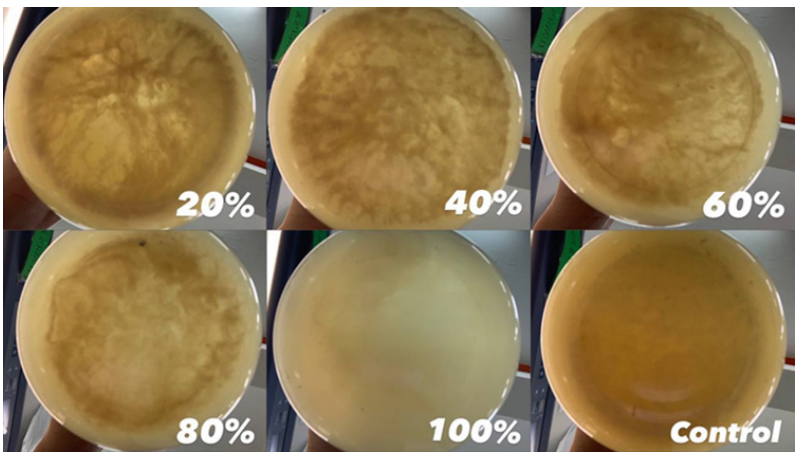

Figure 3: The density of cellulose produced by *Komagataeibacter xylinus* from the broth with different concentrations of rose water at 15 days of fermentation

Total Phenolic and Flavonoid Content

The rose water concentration had the greatest effect on the growth of *K. xylinus* and the total phenolic and flavonoid content in the culture medium. The results revealed that the highest total phenol content was in the treatment medium containing 20% and 40% rose water (Table 1). In these media, the total phenolic content was 0.07 mg/mL, respectively. This was followed by 60%, 40%, and 20% rose water. Results suggested a similar total phenolic content compared to the control in the treatment medium with 60% rose (v/v) water. i.e., 0.05 mg/mL (Table 1).

In terms of total flavonoid content, the results indicated that the highest flavonoid was in the treatment medium of 20% rose water, which was 0.07 mg/mL (Table 1). The total flavonoid content was decreased, and the concentration of rose water used increased. The lowest total flavonoid was in 100% rose water medium (0.02 mg/mL of total flavonoid). The results also revealed that a similar total flavonoid content compared to the control was at a concentration of 60% rose water, which was 0.05 mg/ml of total flavonoid per culture (Table 1).

Table 1: Effects of rose-water concentrations on the total phenolic and total flavonoid content in the broth and final pH after 15-day fermentation with *Komagataeibacter xylinus.*

Treatment with rose water (%)	Final pH	Total Phenolic (mg GAE/ mL)	Total Flavonoid (mg QE/ mL)
20	4.6	0.07 ± 0.00	0.07 ± 0.00
40	4.7	0.07 ± 0.00	0.06 ± 0.00
60	4.9	0.05 ± 0.00	0.05 ± 0.01
80	4.9	0.04 ± 0.00	0.03 ± 0.00
100	4.7	0.03 ± 0.00	0.02 ± 0.00
Control (0)	4.8	0.05 ± 0.00	0.05 ± 0.00

DPPH Radical Scavenging Activity

The result of the radical scavenging activity of various rose water concentrations based on DPPH activity is in Figure 4. The results revealed that rose water at concentrations of 20 to 60% (v/v) was comparable to the control HS medium. On the other hand, rose water at higher concentrations (80 to 100% v/v) significantly reduced ($p < 0.05$) the radical scavenging activity (Figure 4), which was at 88.3% and 82.2% for 80% (v/v) and 100% (v/v) rose water, respectively.

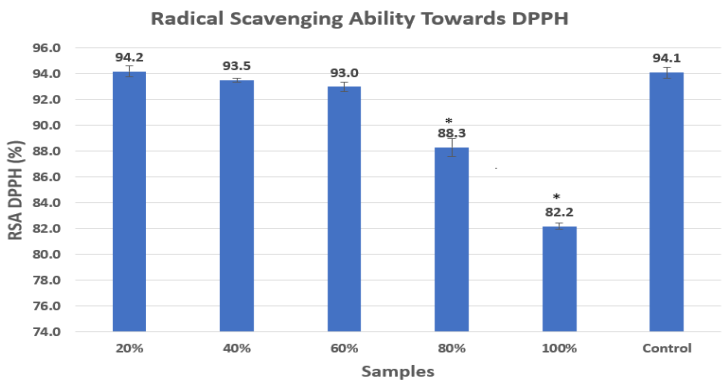

Figure 4: Radical scavenging ability towards DPPH Results expressed in percentage (RSADPPH). The * symbol indicated a significant difference compared to the control ($p < 0.05$) based on the post hoc test.

Discussion

The findings of the current study reveal that *K. xylinus* exhibits cell proliferation and a drastic growth rate in the HS medium after three days of fermentation, which continues until 15 days of fermentation. A previous study by Arikan *et al.* (2020) reported that the *Komagataeibacter* genus dominated the bacterial population in the culture, which is about 95% after ten days of fermentation, and their growth reduced after 15 days of fermentation. This might be due to competition among the bacteria strains used as inoculum, while in the current study, only one strain was used. Moreover, the size of the inoculum can also contribute to the growth behaviour of the microorganism in the medium. In this study, the number of bacteria cells used

as inoculum was estimated at 8 x 10^8 *K. xylinus* cells per millilitre. In terms of the effects of plant extract applied in the culture medium, the rose water concentration exhibits a negative effect on the bacteria cell proliferation (Figure 2). This might be due to lacking nutrients in higher concentrations of rose water and the antibacterial compounds that might be present in the rose water. In addition, rose water was reported to possess an anti-microbial activity (Ren *et al.,* 2016). Moreover, the plant extract application in kombucha production was after the culture attained the lag phase (Leonarski *et al.*, 2021). A previous study by Bodea *et al.* (2022) reported that utilisation of plant extract (rosemary extract) demonstrated visible antibacterial activity against *S. aureus,* although *E. coli* and *C. albicans* in cellulose fermentation.

Interestingly, the results of current findings, Table 1 and Figure 4, suggested that lower concentrations of rose water from Bishop's Castle petals (20% v/v) increased the total phenolic and flavonoid content in the broth higher than in the control (rose water-free medium). The finding suggests that utilisation of rose water at lower than 20% (v/v) might be an elicitor for higher production and accumulation of plant secondary metabolites such as phenolic and flavonoid in the fermented beverages using *K. xylinus.* The concentrations of vitamin C and other advantageous biological active substances in plant extract are beneficial for bacteria growth (Butkevičiūtė *et al.*, 2022). Additionally, flavonoids are a class of phenolic compounds that positively impact human health and work in conjunction with other compounds (Zhao *et al.,* 2021). Previous reports by Zubaidah *et al.* (2018) and Leonarski *et al.* (2021) indicated that plant extract is a beneficial natural resource to enhance the flavour and quality of kombucha. Nevertheless, the growth of bacteria varies depending on the plant extract. In their studies, the bacteria growth was highest after seven days (Zubaidah *et al.*, 2018). In addition, lower rose-water concentration (20% v/v) also promotes the accumulation of bacteria cellulose in the culture medium (Figure 2). The results indicate that *K. xylinus* may highly adapt to the lower concentration of rose water, utilise the nutrient present in the culture medium and release the matrix of cellulose as a by-product (Rahmani *et al.*, 2019).

Phenolics and flavonoids contained in the rose water seemed to influence microbial metabolism. In the case of *K. xylinus*, these chemicals may affect the synthesis of cellulose. Furthermore, the addition of rose water may stimulate bacterial growth or metabolic activity by a certain percentage, resulting in higher cellulose or bacterial growth output. However, the increment of rose-water percentage decreased phenolic and flavonoid content.

DPPH radical is well recognised as a stable synthetic product. It is commonly utilised in exploratory studies on the antioxidant properties of many natural compounds. The higher the concentration of rose extract fermentation, the lower the percentage of RSA. This could be due to various factors, including the presence of inhibitory chemicals, the saturation of active molecules, or modifications in the chemical composition of the extract at higher concentrations (Odriozola-Serrano *et al.*, 2023). During fermentation, the initial pH values for all treatments were decreased. Note that the pH values are consistent with the bacterial cellulose fermentation employed to produce fermentation beverages. During the fermentation process, sugar in the medium is converted into organic acids, particularly acetic acids (Revin *et al.*, 2018).

There may be minimal research on the effect of rose water on cellulose synthesis by *K. xylinus*. Hence, this work could help to address a gap in our understanding of the effect of natural additives on microbial processes and product production. In addition, investigating the impacts of rose water on fermentation outcomes may provide new insights into how natural extracts influence bacterial metabolism, phenolic and flavonoid content, and cellulose synthesis.

Conclusion

In conclusion, the experiment demonstrated that rose water at concentrations of 20% (v/v) managed to increase the total phenolic and flavonoid content in the broth fermented with *K. xylinus*. The rose water ranged from 20% to 60% and also increased the scavenging activity of DPPH radical. Notably, a lower concentration of rose water from Bishop's Castle petals might be useful for fermentation using *K. xylinus* to produce healthy fermented beverages and bacteria cellulose. The study also suggested that rose water should be supplied into the fermentation medium after the culture attained the end of the growth phase.

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