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## The Assessment of Biofilm Formation in Iranian Meat Processing Environments

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**Abstract:** The biofilm is consist of microbial cell clusters with a network of internal channels or voids in the Extracellular Polymeric Substances (EPS) and glycoprotein matrix. Biofilms due to special structure and EPS are more resistant to control measures. Biofilms can remain on various surfaces which may assist the survival of pathogenic and spoilage bacteria in the food processing environment, a contributing factor in foodborne disease outbreaks. In this research bacterial strains were isolated by swabbing method from surfaces of meat processing factory environments. More than 60 different species of bacteria isolated from various segment of meat processing plant and the hydrophobicity of isolates measured by Microbial Adhesion Test to Hydrocarbon (MATH) method for screening of isolates. The quantity of biofilm of isolates with high hydrophobicity was determined using microtiter plate assay method and ELISA reader machine. Results indicated *Bacillus megaterium* and *Staphylococcus epidermidis* with 45 and 33% of hydrophobicity have the highest potential in biofilm formation. Pathogenic *S. aureus* with 30% of hydrophobicity classified under moderately adherent. *B. subtilis* with 22% of hydrophobicity considered as weakly adherent. *Micrococcus varians* and *M. roseus* with 1 and 5% of hydrophobicity were non adherent. The result from this study highlighted the problems of spread of bacteria. In the development of cleaning and sanitization protocol in meat processing environments, an awareness of these biofilm forming bacteria is essential for the meat processing.

**Key words:** Biofilm, meat products, hydrophobicity, microbial attachment, extracellular matrix

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### INTRODUCTION

Biofilm formation creates major problems in many areas such as industrial water systems, health care and the food processing industry (Cunliffe *et al.*, 1999; Gilbert *et al.*, 2003). The hygiene of the process and processing environment is a significant factor in the production of microbiologically safe and good quality products in the food industry. Food residues are accumulated on inert structural surfaces such as floor drains, conveyors and product tote boxes, producing environments which can act as continuous culture systems in which microorganisms reside and multiply (Bower *et al.*, 1996; Chmielewski and Frank, 2003). Development of biofilms on food processing environments is a potential source of contamination of foods that may lead to spoilage or transmission of foodborne pathogens (Wirtanen and Salo, 2003). In addition to product spoilage and possible risks to public health, biofilms create a number of serious problems for industrial fluid processing operations such as mechanical blockages, impedance of heat transfer processes and biodeterioration of the components of metallic and polymeric systems which result in billions of dollars in losses each year (Zottola and Sasahara, 1994; Mittelman, 1998; Neil, 2001; Bryers, 2000; Lee Wong, 1998; Dorthe *et al.*, 2001; Djordjevic *et al.*, 2002; Harvey *et al.*, 2006).

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Cells in a biofilm have been shown to be significantly more resistant to disinfectants than planktonic cells (Joseph *et al.*, 2001; Tompkin, 2002; Trachoo, 2003).

The most common foodborne biofilm producers belong to the genera *Alcaligenes*, *Bacillus*, *Enterobacter*, *Flavobacterium*, *Pseudomonas* and *Staphylococcus* (Bryers, 2000). Recent reports show that bacterial contamination continues to present a significant threat to product quality and systems operations. Several studies were linked the contamination of equipment to the foodborne infection outbreaks (Res *et al.*, 1992; Midelet and Carpentier, 2002).

When contamination of dairy products occurs, evidence suggests that microorganisms on the surfaces of milk processing equipment are a major source (Flint *et al.*, 1997). In dairy processing operations, biofilms have been reported on bends in pipes, rubber seals, gaskets, conveyor belts, floors, plate-heat exchangers and the pasteurized milk section of pasteurizer, etc. (Carpentier and Cerf, 1993; Mittelman, 1998). In an ice cream plant indicated pathogenic bacteria such as *Listeria* and *Shigella* are able to form biofilm in the ice cream production line and in the environment tested (Gunduz and Tuncel, 2006) and in meat processing environments, the presence of biofilm on equipment could not be definitively concluded (Jessen and Lammert, 2003).

Considerably because in Iran, there was little information about biofilm formation in food industry, the objective of this study were to investigate existence, location, type and quantification of biofilm formation on various segments of meat processing environments.

## MATERIALS AND METHODS

### Sampling and Isolation

Biofilm forming bacteria was isolated from samples of meat processing environments. All samples were obtained from a meat processing factory located nearby Isfahan, on January 2006. The capacity of this meat processing plant was about 25 tons of sausage per day. The samples were taken from different segments of meat processing lines, equipment and processing environments from reception to packaging sections. Collection of samples was performed after sanitization treatment and before the meat was taken in for processing using the swab method. Totally 25 swab samples obtained from dead ends, cracks, valves, gaskets and linkage of plants. The segments were: Filler, Cutter, Slicer, Mixer and Mincer. Then samples (Swabs in Ringer solution) were transferred to the laboratory and immediately samples were cultured on Nutrient Agar (NA) and Tryptogase Glucose Yeast Extract (TGYE) agar. Furthermore two NA plate placed for 30 and 60 min for sampling of factory air (Sharma and Anand, 2002a). All plates were incubated at 30°C for 24 h. Single colonies of microorganism further purified and identified according to Bergeys manual (Krieg *et al.*, 1998). Notably, the screening of isolates was performed by hydrophobicity assessment and isolates with higher hydrophobicity than 20% were identified.

### Hydrophobicity Assessment

Cell surface hydrophobicity was measured by the MATH test. The MATH method developed by Rosenberg *et al.* (1980) gives a cell hydrophobicity index ( $A\%$  = percentage of adhesion) and is easy to perform. Cell suspension were classified to 0.5 Macfarland standard and 4 mL of cell suspension were transferred to individual test tubes (diameter, 1.7 cm; length, 15 cm), which contained 1 mL of octane. The test tubes were vortexed at full speed for 2 min and then left to stand for 15 min to allow phase separation. The Optical Density ( $OD$ )<sub>640</sub> of the aqueous phase was determined and partitioning of the bacterial suspension was expressed as the percentage of cells adsorbed by the hydrocarbon phase:

Table 1: Classification of attachment of bacteria based on optical density measured at 492 nm by ELISA reader

Attachment type	OD (nm)
Non-adherent	$OD \leq OD_c$
Weakly-adherent	$OD_c < OD \leq 2OD_c$
Moderately-adherent	$2OD_c < OD \leq 4OD_c$
Strongly-adherent	$4OD_c < OD$

OD = OD mean of tested bacteria,  $OD_c$  = OD mean of negative controls

$$\text{Percentage of partitioning} = \frac{A_1 - OD_{640} \text{ of aqueous phase}}{A_1} \times 100$$

$A_1$  is primary  $OD_{640}$  of cell suspension before adding octane hydrocarbon to it (Mozes and Rouxhet, 1987).

### Microtiter Plate Method

The wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid were filled with 200  $\mu$ L of each bacterial suspension. Negative control wells contained broth only. The plates were covered and incubated aerobically for 24 h at 37°C. Then, the content of each well was aspirated and each well was washed three times with 250  $\mu$ L of sterile physiological saline. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 200  $\mu$ L of 99% methanol per well and after 15 min plates were emptied and left to dry. Then, plates were stained for 5 min with 0.2 mL of 2% crystal violet (bioMerieux) per well. Excess stain was rinsed off by placing the plate under running tap water. Plates were air dried and the dye bound to the adherent cells was resolubilized with 160  $\mu$ L of 33% (v/v) glacial acetic acid (Merck) per well. The OD of each well was measured at 492 nm by using an automated Statfax ELISA reader. For the purposes of comparative analysis of test results, based upon the ODs of bacterial films, adherence capabilities of tested strains classified into four categories: non-adherent (0), weakly (+), moderately (++) or strongly (+++) adherent (Stepanovic *et al.*, 2000) (Table 1).

### Statistical Analysis

Wilcoxon paired test was used to compare OD values obtained in the microtiter-plate tests (SPSS software). p-values of <0.05 were considered significant.

## RESULTS AND DISCUSSION

A total of 60 different species of bacteria were isolated. 72, 6 and 22% of isolated species were Gram positive cocci, Gram negative cocci and Gram positive rods respectively. Isolated aerobic facultative Gram-positive cocci were included *M. roseus*, *M. varians*, *S. aureus* and *S. epidermidis*. Gram-positive rods were included two species of *B. subtilis* and *B. megaterium*. Similar to present observations, Jeong and Frank (1994) suggested that *Staphylococcus*, *Flavobacterium*, *Pseudomonas*, *Bacillus* could form biofilm in the dairy industry. In addition, *Escherichia*, *Enterobacter*, *Streptococcus*, *Lactococcus*, *Citrobacter*, *Proteus*, *Micrococcus*, *Klebsiella*, *Shigella* formed biofilm in a dairy plant (Sharma and Anand, 2002a). In an ice cream plant, *Staphylococcus*, *Bacillus*, *Listeria* lactic acid bacteria and Enterobacteriaceae were also isolated (Gunduz and Tuncel, 2006). Jessen and Lammert (2003) described an indirect way to detect foodborne biofilms on visually clean equipment surfaces of meat processing plants. They suggested that presence of biofilm in meat processing plant could not be definitively concluded. But in our experiments presence of biofilm forming bacteria after sanitation is proved.

Table 2: Type, attachment, site and hydrophobicity of identified isolates from meat processing environments

Isolation site	Hydrophobicity (%)	Type of attachment	Isolated bacteria
Filler	1	Non-adherent	<i>M. roseus</i>
Mincer	5	Non-adherent	<i>M. varians</i>
Mincer	22	Weakly-adherent	<i>B. subtilis</i>
Slicer	30	Moderately-adherent	<i>S. aureus</i>
Mincer	33	Strongly-adherent	<i>S. epidermidis</i>
Air	45	Strongly-adherent	<i>B. megaterium</i>

In this study *M. roseus* from filler, *M. varians*, *B. subtilis* and *S. epidermidis* from mincer, *S. aureus* from slicer and *B. megaterium* from factory air were isolated (Table 2). Isolation of these bacteria (except *M. roseus* and *M. varians* were non-adherent) from meat processing environments after sanitation shows that the cleaning and disinfecting process was not effective. Even with acceptable cleaning systems, bacteria may remain on equipment surfaces and may accumulate and form biofilms (Sharma and Anand, 2002b). Faille *et al.* (2001) also suggested that spores of *Bacillus* forming biofilm could not be removed by normal CIP system. In this research *B. subtilis* and *B. megaterium* were isolated from mincer and factory air, respectively. Den Aantrakker *et al.* (2003) show that contamination of products via air can occur through dust particles (dry air) or via aerosols. Aerosols are, for instance, formed when contaminated floors or drains are sprayed with high-pressure jets, resulting in the formation of droplets that can be suspended in the air. In present study, the isolation of *B. megaterium* from factory air is more likely to be associated with recontamination, perhaps through an unfiltered air supply because *B. megaterium* probably during cleaning of this factory has formed aerosols.

In Table 2, *M. roseus* and *M. varians* were non-adherent, *B. subtilis* with 22% of hydrophobicity considered as Weakly-adherent. *S. epidermidis* and *B. megaterium* with 66 and 45% of hydrophobicity were strongly-adherent. Therefore, these adherent microorganisms could still contribute to the contamination of food products. Biofilm formation on equipment surfaces increases the biotransfer potential that can be described as the ability of microorganisms present on equipment surfaces both before and after cleaning procedures to contaminate a product during processing. If the cleaning was inappropriate, biofilms can form and the biotransfer potential may increase (Wirtanen *et al.*, 1996).

Quantity of biofilm formation tests indicated that *S. epidermidis* and *B. megaterium*, with 0.958 and 0.972 OD, respectively were the highest in biofilm formation. Also, as seen in Table 2 hydrophobicity of these bacteria is high and indicates their ability to form biofilms is very well. *S. aureus* and *B. subtilis* with 0.797 and 0.6 OD were considered as a moderate and weak respectively. Furthermore *M. roseus* and *M. varians* with 0.446 and 0.425 OD classified as a non adherent. In addition, comparison of data related to the hydrophobicity and quality of biofilm of *M. varians*, *B. subtilis* and *S. epidermidis* isolated from mincer (Table 2) indicated that type; attachment and hydrophobicity of bacteria were important factors in biofilm formation. So that whatever hydrophobicity less, either attachment of bacterium on surfaces was less and whatever hydrophobicity more, also attachment of bacterium was greater. Many researchers also have confirmed the role of hydrophobicity in biofilm formation (Cerca and Pier, 2005). For instance, Frank (2001) pointed that when biofilms formed; each biofilm member has been naturally selected by its cell surface properties including the presence of capsules, fimbriae and cell surface hydrophobicity.

Present results indicated that pathogenic bacterium, such as *S. aureus* is able to form biofilm in the meat production line and in the environment tested. This bacterium with 30% hydrophobicity percent and moderately-adherent (Table 2) is a good biofilm former. The release of these bacteria from biofilms has important implications for the safety of meat for the consumers. Proper cleaning and disinfection programs are needed in order to eliminate biofilm forming pathogen bacteria. This study would be useful in establishing critical control points in the cleaning and disinfecting process specific to meat plant with the aim of reducing the rate of contamination.

## CONCLUSION

Results using microbiological methods confirmed that biofilm has formed on various segments of meat processing environments. Isolation of biofilm forming bacteria after sanitation, exhibits sanitation protocol is not efficient in this factory. Also isolation and biofilm formation of pathogenic bacterium, *Staph. aureus* on surfaces (Slicer) is important, especially in public health and disease transmission.

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