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Original Research

Studying optimum conditions for biofilm production from *Leuconostoc mesenteroides*

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ABSTRACT:

A series of experiments were conducted to identify the optimal conditions for biofilm production including physio-chemical substances such as : sucrose, tween 80, glycerin, sorbitol and other physical-chemical factors (temperature, pH). Different degrees of temperature, were used (25, 30, 35 and 40°C), also different pH were used (5.5, 6.0, 6.5, 7.0) in different incubation periods, (24, 48, 72 and 96 h) and at different sucrose concentrations (5, 10 and 15%). Bacterial activity in biofilm production is estimated under each factor by using Microtiter Plate method (MTP). It was found that the highest activity was achieved under this factors: fermentation temperature of 30°C, initial pH of 6.0, incubation period of 72 h, with medium contains sucrose (10%) and 1% of tween 80.

Keywords:

Biofilm, Dextran, Exopolysaccharide, *Leuconostoc mesenteroides*, Microtiter plate method.

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INTRODUCTION

Biofilms are defined as microbial communities characterized by the cells that are attached to each other, embedded in a matrix of Extracellular Polymeric Substances (EPS) they have produced (Triveda and Gomathi, 2016). Many microbial strains could produce biofilm. Extracellular polysaccharides are the most important part of the biofilm matrix, especially extracellular polysaccharides produced by lactic acid bacteria (Adebayo-tayo and Onilude, 2008). Dextran are important in the growth of *Leuconostoc* sp biofilm (Leathers and Bischoff, 2011). Biofilm matrix of *Leuconostoc mesenteroides* contains components dextran, proteins and nucleic acids (Badel *et al*., 2008). The ability of bacteria to produce biofilm is affected by physical and chemical factors, including chemical composition of the medium and other factors such as pH, temperature, etc. (Emanuel *et al*., 2010).

MATERIALS AND METHODS

Biological material

In this study, *Leuconostoc mesenteroides* sub sp *mesenteroides* was isolated from Sauerkraut and then identified by using cultural, microscopical and biochemical tests according to Garvie (1986).

Cultivation conditions

The cultivation of lactic acid bacteria is usually performed in MRS medium (Slížová *et al*., 2015; Salas-Jara *et al*., 2016) which consists of glucose 2.0%, peptone 1%, meat extract 0.8%, sodium acetate trihydrate 0.5%, yeast extract 0.4%, dipotassium hydrogen phosphate 0.2%, triammonium citrate 0.2%, Tween 80 0.1%, magnesium sulfate heptahydrate 0.02%, manganese sulfate tetra hydrate 0.005% (Emanuel et al., 2010). It is possible to use BHI (Brain Heart Infusion) medium (Badel *et al*., 2008).

Physical and chemical factors

Temperature

Temperature is one of the most important factors affecting the formation of biofilm, in this study different temperature are tested (25, 30, 35 and 40°C).

pH

In order to know the best level of pH of biofilm production in this study different pH levels are tested (5.5, 6.0, 6.5 and 7.0).

Incubation period

To get the higher level of biofilm production, Incubation has been in different number of hours (24, 48, 72 and 96).

Sucrose

This type of nutrient is the most important sources for carbon, so the study performed by using different concentrations (5, 10 and 15%).

Effect of using some additives

Some materials were added to the production medium to determine their effect on biofilm production such as tween 80, glycerin, sorbitol.

Determination of the biofilm formation capacity by microtiter plate method

In this method an MTP dish has been used which have 96 flat bottom wells, Phosphate Buffer Solution (PBS) (pH=7.2), an aqueous solution of Crystal Violet dye (CV) 0.1%, ethanol 95%, as well as the media used in the study, which for the production of bio-

Table 1. Determination of the ability to produce biofilm based on OD values

S. No	The absorbance (OD)	Capacity of adhesion	Biofilm production
1	0.120>	Non	Non/weak
2	0.120-0.240	Moderate	Moderate
3	0.240<	Strong	Strong

Absorption is measured for wells and then the control or negative wells from all OD values were deducted (Bose *et al*., 2009; Salman and Khudair 2015)

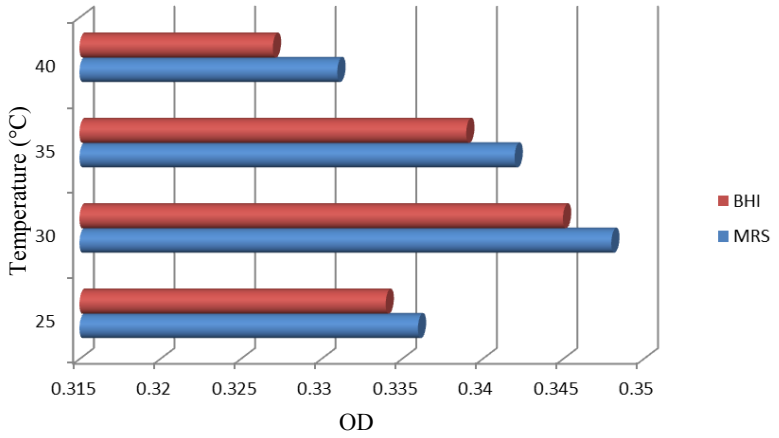

Figure 1. The biofilm formation capacity at different temperature

film under each factor. The bacteria have been activated and this culture was further diluted at 1:100 with the fresh medium. Some of the wells were filled with 0.2 ml of diluted cultures individually. The wells were filled with medium as control, the culture plates were incubated at different conditions, after incubation, unattached bacterial cells were removed by washing the wells three times with PBS or distilled water. After drying at room temperature, the wells were stained by crystal violet dye (0.1%) for 30 min. The stained attached bacterial cells were rinsed three times with distilled water and then allowed to dry at room temperature. Thus, bacterial susceptibility to adhesion can be assessed quantitatively, by evaluation the amount of dye attached to the wells, extraction of the dye attached to the wells by adding 200 µL of 95% ethanol. The absorbance of the wells were measured at 630 nm using ELISA reader (Bose *et al*., 2009; Salman and Khudair, 2015; Wilson *et al*., 2017), the flowing values in Table 1 used to determine the capacity of bacteria for biofilm production under each factor

RESULTS AND DISCUSSION

The results in Figure 1 showed that the higher production of biofilm was at 30°C, so that is the optimization temperature for biofilm production from this isolate, it is agreed with Sawrat *et al*. (2008) who studied the effect of temperature on dextran production from

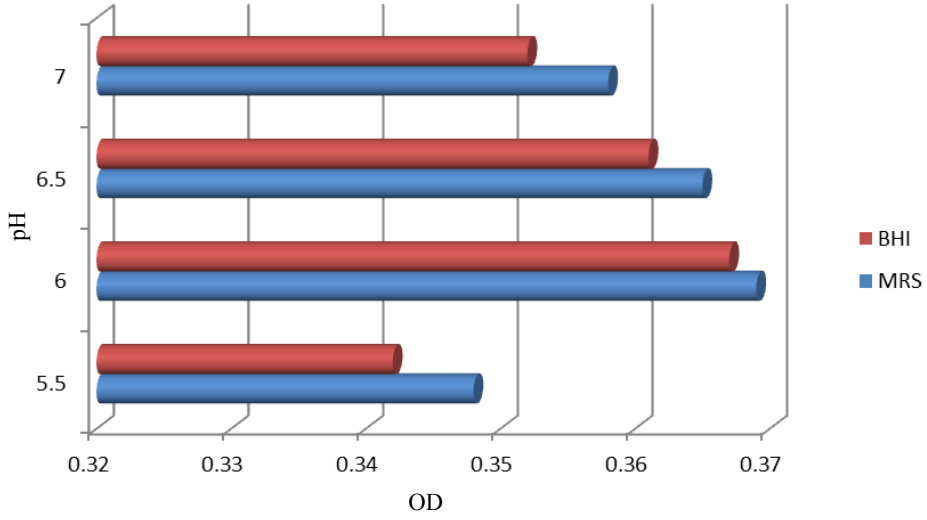

Figure 2. The biofilm formation capacity at different pH

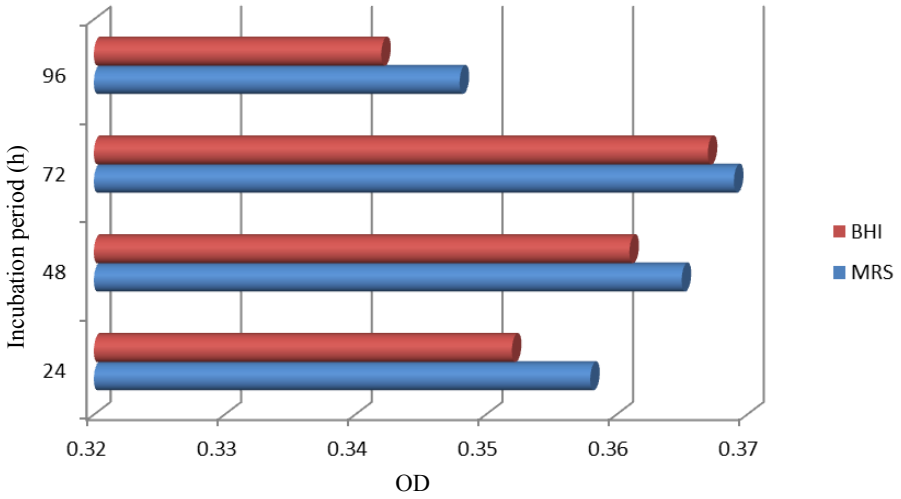

Figure 3. The biofilm formation capacity at different incubation period (h)

L. mesenteroides at various temperature and they observed the optimum temperature for dextran (exopolysaccharide) production was at 30°C. In contrast, Onilude *et al*. (2013) concluded that the production of dextran would be decreased gradually when the temperature is below 25°C.

The results in Figure 2 showed the higher production of biofilm was at pH value 6.0, It is a close result to the results of Onilude *et al*. (2013) who studied the effect of different pH on dextran production and found that the production was progressively increased until reached maximum production at 6.5, while Joshi and Koijum (2014) reported that pH 6.5 is the best for EPS production from *Leuconostoc lactis*. Moreover, Dogan *et al*. (2015) reported that the best pH level for EPS production from *Bacillus licheniformis* was at 6.0-7.0.

The results in Figure 3 showed that a 72 h incubation period is more adequate for *L. mesenteroides* for the highest production of biofilm, It is close result with Slížová *et al*. (2015) and this result corresponds to the study of Oliveira *et al*. (2007) who that reported a 72 h incubation period which is more adequate for *Staphylococcus epidermidis* in order to produce biofilm.

Figure 4 showed the higher production of biofilm by adding sucrose to the production medium at 10%, this is confirmed by a study Lule *et al*. (2015) who found the higher production of biofilm from bacteria

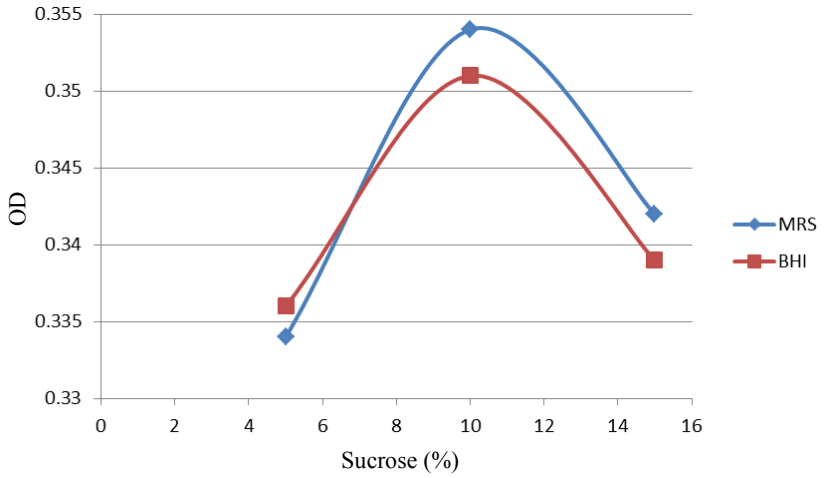

Figure 4. The biofilm formation capacity at various concentration of sucrose

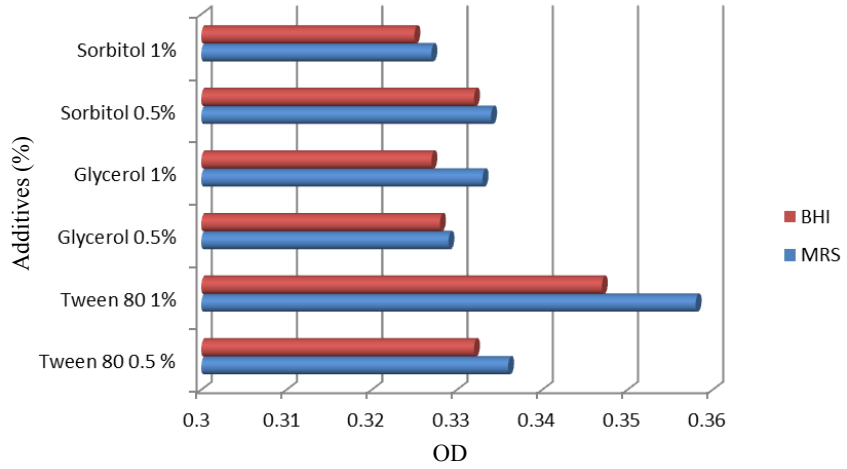

Figure 5. The biofilm formation capacity at various concentration of additives

Leuconostoc mesenteroides using medium containing 10% sucrose while Sarwat *et al*. (2008) found the maximum dextran produced with 15% sucrose from bacteria *Leuconostoc mesenteroides* CMG713.

Aman *et al*. (2012) reported that sucrose is the substrate capable of inducing dextransucrase enzyme production. Sarwat *et al*. (2008) mentioned higher concentration of sucrose in the fermentation medium that led to a decrease in biofilm production, thus, had substrate inhibitory effect, therefore, decreased dextran production. Figure 5 showed the higher production of biofilm by adding tween 80 to the production medium was at the concentration 1%, thus, tween 80 is a factor the most supported in biofilm production. Tween 80 is a growth promoter, the increase in dextran production is directly proportional to the increase in the concentration of tween 80 in medium of production, it is close to the results of Purama and Goyal (2008) and Shukla and Goyal (2011) who used concentrations ranging from 0.1% to 0.5% .

This substance is a catalyst for dextransucrase enzyme. As the presence of this substance in the medium of production leads to a change in the components of the cellular membrane especially fatty acids, it improves the excretion of dextransucrase enzyme and increase its effectiveness and then increased dextran production. This is confirmed by the study Sato *et al*. (1989) and Goyal and Katiyar (1997). Purama *et al*. (2010) reported that tween 80 also used as a stabilizer for the enzyme to protect it from the loss of efficiency at low or high temperature.

There was a slight increase in biofilm production by the addition of glycerol to production medium, which corresponds to the results of Shemesh and Chai (2013). Glycerol is unique in stimulating biofilm formation by *Bacillus subtilis* B_1 strain while Hamon and Lazazzera (2001) mentioned glycerol as a carbon source which did not support cells adherence to the microtiter plates.

There was a slight increase in the biofilm production by adding sorbitol to the production medium at the concentration 0.5% which corresponds to the results of Söderling *et al*. (1987). They reported that sorbitol increased the production of soluble polysaccharides and the amount of insoluble carbohydrates associated with the cell mass produced by *Streptococcus mutans*.

CONCLUSION

The biofilms could be used in the synthesis of biopolymers which can be introduced into many food industries with various applications. Natural media supported by sucrose can also be used in biofilm production.

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