CHEMICAL ENGINEERING TRANSACTIONS
VOL. 56, 2017
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Jiří Jaromír Klemeš, Peng Yen Liew, Wai Shin Ho, Jeng Shiun Lim

ISBN 978-88-95608-47-1; **ISSN** 2283-9216
DOI: 10.3303/CET1756086

Growth of *Bacillus Coagulans* Using Molasses as a Nutrient Source

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Bacillus coagulans (*B. coagulans*) can be utilized as microbial inoculant to speed up the bioremediation of wastewater. The effectiveness of the microbial inoculant for treatment is highly selective and relying on the characteristics of the wastewater. A feasible carbon source must be first chosen to pre-culture the microbe prior to the bioremediation. Commercial nutrient broths are efficient to grow the microbial; they are costly for the treatment of large volume of wastewater treatment. This study aims to evaluate the growth rate of *B. coagulans* using a cheaper nutrient source, i.e. molasses. The commercial Man, Rogosa and Sharpe (MRS) media was used as a benchmark. The growth rate of *B. coagulans* was conducted at different concentrations of molasses (1 %, 3 % and 5 % (w/v)) and in the MRS broth as benchmark. *B. coagulans* grown in the MRS has shown a much higher maximum specific growth rate (0.69 h^{-1}) compared to that grown in 1 % (w/v) molasses (0.14 h^{-1}). No growth was observed in the higher concentrations of molasses (3 and 5 % (w/v)). Measuring the colony-forming units of *B. coagulans* in both the MRS and molasses mediums validated the results. Molasses is a desirable carbon source as it is relatively cheaper and easily available. More studies are needed to improve the maximum specific growth rate of *B. coagulans* in 1 % (w/v) of molasses.

1. Introduction

Bioremediation is a process using microorganism to breakdown the environmental pollutants (Kumar et al., 2011). It is relatively low-cost and environmental-friendly and has received good public acceptance (Mary, 2011). To ensure effective bioremediation, a suitable microorganism must be selected based on its ability to degrade contaminants. *Bacillus coagulans* was originally introduced as a probiotic bacterium, often marketed wrongly as '*Lactobacillus sporogenes*' (Food info, 2016). *B. coagulans* has been applied for the bioremediation of wastewater due to its ability to digest contaminants (nitrogen, phosphorus, heavy metals) and to convert it to biomass (Kotay and Das, 2007). *B. coagulans* has been used for the production of L-lactic acid (Ma et al., 2014), and lactic acid (Kumar et al., 2005). The microbes were cultured in a nutrient medium to a desired quantity before being applied to the wastewater. Several types of carbon sources such as glucose, fructose and lactose were used for the wastewater treatment, but the usage of such pure or mixed media at industrial scale would incur a high cost (Michailides et al., 2015). There is a need to find a more economical carbon source for the industrial application.

Molasses is an important agro-industrial by-product that contains high sugar contents (48–50 %) (Quan et al., 2005). Molasses can be used as a cheaper nutrient source for microbial growth. Zhang et al. (2008) used molasses as a carbon source for the remediation of selenium; for the sulphate reducing bacteria (Telcu et al., 2009). Molasses have been used for the production of cellulose (Bae et al., 2005); fermentation of amylolytic enzyme (Najafpour and Shan, 2003); and for the biological removal of Cr(VI) in the wastewater (Michailides et al., 2015). The Malaysian market price of the liquid molasses is around MYR 27/ kg, whereas the common

Please cite this article as: Ahmad Sanadi N.F., Fan Y.V., Leow C.W., Wong J.H., Koay Y.S., Lee C.T., Chua L.S., Sarmidi M.R., 2017, Growth of bacillus coagulans using molasses as a nutrient source, Chemical Engineering Transactions, 56, 511-516 DOI:10.3303/CET1756086

nutrient broth such as de Man, Rogosa and Sharpe (MRS) broth (Merck, Germany) is MYR1,000/ kg. Different types of substrates will affect the microbial growth rate. Different concentrations and quality of the molasses might affect the growth rate of the inoculum. This study aims to evaluate the growth rate of *B. coagulans* using molasses as the nutrient source and comparing the growth rates against those grown in the commercial MRS broth as a benchmark. Comparison of properties for molasses and MRS broth as the growth media for bacterial is shown in Table 1.

Table 1: Comparison of properties for molasses and MRS broth as growth media

Properties	Molasses	MRS broth
Selectivity	All bacteria	Lactobacilli sp. Bacteria
Price (MYR/ kg)	27	1,000
Health factor	None	Can cause skin and eye irritation if the expose limit is exceeded (OSHA, 2012)
Environmental factor	None	Hazardous and must be disposed according to standards (OSHA, 2012)

Table 2 indicates that molasses produced from different sources has approximated 4-5 g/L of sugar content.

Table 2: Sugar composition of molasses (g/ L) from different sources (Dionex, 2003)

Components	Sugar beet molasses	Sugar cane molasses
Glucose	-	0.439
Fructose	-	0.667
Sucrose	4.77	3.08
Raffinose	0.210	-

2. Materials and Methods

Unless otherwise stated, all experiments were duplicated and the respective standard of deviations calculated using the Microsoft Excel 2010 statistic tool.

2.1 Preparation of inoculum

A single colony of *B. coagulans* from ATCC 7050 (Arachem) was picked from the overnight nutrient agar plate and cultured in the 100 mL MRS broth (1.10661.0500, Merck, Germany) for overnight in the incubator shaker at 50 °C and 200 rpm (Zhang et al., 2014). All media and apparatus were autoclaved at 121 °C for 15 min prior to the addition of the colony.

2.2 Culture Medium

Two types of culture media were used, i.e. the MRS broth (1.10661.0500, Merck, Germany) and the molasses purchased from EMRO Private Limited (Johor, Malaysia). Molasses was prepared at different concentration (1 %, 3 %, 5 % (w/v)). The growth rate of *B. coagulans* in the MRS broth was used as a benchmark to evaluate the growth of *B. coagulans* in molasses (Hyronimus et al., 2000).

Table 3: Composition of MRS broth (Merck, Germany)

Composition (g/ L)	Components
2	Dipotassium hydrogen sulphate
0.2	Magnesium sulphate
20	Heptahydrate
0.05	Glucose
8	Maganous sulphate
10	Tetrahydrate
5	Meat extract
4	Yeast extract

2.3 Growth rate study by optical density

One mL of *B. coagulans* prepared with optical density (OD_{600}) 2.72 was inoculated in a 250 mL conical flask containing 100 mL of medium at different concentrations (1 %, 3 %, 5 % (w/v)). The respective conical flasks were cultured in an incubator shaker at 50 °C and 200 rpm. The cell growth was monitored by measuring the optical density of the samples at the wavelength of 600 nm (OD_{600}) (Soleimaninanadegani and Manshad, 2014) using a spectrophotometer (Jenway 7305, United Kingdom). The samples were taken at 2 h interval.
B. coagulans undergoes the exponential growth phase where the rate of cell production is proportional to the number of cells present at any particular time (Maier et al., 2000). The maximum specific growth rate (μ_{max}) is defined as the maximum rate of microbial growth or maximum substrate conversion by the *B. coagulans*. The maximum specific growth rate for each nutrient source has been determined using Eq(1):

$$\mu_{max} = \frac{dX}{dt} \quad (1)$$

where $\frac{dX}{dT}$ is the rate of microbial growth in the exponential phase per h.

2.4 Growth by Colony-forming-unit (CFU)

CFU is a unit used to measure the number of viable *B. coagulans* in the samples. One mL of each sample was diluted in series (10^2-10^7) and inoculated every 2 h into a MRS agar plate (1.1106600500, Merck, Germany). The MRS agar plate was placed in an incubator for 24 h at 50 °C. The CFU was calculated using Eq(2):

$$\frac{\text{Bacteria}}{\text{mL}} = \frac{\text{no. of colonies x dillution factor}}{\text{volume of inoculant (mL)}} \quad (2)$$

2.5 Morphological of *B. coagulans* after 24 h

Gram-positive staining method was used to visualize the morphology of *B. coagulans* (Barile, 2012) using the light microscope under 100x magnification (Olympus CX21 Microscope, Japan). The standard glass slide was immersed in alcohol and dried before use. One colony of *B. coagulans* was picked from the nutrient agar and smeared on the glass slide. A drop of crystal violet (Merck, Germany) was placed on top of the smear and left for one min before rinsed with tap water. A drop of iodine (Merk,Germany) was added on the smear and left for one min before rinsed with tap water. Decolorized ethanol was added drop wise until the crystal violet color was merely seen with a bright blue color retained.

3. Results and Discussion

3.1 Growth rate of Bacillus *coagulans* by Optical Density

B. coagulans was grown in different concentrations of molasses (M). The growth in the commercial MRS broth was used as a benchmark. Measurement of optical density (OD) is a rapid method to monitor the cell growth. From Figure 1, only the 1 % (w/v) molasses has indicated the cell growth (OD_{600}= 0.32) at 24 h while the other two concentrations (3, 5 % (w/v)) did not recorded any OD_{600}. The MRS broth has shown the highest growth (OD_{600}=2.27) at 24 h. This results shows that 1 % (w/v) molasses serves as a viable concentration to grow *B. coagulans* with further optimization work required. Molasses has a substantial amount of sugar content to serve as a nutrient source. The growth of the microbial will be inhibited at high sugar content due to substrate inhibition possibly via the osmosis effect (Lodish et al., 2000). The growth rate of *B. coagulans* could be inhibited by the initial glucose concentration between 0.4 % (w/v) to 2 % (w/v) using glucose mineral salt media (GMSM) as the growth media (Das and Sen, 2011).
As summarized in Table 4, MRS broth recorded the highest maximum specific growth rate (μ_{max} =0.69 h^{-1}) of *B. coagulans* followed by the growth in 1 % (w/v) molasses (μ_{max} =0.14 h^{-1}) at 24 h. Higher molasses concentrations (3 and 5% (w/v)) did not shown any growth till 24 h. The μ_{max} achieved in 1 % (w/v) of molasses was much lower as compared to the MRS broth. It was likely that the high growth rate achieved in MRS broth was promoted by high concentration of minerals as shown in Table 3.
Measurement of OD is a convenient and rapid method to monitor the cell growth, the value might be interfered by the death cell. Measurement of colony-forming unit (CFU) serves as a complementary method to validate the growth of the living cells.

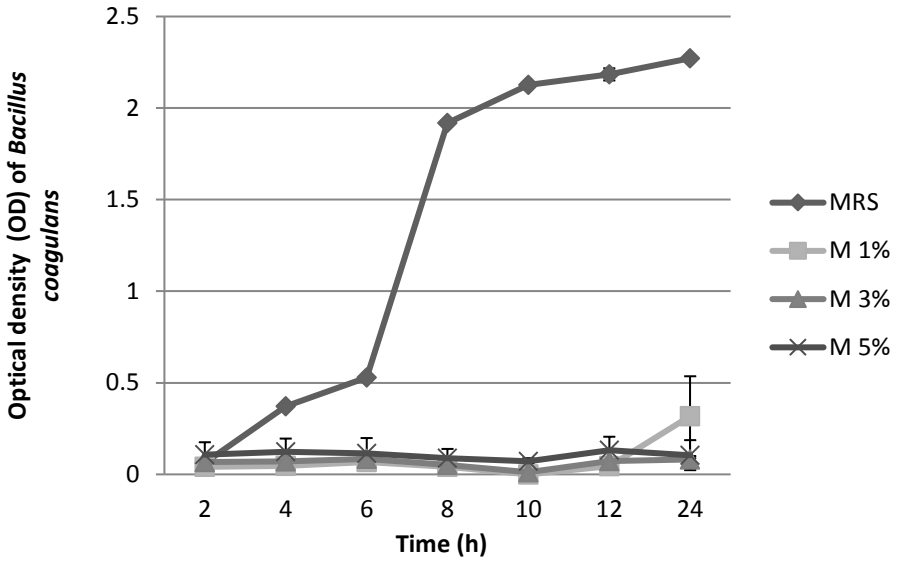

Figure 1: OD_{600} of B. coagulans in 1, 3, 5 % (w/v) of molasses (denoted as M 1 %, M 3 %, M 5 %) and MRS broth (MRS) as benchmark

Table 4: Specific growth rate of B. coagulans

Sample	Specific growth rate, μ_{max} (h^{-1})
MRS	0.65
M 1 %	0.14
M 3 %	-
M 5 %	-

3.2 Growth of *Bacillus coagulans* by Colony-Forming Unit (CFU)

As shown in Table 5, there was a significant increase in CFU for the *Bacillus coagulans* samples grown in MRS between 2 to 24 h. High CFU (too numerous to count, TNTC) was recorded in the MRS sample from 2 h onwards. The M 1 % samples have recorded a significant increase in CFU between 12 to 24 h. Consistent to the OD_{600} results in Section 3.1, CFU was not detected (ND) for the M 3 % and 5 % samples, an indication of the poor growth rate of the cells up to 24 h. The results validated that *B. coagulans* could grow well in M 1 % and MRS and could not grow well in M 3 % and M 5 % possibly due to high concentration of sugar. Comparing to Figure 1, there is a positive correlation between the OD and the CFU results.

Table 5: CFU of B. coagulans in different growth media

Sample	2 h	4 h	6 h	8 h	10 h	12 h	24 h
MRS	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
M 1 %	5,000	6,260	9,880	122,000	146,200	306,000	4,850,000
M 3 %	ND	1,600	2,600	ND	ND	ND	40
M 5 %	200	7,200	1,000	2000	ND	ND	ND

3.3 Morphology of *B. coagulans*

To verify that the pure culture of *B. coagulans* was grown in all media, the morphology of *B. coagulans* was examined by the microscope. The rod-like morphology of *B. coagulans* has been validated and shown in Figure 2.

B. coagulans originates from the Bacillus genus. The blue colour of the stained *B. coagulans* in Figure 2 indicates that the bacterium is a gram-positive strain. If the bacteria are gram-negative, it will appear as stained orange-red. The extent of decolourisation is a crucial step and must be standardized (Barile, 2012).

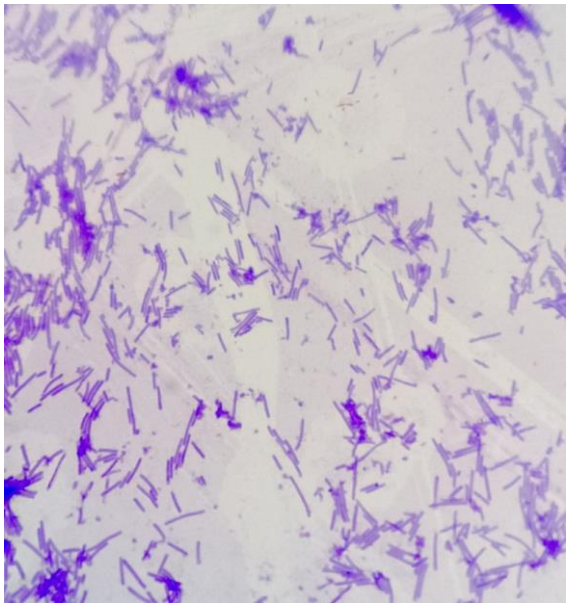

Figure 2: Rod-like morphology of B.coagulans at 24 h under 100x light microscope lens.

4. Conclusion

Based on the OD and CFU data, molasses has been shown as a potential media for the growth of *B. coagulans* as benchmarked against the MRS broth. Further optimization would be conducted to enhance the growth of *B. coagulans* in molasses, notably the alternation of operating temperature and addition of minimal amount of minerals. This result is desirable as molasses serves as a cheaper and easily available nutrient source for culturing *B. coagulans* as microbial inoculant. Molasses could also be considered to culture more other strains for industrial applications.

Acknowledgement

The authors acknowledge research grants from the Ministry of Higher Education (MOHE) Malaysia with grant no. 7301.4B145 and 2546.15H25; and from Universiti Teknologi Malaysia with the grant no. 2546.14H65 and 2501.10H28. The authors also acknowledge the funding and support from JICA-JST-SATREPS entitled “Development of Low Carbon Scenarios in Asian Region”.

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