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Research Article

Biofilm and Growth Effect of Sugar Beet Molasses (SBM) on *Pseudomonas aeruginosa*

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Abstract

Since many agricultural and biological wastes are given to the environment, they cause environmental problems. One of them is a molasses. Biofilm tubes were washed four times with 1 x phosphate buffered saline (PBS) to eliminate any remaining cells. Cells attached to the tubes were then fixed with ethanol (99%) for 15 min room temperature and stained with 1% crystal violet. After staining, excess crystal violet was eliminated with water, and 33% acetic acid was used to dissolve the remaining dye. In our study, biofilm, BFI production and positive growth results were obtained by using molasses under different ventilation conditions. As a result, increases in biofilm, number of viable cells and biomass formation up to 9, 1511 and 3,5- fold formation were observed, respectively. We think that this study will contribute to the literature and will be guiding.

Keywords: *P. aeruginosa*; Molasses; Biofilm; Growth; BFI

Introduction

Agricultural raw materials such as wheat bran, corn yellow liquor, rapeseed meal, starch waste water and molasses have been used as bio-refinery raw materials for many industrial products, such as traditional chemicals. These wastes usually contain sucrose, glucose and fructose [1]. SBM is a by-product of the sugar industry and is widely used as an economical and useful carbon source for microbial fermentation due to high sugar concentration. SBM; sucrose, glutamate and betaine, and is a wastewater produced by *P. denitrificans*. Therefore, beet molasses can potentially be considered an efficient and low-cost substrate in the production of many metabolites [2]. In the sugar industry, many by-products such as molasses, pulp and fiber cakes are produced; molasses are the most important among them. The molasses contain about 50% saccharin; therefore, it has a high commercial value because it is used as a carbon source in various fermentations. However, after the use of molasses as a raw material for fermentation's such as alcohol and amino acid production, a large amount of colored material remains in the fermentation as waste after recovery of the product. SBM is one of the most difficult waste products to discard due to low pH and dark brown color. High COD content can be reduced to a certain extent by methane fermentation and activated sludge treatments, but remains a dark problem, which requires a pre-treatment before being disposed of safely in the environment. These compounds have antioxidant properties which make them toxic for aquatic micro and macro organisms [3]. Molasses has been widely used as a carbon source for the commercial production of baker's yeast [4-5].

P. aeruginosa is a gram-negative bacterium that can live in many ecological niches including soil, water and plants. It can produce many metabolites (such as biofilm, rhamnolipid) that provide competitiveness and survival [6].

Pseudomonas spp. is capable of using a variety of renewable resources, in particular agricultural industrial wastes. This leads to the possibility of reducing the pollution caused by wastes. Molasses is used as a carbon source because it is cheap. The sweet, dark brown, molasses produced in the beet process offer a high concentration of sucrose, a high concentration of sucrose, other important substances and a low cost [6,7].

The aim of this study, cheap carbon and molasses were used as energy sources for the production of the production of biofilm, total cell mass, movements and cfu/ml sugar beet molasses of P. aeruginosa. This will reduce the environmental pollution caused by these materials.

Materials and Methods

Solvents and chemicals

NaCl, $Na_2HPO_4.2H_2O$, KH_2PO_4, Crystal violet (Merck); KCl (Carlo Erba); EtOH (Tosel); Acetic acid (Sigma-Aldrich) were used in the study. All chemicals used were of analytical grade.

Microorganism

P. aeruginosa (ATCC 27853), obtained from the ATCC and used in this study.

Sugar beet molasses

Sugar beet molasses (SBM) was collected from Malatya Sugar Factories in Malatya, Turkey. This waste was filtered for removing crude impurities and then, SBM autoclaved, and then used.

Growth conditions

P. aeruginosa was cultivated in Luria- Bertani (LB) broth medium (g l^{-1}); peptone 10, NaCl 10, and yeast extract 5. The final pH values of broth media was adjusted to 7.0. The same amounts of cells were grown at 37°C, 0 rpm on incubator for overnight (O/N). 100 µl of over night cultures was setting OD_{600} nm of 0,2-0,3 grown tube and filled with 5 ml in 10 ml tubes was inoculated, and incubated for 24 h of time. Phosphate-buffered saline (PBS buffer) (gl^{-1}: 8,0 NaCl; 0,2 KCl; 1,44 Na_2HPO_4; 0,24 KH_2PO_4 and pH 7,4) and PBS+10 % SBM. These cultures were subsequently incubated on an orbital shaker at 0, 100 rpm, 200 rpm and 37°C for 24 h.

Biofilm formation

After the incubation, the supernatant was removed. Biofilm tubes were washed four times with 1 x phosphate buffered saline (PBS) to eliminate any remaining cells. Cells attached to the tubes were then fixed with ethanol (99%) for 15 min room temperature and stained with 1% crystal violet. After staining, excess crystal violet was eliminated with water, and 33% acetic acid was used to dissolve the remaining dye. Biofilm mass was finally determined as a function of the concentration of this dye based on the absorbance at 570 nm [8-11].

Biofilm formation was standardized to growth with the biofilm index (BFI), which was calculated. The extent of biofilm formation was determined by applying this formula: "BFI = (AB - CW)/G in which BFI is the "Biofilm Formation Index was defined as the average percentage of bacteria grown as biofilms" [12], AB is the OD_{570} nm of stained attached bacteria and CW is the OD_{570} nm of stained control tubes containing only bacteria-free medium, G is the OD_{600} nm of cells growth in suspended culture" [13,14]. OD600 and OD_{570} were measured using a spectrophotometer directly from tubes. Growth curves were established by plotting the $\log_{10}$ cfu/ml as a function of time. Bacterial growth was determined by measuring the absorbance at 600 nm (OD_{600}) by a spectrophotometer. Each value is the average of three independent experiments.

Results

Biofilm

When we look at the OD_{570} values the greatest increase was at 4,65 at 200 rpm ventilated runs while the lowest rate was achieved at 3,06 at 100 rpm ventilation conditions. The addition of molasses caused the biofilm formation to increase at all ventilation conditions. While this increased is resulted in 9,4; 1,7 and 2,2- fold 0, 100 and 200 rpm, respectively (Figure 1). This value is an indication that the contribution of molasses to biofilm formation is high. Overall, biofilm 10% SBM addition at 0 rpm and 200 rpm resulted in a 1,3- fold increase in biofilm formation and at 0 rpm and 100 rpm resulted in a 1,2- fold decrease in biofilm formation (Figure 1). In another unpublished study, NB the rich media medium, was used. In the end (OD_{570}); 0 rpm 0.066, 100 rpm 0.222, and 0.389 at 200 rpm was obtained (N.A), respectively. This work was done to control.

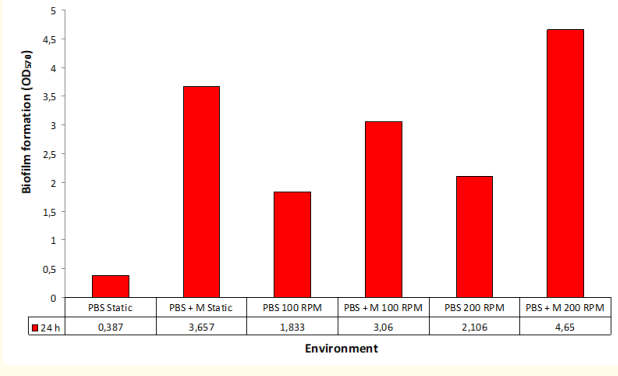

Figure 1: Biofilm formation on tubes by *P. aeruginosa* with different gentle swirling at 37°C for 24 h.

BFI

In all experimental conditions, the addition of molasses to controls resulted in a 1.5- fold decrease. The lowest BFI value has 0,63

0 rpm and while the highest value was 0,73 at 200 rpm shaking conditions with addition of molasses. Given the experimental conditions, molasses addition resulted in a reduction of up to on average 63% in BFI formation (Figure 2).

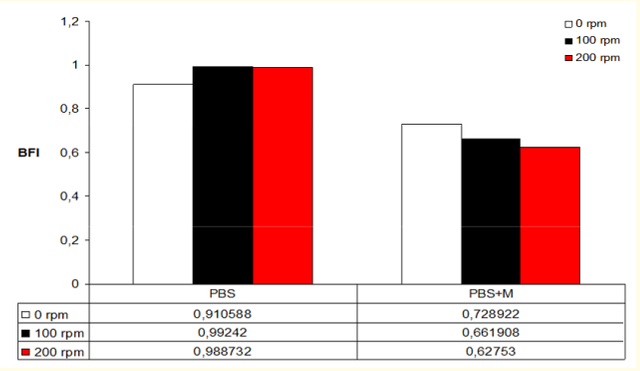

Figure 2: Biofilm index. Adhesion to glass surfaces of *P. aeruginosa* with different gentle swirling at 37°C for 24 h.

cfu

When the PBS medium was not taken into account, the increase in cell count in the molasses-added medium was 9,07x10^8 at ventilated runs of at most 200 rpm, while the lowest increase occurred at 0 rpm with 5,87x10^8 non-agitated media. As can be seen, as the ventilation conditions increase, the number of cells increases. However, the difference between 0 rpm and 200 rpm was only 1,5 times (Figure 3). When the 24th and the final run were compared, the highest difference was observed at 200 rpm shaking conditions, with the highest difference being 1511 times, while the lowest difference was observed at 601 times at 100 rpm shaking conditions.

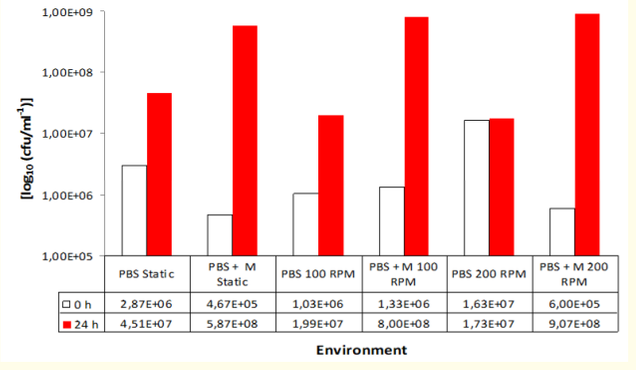

Figure 3: Microbial counts of *P. aeruginosa* with different gentle swirling at 37°C for 24 h.

When we look at the difference; the cell count increased by as much as 80 times in 0 rpm conditions, 31 times in 100 rpm shaking conditions, and 1426 times in 200 rpm shaking conditions. The biggest difference was realized at 151167% at 200 rpm. This was followed by 100 rpm with 60150% and 0 rpm with 125696% (Figure 4).

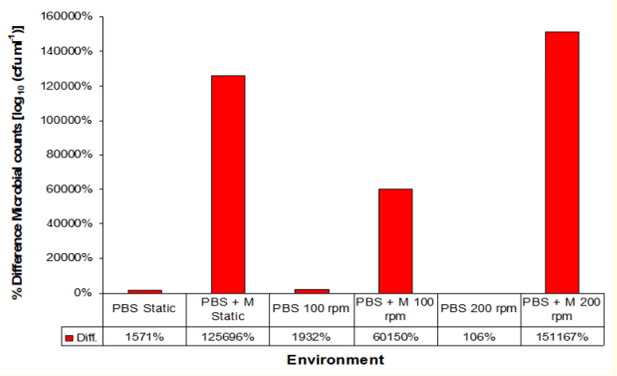

Figure 4: OD$_{600}$ values of *P. aeruginosa* with different gentle swirling at 37°C for 24 h.

Bacterial density (OD$_{600}$)

When we look at the OD600 values that we call biomass when adding molasses; again the greatest increase was at 2,76 at 200 rpm ventilated runs while the lowest rate was achieved at 1,36 at 0 rpm ventilation conditions. The difference between 0 rpm and 200 rpm was only 2 times. The 24th time that the start and end of the run was compared when the time was compared to 3,5 times with 200 rpm shaking conditions while the lowest rate was 1,3 times with 0 rpm without agitation. As a different point of view; the highest rate was observed at 3,5 folds with 200 rpm shaking conditions, while the lowest rate was achieved with 1,8 times decrease at 0 rpm non-shaking conditions (Figure 5).

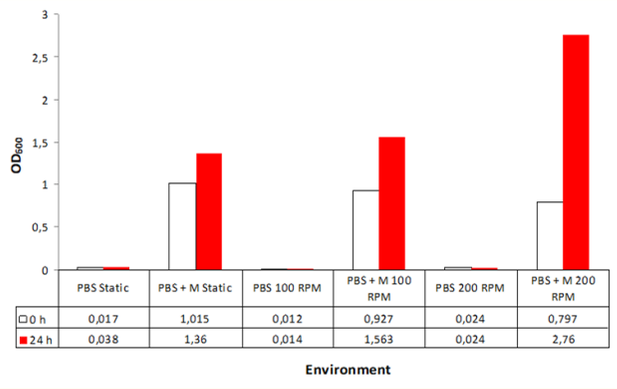

Figure 5: Percentage of the difference between cell numbers at initial and after 24 hours.

When we look at the difference; the largest headlights were followed by 346% at 200 rpm followed by 169% at 100 rpm and 134% at 0 rpm, respectively. Addition of SBM resulted in an increase in biomass values of up to 197 fold when the initial value of 0.014 was considered (Figure 6).

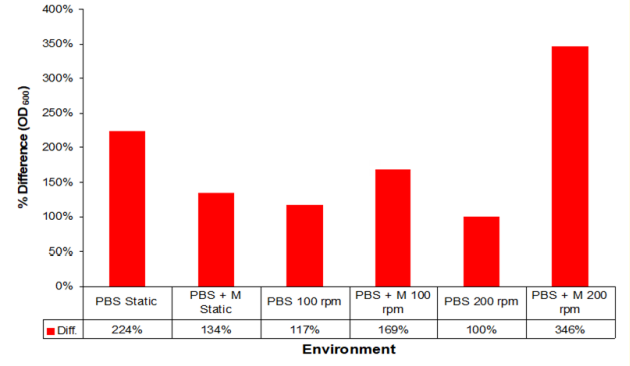

Figure 6: Percentage of the difference between cell density values at initial and after 24 hours.

Discussion

Studies on biofilm production have not been found in the literature. In the studies, B vitamins, bio-plastic, ethanol and biosurfactant was related to the production [1,2,4,6,7,15]. SBM contributed to the formation of cfu/ml, OD_{600} and biofilm, while causing a decrease in BFI values. In another published work we did, we used waste oil and whey. When we compared the study with the molasses trial, we reached higher values of cfu, OD_{600} and biofilm with this study. The addition of SBM and increased ventilation (rinse) has resulted in increased value in all experimental conditions. Although the legacy BFI values also decreased, this decrease caused the decrease to be below the control conditions. Therefore, the addition of SBM did not have a significant effect on BFI. The highest biofilm production occurred at 200 rpm, the highest number of cells; the cell density (live + dead) at bacterial density does support this.

Only 24 hours of time are used in our work. We interpreted the results by limiting it to this time slice. However, in the case of some similar works, over time zones of 72 hours were used. For this reason, it was not possible to make a comparison. These studies have shown that molasses may be relatively better substrates for commercial production of biofilm and management of these molasses. There are also many studies supporting this.

Conclusion

As a main by-product in the sugar industry, if molasses are not used in time and in full, they cause a serious liquidation problem and cause environmental pollution. Therefore, the use of molasses to produce high-value bio-products are considered is a profitable alternative. Although the price of beet molasses is very low, our result shows that molasses is an excellent substrate for *P. aeruginosa* fermentation and is an enriched and useful nutrient composition for cell growth and biofilm biosynthesis [2].

P. aeruginosa in this study, as a source of carbon and energy grown with molasses and biofilm production successful results were obtained. Few studies have been published on the use of wastes as substrates for biofilm production. Therefore, *P. aeruginosa* showed the ability to use molasses for growth and biofilm production and ultimately did not require additional nutrients, and fermentation costs were significantly reduced.

Conflict of Interest

No conflict of interest was declared by the authors.

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