Development of Selective Media for *Komagataeibacter intermedius* and *Dekkera bruxellensis* from a Mixed Culture

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ABSTRACT

*Komagataeibacter intermedius* and *Dekkera bruxellensis* are some of the most commonly found bacteria and yeast, respectively, during Kombucha fermentation. The development of selective media for separating both species is essential to make an accurate cell enumeration. This study aimed to develop a selective media for the enumeration of *K. intermedius* and *D. bruxellensis* from a mixed culture sample. Hestrin and Schramm (HS) agar and Potato Dextrose Agar (PDA) were chosen as the enumeration media for *K. intermedius* and *D. bruxellensis*, respectively. To select for *K. intermedius*, HS agar was added with various concentrations of cycloheximide (0, 1, and 10 mg/mL), NaCl (2% and 5%), and acetic acid (1%). To select for *D. bruxellensis*, PDA was added with various concentrations of chloramphenicol (0, 0.3, and 3 μg/mL) and NaCl (2% and 5%). Each culture (≈10^4 log CFU/mL) was serially diluted and plated on the respective agar media, followed by incubation at 30°C for 1-6 days. The results showed that PDA containing 2% NaCl could completely suppress *K. intermedius* growth while permitting complete recovery of *D. bruxellensis*. However, it took 6 days until visible growth was observed by *D. bruxellensis*. Moreover, HS with the addition of 1% acetic acid successfully inhibited *D. bruxellensis* while allowing the complete recovery of *K. intermedius*. The findings suggested that the two media were suitable for separating *K. intermedius* and *D. bruxellensis* on agar media, thus allowing a more accurate cell counting in studies involving the mixed cultures of both species.

Keywords: Selective media, Dekkera bruxellensis, Komagataeibacter intermedius

Introduction

Kombucha is a sugar tea drink fermented by a symbiotic culture of bacteria and yeasts (SCOBY). These symbionts act in concert during the fermentation, converting sugars into a range of secondary metabolites and bioactive compounds with potential health benefits (Chakravorty et al., 2016). The fermentation also results in the production of bacterial cellulose, which finds applications in various industries, including textile, food, medical, and pharmaceutical industries (Azeredo et al., 2019; Ul-Islam et al., 2020). Since bacteria and yeast play a vital role in the formation of these by-products, studies focusing on the interaction between them during fermentation are paramount.

Previously, Angela et al. (2020) have isolated bacteria and yeast predominant during Kombucha fermentation, identified as *Komagataeibacter intermedius* and *Dekkera bruxellensis*, respectively. The high bacterial cellulose-producing capacity of *K. intermedius* has been reported in several studies (Angela et al., 2020; Fernández et al., 2019; Lin et al., 2016). Unlike *Acetobacter xylinum*, the most commonly used bacterial cellulose producer, *K. intermedius* is able to produce bacterial cellulose within a wide pH range (pH 4-9) (Lin et al., 2016). A mixed culture of *K. intermedius* and *D. bruxellensis* was also shown to increase the production of glucuronic acid by ~184% compared to the use of *K. intermedius* alone (Nguyen et al., 2015). Understanding the dynamics of both microbes resulting from their interactions could allow better control of fermentation (May et al., 2019).

One way of observing the microbes’ dynamics during fermentation is by measuring their viable cell count. However, as *K. intermedius* and *D. bruxellensis* colonies’ morphology look nearly identical, cell counting results would be unreliable when they are grown at the same time. Therefore, for the study,

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both cultures need to be grown on selective media. A common strategy for allowing selective growth in microbes would be to use antibiotics. An antifungal agent when bacterial growth is desired and an antibacterial agent when fungal growth is desired (Bonnet et al., 2020). Cycloheximide is an example of an antifungal agent usually used to suppress eukaryotic growth and more importantly, fungi from mixed cultures (Chauhan & Jindal, 2020). While chloramphenicol is a common antimicrobial agent due to being effective towards a wide spectrum of bacteria (Anderson et al., 2012).

Besides antibiotics, potentially effective alternatives were also considered that were less expensive and safer to handle. *K. intermedius* is known for its high tolerance to acetic acid (Andrés-Barrao et al., 2016), while several yeasts are known for their susceptibility to certain concentrations of acetic acid (Moktaduzzaman et al., 2016). This information suggests a possibility to use acetic acid to select for *K. intermedius* from its co-culture with *D. bruxellensis*. NaCl is another potential selective agent that can be used. *D. bruxellensis* is known for its ability to survive osmotic stress induced by salt at low concentrations, including NaCl (Zemančíková et al., 2018). Meanwhile, to date, there is no study reporting Komagataeibacter tolerance for NaCl-induced osmotic stress. In this study, acetic acid and NaCl of varying concentrations were added into the isolation media, and their effectiveness in separating *K. intermedius* and *D. bruxellensis* was evaluated.

**Material and Methods**

**Culture and media preparation**

*K. intermedius* and *D. bruxellensis* were previously isolated by Angela et al. (2020) from kombucha tea provided by PT. Tujju Kombucha (Jakarta, Indonesia). *K. intermedius* was maintained on Hestrin Schramm (HS) agar at 4°C, while *D. bruxellensis* was maintained on Potato Dextrose Agar (PDA) at 4°C. HS broth and Potato Dextrose Broth (PDB) were prepared to culture the microbes for the selective media test. HS media consisted of 20 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 2.7 g/L Na$_2$HPO$_4$ and 1.15 g/L citric acid. NaCl was directly added into the agar before being autoclaved; acetic acid and antibiotics were added to the agar after autoclave following filter sterilization using 0.22 µm membrane.

**Evaluation of selective agents on the enumeration of tested strains**

First, *K. intermedius* and *D. bruxellensis* were cultured in HS broth and PDB, respectively, at 30°C for 24 hours. Both broth cultures were then diluted serially using a 0.85% NaCl (w/v) solution and plated on the selective agar using the Miles and Misra method (Miles et al., 1938). To select for *K. intermedius*, HS agar was added with either cycloheximide (1 mg/mL and 10 mg/mL) or 1% (v/v) acetic acid. Then, to select for *D. bruxellensis*, PDA was added with either chloramphenicol (0.3 µg/mL and 3 µg/mL) or NaCl (2% and 5% w/v). As a negative control, *K. intermedius* and *D. bruxellensis* were also grown on HS agar and PDA, respectively, in the absence of selective agents. The plates were then incubated at 30°C. The CFU of both microbes were observed after 48 and 144 hours of incubation.

**Results and Discussion**

In this study, selective agents to separate *K. intermedius* and *D. bruxellensis* from a mixed culture during plate counting were explored. The results are reported in Table 1 and Table 2, respectively. Both microbes were found to be unaffected by antibiotics but fully inhibited by 5% NaCl.

As shown in Table 1, the CFU count of yeast grown in HS media with cycloheximide was not significantly different (p>0.05) from the CFU count of the yeast grown in HS media without cycloheximide at 3±0.4 x 10^6 CFU/mL (Table 1). Although the exact mechanism is still unclear, cycloheximide inhibits cytosolic protein synthesis by binding to the large 80s ribosome subunit (Stevens et al., 2001). According to Ciani and Comitini (2014), *D. bruxellensis* is able to tolerate concentrations up to 1 mg/mL of cycloheximide in some cases. Moreover, multiple studies have confirmed the resistance of *D. bruxellensis* towards cycloheximide (Steensels et al., 2015; Morneau et al., 2011). A further increase in cycloheximide concentration to 10 µg/mL confirmed the inability of cycloheximide to inhibit *D. bruxellensis* growth. It is also noted that this concentration is 100 times higher than the working concentration.
The exact mechanism of resistance in *D. bruxellensis* is also still unknown, but some studies have found several ways other fungi resist cycloheximide which *D. bruxellensis* may utilise. In *Candida albicans*, the expression of two certain ABC transporters (Cdr1 and Cdr2) are largely responsible for resistance towards different azole antibiotics, including cycloheximide (Ali et al., 2015). Additionally, the deletion of Cdr1, Cdr2, and other homologues have been found to result in sensitivity towards the azole antibiotics in yeasts (Sanglard et al., 2009). Another method of resistance was observed in *Chlamydomonas reinhardtii* by the replacement of a proline residue with glutamate in the L41 protein of the 80s ribosomal subunit (Stevens et al., 2001; Ali et al., 2015). Besides that, *D. bruxellensis* is known for having more phenotypic diversity which may allow it to adapt to harmful environments (Avramova et al., 2018). Consequently, due to this resistance, other alternatives are usually recommended to suppress *D. bruxellensis*’ growth in different kinds of industries, such as winemaking and ethanol production (Zuehlke et al., 2013; Beckner et al., 2011). Continuing to use antifungals to suppress *D. bruxellensis* may be ineffective as another study by Rodrigues et al. (2001) found that different strains may be resistant to other antifungals as well.

Compared to the negative control, *D. bruxellensis* needed 6 days to grow in 2% NaCl before the colonies were visible enough to count. Nonetheless, *D. bruxellensis* was found to be able to grow in the presence of 2% NaCl with a CFU count of 1.24±0.2 x 10⁶ CFU/mL. *D. bruxellensis* is known for being able to tolerate up to 3.38% of NaCl (Stratford et al., 2019). The sulphite tolerance gene (SSU1) and the HOG-kinase pathway, found in most other fungal species, seem to be responsible for *D. bruxellensis*’ slight osmotolerance (Curtin et al., 2014). However, compared to other yeasts, *D. bruxellensis* is considered to be sensitive towards NaCl which explains why a longer incubation period was required (Bubnová et al., 2014; Zemančíková et al. 2018). On the other hand, 1% acetic acid was found to fully inhibit the growth of *D. bruxellensis* with no growth observed even after 6 days of incubation. Although *D. bruxellensis* is an acetic acid producer, it could only produce a maximum of 0.3% acetic acid (Freer, 2002). Furthermore, *D. bruxellensis* is susceptible to around 0.6% to 0.8% acetic acid (Uscanga et al., 2000). The presence of acetic acid not only inhibits *D. bruxellensis*’ glucose uptake, but also causes oxidative stress in the cells and can disrupt its internal pH (Moktaduzzaman et al., 2015).

Table 1. The effect of a selective agent towards yeast growth

| Media   | Selective agent | The concentration of selective agent | The incubation period (days) | Yeast count (CFU/ml) |
|---------|----------------|--------------------------------------|-------------------------------|---------------------|
| HS      | Cycloheximide  | 0**                                  | 2                            | 3.5±0.3 x 10⁶       |
|         |                | 1 mg/ml                              | 2                            | 3.9±0.5 x 10⁶       |
|         |                | 10 mg/ml                             | 2                            | 3±0.4 x 10⁶         |
|         | Acetic acid    | 1%                                   | 6                            | No growth           |
|         | NaCl           | 2%                                   | 6                            | TNTC                |
|         | NaCl           | 5%                                   | 6                            | No growth           |
| PDA     | NaCl           | 2%                                   | 6                            | 1.24±0.2 x 10⁶      |
|         |                | 5%                                   | 6                            | No growth           |

* Student’s t-test was performed at 95% confidence interval to analyze the effect of cycloheximide at different concentration levels toward yeast count **negative control
Control | Cycloheximide | NaCl | Acetic acid  
--- | --- | --- | ---  
HS only | 1 mg/mL | 10 mg/mL | 2% | 5% | 1%  

Figure 1. Growth of *D. bruxellensis* in the presence of different selective agents. Control and cycloheximide plates were observed after 2 days of incubation while NaCl and acetic acid plates were observed after 6 days of incubation.

As displayed in Table 2, the CFU count of the bacteria with or without chloramphenicol showed no significant difference (p>0.05). Chloramphenicol is a protein synthesis inhibitor which by reversibly binding to the 50s subunit of the 70s ribosome (Balbi, 2004). The concentration of chloramphenicol used in this study is considered to be effective towards Acetobacter which is in the same family as Komagataeibacter (Haghshenas et al., 2014). However, the current study showed that *K. intermedius* was able to grow unhindered in the presence of chloramphenicol as shown in Figure 1. A higher concentration may be required in order to suppress *K. intermedius*’ growth as a previous study reported a higher concentration of 300 μg/mL and 100 μg/mL to be effective towards *Komagataeibacter hansenii* and *Komagataeibacter xylinus* respectively (Varley, 2017). Both concentrations are considered high and known resistant bacteria are also only affected by similar concentrations (Fernández et al., 2014). Another study also found that *Komagataeibacter melaceti* and *Komagataeibacter melebonus* are resistant towards chloramphenicol as they possess a homologue for chloramphenicol acetyltransferase which inhibits the antibacterial activity (Marić et al., 2020).

As shown in figure 2, *K. intermedius* was found to be unable to grow in the both concentrations of NaCl even after 6 days of incubation. *Komagataeibacter* are known for being unable to tolerate more than 0.5% NaCl (Prudnikova & Shidlovsky, 2017). It is likely that the sodium ion affected the bacteria’s plasma membrane properties and therefore the bacteria’s viability (Gandhi et al., 2014). Meanwhile, *K. intermedius* was found to be able to grow in the presence of acetic acid at 1% with a countable CFU after only 2 days of incubation (9.36±2.5 x 10⁶ CFU/mL) (Figure 2). *Komagataeibacter* are able to produce up to 20% of acetic acid, indicating its high tolerance to acetic acid (Andrés-Barrao et al., 2016). Additionally, *K. intermedius* has also been found to tolerate around 6% of acetic acid (Gomes et al., 2018). This resistance may be possible as it was found that an increase in concentration of acetic acid causes the Komagataeibacter to adapt using several strategies: an increase in cell surface area, an increase in efflux-pump systems, and modification of the protein and lipid membrane (Barja et al., 2016).
Table 2. The effect of a selective agent towards bacteria growth

| Media | Selective agent | Concentration of selective agent | Incubation period (days) | Yeast count (CFU/ml) |
|-------|----------------|----------------------------------|--------------------------|---------------------|
| PDA   | Chloramphenicol| 0**                              | 2                        | 8.53±1.6 x 10^6     |
|       |                | 3 μg/ml                           | 2                        | 4.6±0.5 x 10^7      |
|       | NaCl           | 2%                               | 6                        | No growth           |
|       |                | 5%                               | 6                        | No growth           |
| HS    | NaCl           | 2%                               | 6                        | No growth           |
|       |                | 5%                               | 6                        | No growth           |
|       | Acetic acid    | 1%                               | 2                        | 9.36±2.5 x 10^6     |

*Student’s t-test was performed at 95% confidence interval to analyse the effect of chloramphenicol at different concentration levels toward bacteria count ** negative control

Control

| Media | Chloramphenicol | NaCl | Acetic acid |
|-------|-----------------|------|-------------|
| PDA only | 3 μg/mL | 2% | 1% |
| 30 μg/mL | 5% |

Figure 2. Growth of *K. intermedius* in the presence of different selective agents. Control, chloramphenicol and acetic acid plates were observed after 2 days of incubation. Plates containing NaCl were observed after 6 days of incubation.

Conclusion

In this study, we have developed selective media for *Komagataeibacter intermedius* and *Dekkera bruxellensis* from a mixed culture. Antibiotics at the concentrations tested were found ineffective towards the microbes tested in this study, suggesting either the need to use higher concentrations or alternatives. The results of this study showed that NaCl 2% can be used as a selective agent for *D. bruxellensis* from this mixed culture, albeit the slower growth. Furthermore, 1% acetic acid was found to be an effective selective agent for the bacteria as it was able to fully inhibit the growth of *D. bruxellensis* and did not hinder the growth of *K. intermedius*. 
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