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The Determining of the Biofilm Form by Two Industrial Strains on Plastic Composite Supports

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Abstract

In this paper, the biofilm formation of industrial yeasts *Candida intermedia* NRRL Y-8278 and *Kluyveromces marxianus* NRRL Y-8281 yeast were grown on various plastic composite supports (PCS) to analyze colony forming unit (CFU). The biofilm was performed using Stripping-Sand method for yeasts on the PCS (PCS_1, PCS_2, PCS_3, PCS_4). The biofilm formation on the yeasts was observed on the PCS. *K. marxianus* and *C. intermedia* took place the apparent the biofilm population CFU of each yeast on plastic supports. The best biofilm population was performed 2.9×10^8 CFU g^{-1} by *K. marxianus* on PCS_2 support than *C. intermedia*. The calculation of biofilm in CFU mL^{-1} on each yeast also was analyzed 1.6×10^{10} by *K. marxianus* on the PCS_2. *K. marxianus* had better the biofilm values on the PCS than *C. intermedia*. The results of this work will be useful on the some areas that this supports (polypropylene) used in such as food, biomedical and industrial companies and to realize about structure of yeasts and polypropylene supports in the literatures.

Keywords: Biofilm, biopolymer, CFU, PCS, yeast

Plastik Kompozit Destek Üzerine İki Endüstriyel Şus ile Oluşturulan Biyofilm Yapılarının Tespit Edilmesi

Özet

Bu çalışmada *Candida intermedia* NRRL Y-8278 ve *Kluyveromces marxianus* NRRL Y-8281 endüstriyel mayalarının farklı PCS destekler üzerindeki birim alandaki koloni sayıları (CFU) geliştirilip, hesaplanmıştır. Farklı destekler üzerindeki analiz yöntemi olarak Stripping-Sand metodu kullanılmıştır. Her bir destek için (PCS_1, PCS_2, PCS_3, PCS_4) hesaplamalar yapılmıştır. Kullanılan kültürlerin, plastik destekler üzerinde biyofilm yapıları meydana getirdiği tespit edilmiştir. *K. marxianus'*un PCS_2 desteği üzerinde 2.9×10^8 CFU g^{-1} ve 1.6×10^{10} CFU mL^{-1} değerlerinde koloni oluşturduğu ve *C. intermedia'*dan daha iyi biofilm performansı gösterdiği gözlemlendi. Kullanılan plastik desteklerin içerdiği polipropilen gıda, biyomedikal, hastane ve sağlık laboratuvarları gibi birçok yerde bazı cihazların yapısında bulunmaktadır. Ayrıca çalışmamızın sonuçları, polipropilen gibi bazı desteklerin ve endüstriyel mayaların kullanıldığı gıda ile biomedikal teknolojileri ve endüstriyel birçok alanda yararlı olacağı aynı zamanda bu materyallerin önem arz ettiği kanısına varılmıştır.

Anahtar kelimeler: Biyofilm, biyopolimer, CFU, PCS, maya

INTRODUCTION

The yeast's structure is better defined by the growing technology. One of these definitions is form of yeast. These structures are explained by biofilm properties and it described as a population of living cultures with a matrix such as extracellular substances. This matrix is known to consist of mainly polysaccharides, besides of proteins, nucleic acids, lipids, mineral ions. Microorganisms must have some necessary nutrients for life and growing on surfaces (inside or outside) of film (Aktas, 2003). More organism growth in this process therefore it is useful a producing higher organic material yield likes antibiotic, biofuels, enzyme at the same time refining can be provided especially with these forms (Qureshi et al., 2005). Also, these complex forms of microorganisms have a resistance structure due to the biofilm colonies. That is significant results in some areas. This forms also causes for increasing the hydrophobicity of the surface material. The biofilm populations growths more rapidly on teflon and plastics than glass or metal. Because this is due to differences

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in hydrophobicity of the surfaces and ionic charges (Holzapfel et al., 1998). The form of the biofilm is significant clinically as well as industrially. Clinically, the biofilms are important as the source of persistent infections. They cause the dental caries and nosocomial infections, as well as a variety of other infections and diseases (Costerton et al., 1999) and the biofilms are harmful in many places but these forms of cultures are useful in the industry. For example, natural the biofilms can reduce heat transfer by cooling towers, foul reverse osmosis membranes and infect the food processing equipment (Carpentier and Cerf, 1993; McDonogh, 1994; Mortensen and Conley, 1994). These forms also are used in industrially to achieve several aims including the treatment of wastewater for removal of organics (Hall et al., 1987; Taras et al., 2005) and heavy metals (Meyer and Wallis, 1997).

Detecting of the biofilm form is quite difficult. Some models were used with different substrates-devices. Especially in vitro models are useful on the basis of different surfaces. These methods were employed to analyze the effect of including flow, growth phase, nutrients and physiological conditions on culture architecture, morphology and the biofilm formation (Nett and Andes, 2006; Chandra, 2008; Uppuluri and Lopez-Ribot, 2010). In our study, biofilm analyzed with some supports that include polymer and agriculture materials. Polymers are easily shapeable light and cheap organic compounds. They draw attention of not only chemists but various kinds area likes textile, industrial and physic engineering, mechanical, and chemical. The importance of polymers is also big in terms such as molecular biology, biochemistry and medicine (Saçak, 2004). The PCS disc that consistently demonstrated the highest performance contained 50 % polypropylene (PP), 35 % soybean hulls (SH), 5 % soybean flour (SF), 5 % yeast extract (YE), % dried bovine albumin (BA), and mineral salts (MS) (Yönten, 2010).

The aim of this paper is to calculate the biofilm form of *K. marxianus* and *C. intermedia* on the plastic composite supports that include PP and some agriculture materials.

MATERIAL AND METHOD

Lactose, glucose, potassium phosphate, sulfate, sodium chlorine, calcium chlorine, magnesium phosphate, sulphuric acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Ferrous chloride (Sigma, Aldrich, USA) agar, peptone, yeast and malt extracts were purchased from Acumedia (Michigan, USA). PCS were donated by Demirci, Penn State University.

Culture and Maintenance

Yeast strains, *C. intermedia* NRRL Y-8278 and *K. marxianus* NRRL Y-8281 were donated by NRRL (Northern Region Research Laboratories) culture collection (Peoria, IL, USA). These yeasts were chosen because they exhausted the lactose instead of carbon material and they were useful in industrial area. Lyophilized culture was re-activated in yeast malt extract medium (in 0.5 mL, both at 3 g L^{-1} concentration) for 2-3 min., then culture was aseptically expand on solid agar slants involving 3 g L^{-1} malt extract, 10 g L^{-1} glucose, 3 g L^{-1} yeast extract, 5 g L^{-1} peptone and 20 g L^{-1} agar in distilled water. The solid medium was incubated at 30 °C for 4 days for appropriate growth and stored at 4 °C for further uses.

Supports

The supports was illustrated Table 1. They consisted of PP and some agriculture materials (Ho et al., 1997). PCS was cut to the discs (3.2 mm I.D., 12.7 mm O.D.) by methods were given by (Demirci et al., 1997). The PCS pipes were prepared at Penn State University and sent to our laboratory.

Table 1. The consist of plastic composite supports (Demirci et al., 1997)

	weight % (w/w)						
Support	PP	SH	SF	YE	RB	Ba	MS
PCS1	50	40	10	–	–	–	+
PCS2	50	35	5	5	–	5	–
PCS3	50	40	5	5	–	–	+
PCS4	50	35	5	5	5	–	+

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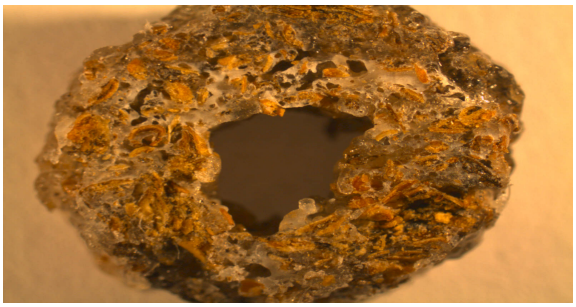

Figure 1. The image of plastic composite support disk (3 mm)

The supports were prepared approximately 3 mm slices as shown in Figure 1.

Batch Tube Fermentations

The experiments were used to analyze of the biofilm. 2.0 g supports were chosen to 25x150 mm tubes. Chosen supports sterilized at 121 °C for 30 min. After sterilization 10 mL sterile 5 % glucose medium 0.6 % YE added to sterile tubes. The tubes were incubated to perform a balance with shaking at 30 °C and 130 rpm for 24 h. The decantation was achieved aseptically to move some particulars. 10 mL sterile GM-YE medium was taken to the tubes. Then inoculated with 0.1 mL of yeasts at 30 °C for 24 h. The precipitation was performed many times in a day. This system was performed for each 6 days. After this period 5 PCS disks were taken to determinate. As a result of this process, the biofilm formations on PCS disks were analyzed by the Stripping-Sand method (Ho et al., 1997).

The Biofilm Analyzes

The five pieces of support were taken to 100 mL sterile 0.1 %peptone-waters for washing. Washing period were performed by the diversion of the tubes. Then mixture (5 g sand + 9 mL 0.1 % peptone-waters) added to the tubes. The tube was vortexes at 30 second for a total of 1.5 minutes. The complex was serially diluted, and CFU of the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were analyzed by using yeasts agar spread plates. CFU for the stability of each planting was done 2-3 times. Then plates were taken to oven to inoculate at 30 °C for 48 h. CFU of culture were calculated. As shown in Figure 1 the colony count of microorganisms illustrated in the plates. The number of living cells was calculated by the formula (Halkman, 2005).

$$\frac{Count(CFU)}{ml} = \frac{CN.DF}{V} \quad (1)$$

$$\frac{DF}{1} = \frac{1}{DR} \quad (2)$$

$$\frac{Count(CFU)}{g} = \frac{CN.DF}{w} \quad (3)$$

In Equation 1 CN is colony count, DF is dilutions coefficient and V is the volume (mL). In Equations 2 and 3 DR is the dilution ratio, w is the weight of five pieces of of disks. According to Halkman 2005, CFU must be between 50-250 as shown Figure 2. After this period, five pieces of PCS weighed on precision scales for the account of PCS/CFU per g.

RESULT AND DISCUSSIONS

The Counting of the Biofilm Formation

The biofilm formation by methods used by Demirci et al., 1997 for microorganisms reproduction environments of which are optimized was calculated as PCS and analyzed. These analyses were noticed as repeated tube fermentation and then the biofilm form was recorded after checking PCS analyses (Demirci et al., 1997; Halkman, 2005). The form of the biofilm colonies per unit amount and milliliter volume was given in Table 2 for *C. intermedia* culture. The biofilm analyses occurring in terms of unit volume and weight again for *K. marxianus* culture were given in Table 2.

Table 2. The biofilm form of yeasts as CFU g^{-1} and CFU mL^{-1} on the plastic composite supports

Supports	Yeast Cultures			
	C. intermedia	*K. marxianus*	*C. intermedia*	*K. marxianus*
	CFU g^{-1}		CFU mL^{-1}	
PCS1	1.0×10^{7}	1.8×10^{8}	6.8×10^{7}	1.4×10^{9}
PCS2	1.9×10^{8}	2.9×10^{8}	1.2×10^{9}	1.6×10^{10}
PCS3	1.7×10^{7}	2.6×10^{8}	1.0×10^{8}	1.5×10^{9}
PCS4	2.4×10^{7}	2.7×10^{7}	1.5×10^{8}	1.3×10^{8}

The best count of colonies observed between 10^{9} and 10^{8} on PCS.

It was observed that *K. marxianus* created best colonies for the biofilm and most culture concentration in both Figure 3 and Figure 4.

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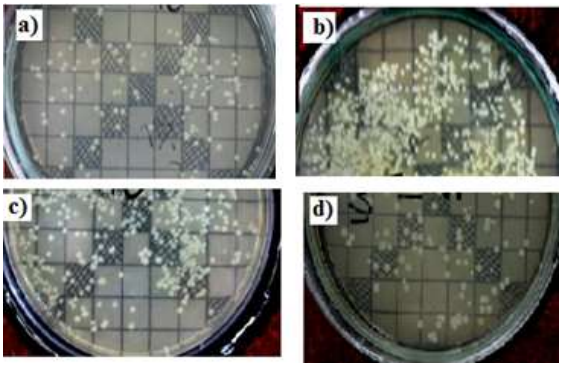

Figure 2. The image of yeast colonies on the glass plates. **a)** 10^4 times dilution **b)** 10^6 times dilution **c)** 10^5 times dilution **d)** 10^3 times dilution

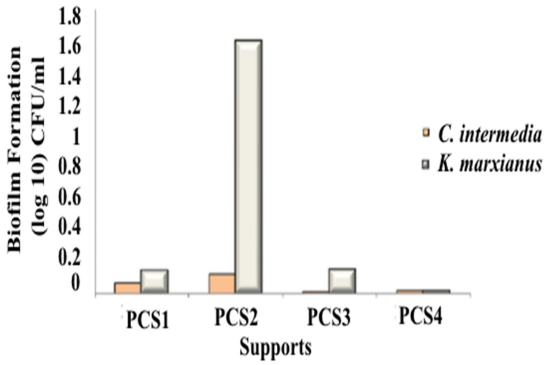

Figure 3. The biofilm formation of *C. intermedia* and *K. marxianus* on PCS.

As shown in Figure 3 the decent biofilm form in liquid volume was analyzed to be arising from *K. marxianus* of PCS_2 as 1.6×10^{10} CFU mL^{-1}. In a same work, the biofilm properties of *Klebsiella* culture were observed to be between 2×10^7 CFU mL^{-1} and 8×10^8 CFU mL^{-1} (Maldonado et al., 2007). On the other hand *C. intermedia* formed a biofilm form with a value of 1.2×10^9 CFU mL^{-1} again on PCS_2 support. So it was observed the biofilm formation of *K. marxianus* is better than *C. intermedia* yeast per milliliter. The cell concentrations after detachment from the biofilms were 2.3×10^7 CFU mL^{-1} for *Candida glabrata* strains in another work (Hala, 2014) and the biofilm form of *C. parapsilosis* was founded 1×10^7 CFU mL^{-1} on the teflon supports (Estivil et al., 2011) decent the biofilm population per unit weight amount was found to be *K. marxianus* yeast on PCS_2 support as 2.9×10^8 CFU g^{-1} in Figure 4. Other yeast *C. intermedia* noticed it's best the biofilm on PCS_2 support as 1.9×10^8 CFU g^{-1}. So *K. marxianus* yeast formed a better culture the biofilm population compared to *C. intermedia.*

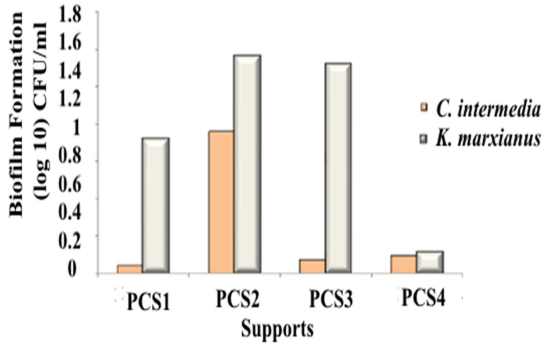

Figure 4. The biofilm formation of *C. intermedia* and *K. marxianus* on PCS

The counting of the biofilm formation obtained in a study was found to be similar to the results of this work. Here the PCS were used to produce lactic acid in the fermentation process. During this process the biofilm formation on supports were analyzed by notice colony numbers and found to be 1×10^8 CFU g^{-1} (Estivil et al., 2011). In a study, the number of colonies formed by *L. casei* culture on PCS was analyzed to be 5×10^8 CFU g^{-1} (Ho et al. 1997). *L. lactis* culture the biofilm was formed with the same supports to product nisin material in a study and CFU was calculated as 1×10^9 (Bober et al., 2007). Result of this work was compared to the other studies in the literature and various supports were chosen to immobilize the culture to these supports in Table 3.

The biofilm population was performed using CFU and OD system on some supports likes dairy equipments, polyethylene terephthalate, polystyrene, teflon, PVC, titanium, stainless steel and polypropylene as shown in the Table 3.

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Table 3. The analyses of some culture's the biofilm in literature and comparison of them to our study

Cultures	Supports	Counting unite	References
Candida tropicalis	Polyurethane	5.75×10^{5} CFU mL^{-1}	Estivill et al., 2011
Candida parapsilosis	PVC	8.0×10^{6} CFU mL^{-1}	Estivill et al., 2011
Candida parapsilosis	Teflon	1.5×10^{6} CFU mL^{-1}	Estivill et al., 2011
Candida albicans	Titanium	9.588×10^{8} CFU/disk	Li et al.,2012
Candida albicans	Polyethylene terephthalate	9.108×10^{8} CFU/disk	Li et al., 2012
Staphylococcus aureus	Polystyrene	0.405 (OD590)	Ciccio et al., 2015
Staphylococcus aureus	Stainless steel	0.486 (OD590)	Ciccio et al., 2015
Staphylococcus epidermdis	Polystyrene	0.294 (OD590)	Ciccio et al., 2015
Staphylococcus epidermdis	Stainless steel	0.145 (OD590)	Ciccio et al., 2015
Lactobasillus casei	Plastic composite supports	1.6×10^{10} CFU g^{-1}	Ho et al., 1997
Klebsiella	Polystyrene	8×10^{8} CFU mL^{-1}	Maldonado et al., 2007
Lactic Acid Bacteria	Glass cover slips	1×10^{9} CFU mL^{-1}	Kubota et al., 2008
Bacillus species	Equipment in the dairy industry	10^{6}-10^{8} CFU mL^{-1}	Ronit et al., 2014
Lactobasillus casei	Plastic composite supports	$7.5\text{-}8.0\times10^{9}$ CFU disk	Demirci et al., 2003
Candida intermedia	BCS2	1.2×10^{9} CFU mL^{-1}	**In this study**
Kluyveromces marxianus	BCS2	1.6×10^{10} CFU mL^{-1}	**In this study**

The Analysis of Culture's Concentrations on the Biofilm Formation

The yeasts were subject to treatment with PCS's for 6 days in order to allow them form the biofilm on PCS. During this time period the changes in concentrations were calculated for each day according to the calibration curve as shown in Figure 5.

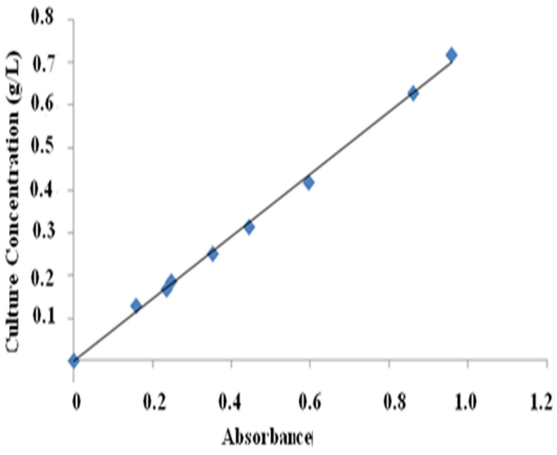

Figure 5. The graphic of calibration curve (culture concentration-absorbance values)

Examples of yeast from the fermentation broth were washed twice with distilled water.

The mixtures were analyzed the absorbance spectrophotometer at 500 nm wavelength. Then the mixture of cultures was taken to the oven at 70 °C temperature. The calibration against absorbance values measured by calculating the concentration of the yeast weight charts has been created.

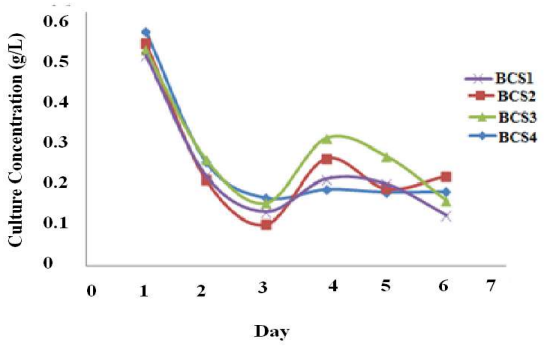

Figure 6. The culture concentration of *C. intermedia* on biopolymer composite supports in the biofilm medium

In Figure 6 biomass changing was given for *C. intermedia* culture. As understood from the graph, biomass concentrations were calculated in decants in 4 different PCS environments. These concentrations should be considered different from yeast culture concentrations forming the

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biofilms. Because biomass concentrations decrease in decants within time. So the yeast culture existing in liquid phase is changed every day and released out. While the biomass concentration in first day was approximately 0.55 g L^{-1} this value decreased in 2nd and 3rd days. After 3rd day it increases because the culture is growing in the medium conditions among 3rd and 4th day. The reason of this is that the 0.1mL inoculation applied the first day was aseptically decanted from suspension environment in the second day. This means the removal of almost all culture except the ones forming the biofilms. Biomass concentration for each PCS environment started increasing again around the end of fourth day and decreased again in fifth and sixth days.

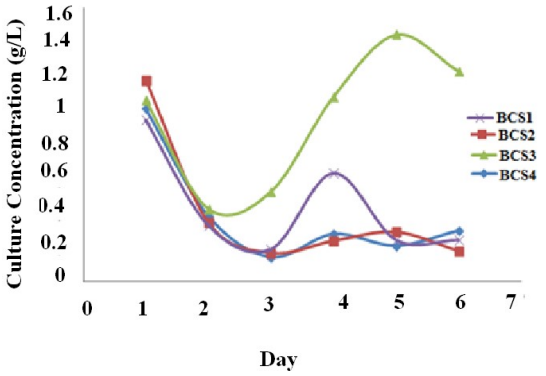

Figure 7. The culture concentration of *K. marxianus* on biopolymer composite supports in the biofilm medium.

In Figure 7 the biomass concentration in liquid phase was given for *K. marxianus* culture. In this graph the biomass in the biofilm changed in same amount for the first 2 days and started to increase after third day and took a separate value for each culture. Biomass concentrations decrease in decants within time likewise Figure 6. So the yeast culture existing in liquid phase is changed every day and released out. After 3rd day it increases because the culture is growing in the medium conditions among 3rd and 4th day. In another study where PCS's were used the biomass concentration was found to be 2.1-2.7 g L^{-1} (Velazquez et al., 2001; Demirci et al., 2003).The biofilm densities in the environment, where supports are located, approximately varies between 0.2 and 0.3 g L^{-1}.

CONCLUSIONS

K. marxianus and *C. intermedia* occurred visible biofilm formations CFU g^{-1} and CFU mL^{-1} of each yeast on PCS. The best biofilm population were performed 2.9×10^{8} mL CFU g^{-1} by *K. marxianus* on PCS_2 support than C. *intermedia*. The calculation of the biofilm in CFU mL^{-1} on each yeast was analyzed 1.6×10^{10} by *K. marxianus* on the PCS_2. Results show that the biofilm formation of yeasts may analyze on PCS and it will be useful to use on form of microorganism and as a result of this work acquired resistance in medicine area. For example, the number of antibiotics increases day by day but they are ineffective against microorganism's resistant biofilm. So the biofilm forms would noticed by peoples in the health sector. On the other hand, microorganisms have important and costly effects of corrosion on some valuable devices in the clinics, offices, and factories and the biofilm cause rotting on the devices. So, this paper will be usefully on the formation of microorganisms in this area.

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