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Maciel PAULO, Elinalva; Pinho VASCONCELOS, Murilo; Santiago OLIVEIRA, Ivelise; de Jesus AFFE, Helen Michelle; NASCIMENTO, Rosely; Soares de MELO, Itamar; de Abreu ROQUE, Milton Ricardo; Aparecida de ASSIS, Sandra

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An alternative method for screening lactic acid bacteria for the production of exopolysaccharides with rapid confirmation

Método alternativo de triagem de bactérias lácticas produtoras de exopolissacarídeos com confirmação rápida

Elinalva Maciel PAULO¹, Murilo Pinho VASCONCELOS¹, Ivelise Santiago OLIVEIRA¹,
Helen Michelle de Jesus AFFE¹, Rosely NASCIMENTO², Itamar Soares de MELO²,
Milton Ricardo de Abreu ROQUE³, Sandra Aparecida de ASSIS^{4*}

Abstract

The accumulation of exopolysaccharides (EPS) produced by microorganisms occurs in the presence of excess substrate and limiting conditions of elements that are essential to growth, such as nitrogen, phosphorus, sulfur, and magnesium. The presence of EPS produced by bacterial cells contributes to slime colonies formation in solid medium and increased viscosity in liquid medium. This paper proposes an alternative method for screening EPS-producing lactic acid bacteria using solid medium-containing discs of filter paper that are saturated with active cultures. The screening was carried out under different culture conditions varying the type of sugar, pH, and temperature. EPS production was visualized by the presence of mucoid colonies on the discs, which was confirmed by the formation of a precipitate when part of this colony was mixed with absolute alcohol. The established conditions for obtaining a high number of isolates producing EPS were 10% sucrose, pH 7.5 and 28 °C. This method proved to be effective and economical because several strains could be tested on the same plate, with immediate confirmation.

Keywords: screening; exopolysaccharides; lactic acid bacteria.

Resumo

Em geral, o acúmulo de exopolissacarídeos (EPS) produzidos pelos microrganismos ocorre na presença de excesso de substrato e condições limitantes de elementos essenciais ao crescimento, como nitrogênio, fósforo, enxofre, ou magnésio. A presença de EPS associada com células bacterianas é reconhecida em meio sólido para a formação de colônias mucoides e no meio líquido pelo aumento da viscosidade. Este trabalho propõe um método alternativo para realizar triagem de bactérias lácticas produtoras de EPS utilizando meio sólido contendo discos de papel de filtro impregnado pela cultura. A seleção foi realizada em diferentes condições de cultivo, variando o tipo de açúcar, pH e temperatura. A produção de EPS foi visualizada pela presença da colônia mucoide sobre os discos, que foram confirmadas pelo precipitado, quando parte desta colônia foi misturada com álcool absoluto. As condições de cultivo em que houve maior produção de EPS foram: sacarose 10%, pH 7,5 a 28 °C. Este método provou ser eficaz e econômico, uma vez que várias cepas podem ser testadas na mesma placa, sendo possível realizar uma prévia confirmação da produção do EPS.

Palavras-chave: triagem; exopolissacarídeos; bactérias lácticas.

1 Introduction

Microbial exopolysaccharides (EPS) are a wide group of secreted polymers that can be tightly attached to the cell surface (capsular polysaccharides) or released as extracellular slime in the surroundings of the cell (KLEEREBEZEM et al., 1999; SANCHEZ et al., 2006). In nature, polysaccharides may be associated with virulence and cell protection against desiccation, osmotic stress, antibiotics, toxic compounds, and bacteriophage or protozoa attack (SANCHEZ et al., 2006). EPS produced by BAL include the *Lactobacillus* genus *Streptococcus*, *Leuconostoc*, and *Lactococcus* (DE VUYST; DEGEEST, 1999).

EPS produced by lactic acid bacteria may be of two types: a mucus layer that forms a colloidal aggregate as an amorphous,

mucilaginous substance around the cell, with little or no cell adherence, or a capsule form that is cohesive and adheres to the cell (BOELS et al., 2001; PORTILHO, 2002). EPS produced by lactic acid bacteria are used to improve the rheological features of various food products. These bacteria produce a wide variety of EPS that have applications in various industrial sectors (SUTHERLAND, 1998; BOELS et al., 2001).

EPS associated with bacterial cells are responsible for the formation of mucous colonies in solid medium or for the formation of long filaments when the colony is extended with an inoculation loop (RUAS-MADIEDO; REYES-GAVILÁN, 2005). In liquid medium, EPS can be detected by the high resistance

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¹ Departamento de Biologia, Universidade Estadual de Feira de Santana – UEFS, Av. Transnordestina, s/n, BR 116, Bairro Novo Horizonte, CEP 44036-900, Feira de Santana, BA, Brasil

² Empresa Brasileira de Pesquisa Agropecuária – Embrapa Meio Ambiente, Jaguariúna, SP, Brasil

³ Instituto de Ciências da Saúde, Universidade Federal da Bahia – UFBA, Vale do Canela, Salvador, BA, Brasil

⁴ Departamento de Saúde, Universidade Estadual de Feira de Santana – UEFS, Av. Transnordestina, s/n, BR 116, Bairro Novo Horizonte, CEP 44036-900, Feira de Santana, BA, Brasil, e-mail: sandassi@hotmail.com

*Corresponding author

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when flowing through serological pipettes, viscous strand formation during free fall from the pipette, or upon increasing the viscosity of the culture medium. EPS can also be detected using analytical methods or chemical analysis (GÓMEZ, 2006). The EPS polymers are highly hydrated, containing 99% water, though they can be visualized by light microscopy when stained with appropriate dyes, such as India ink or cationic dyes, such as ruthenium red and alcian blue, due to the anionic nature of many EPS (DECHO, 1990; SUTHERLAND, 2001). Through the use of scanning electron microscopy and transmission electron microscopy, EPS have been revealed as an array of fiber polysaccharides (WHITFIELD; ROBERTS, 1999). The PCR technique is also used to detect genes encoding homopolysaccharide (*gtf* and *lev*) and heteropolysaccharide (*epsA*, *epsB*, *epsD/E*, *epsGTF*) (PALOMBA et al., 2012).

The EPS can be recovered in their crude form by directly drying the viscous extract (VIEIRA, 2003) or may undergo an initial purification using techniques such as insolubilization of the polymer (formation of a precipitate) with the addition of certain salts or pH change. They may also be recovered by the process of precipitation after treatment with organic solvents soluble in water. The most commonly used are ethanol, acetone, methanol, and isopropanol (CERNING et al., 1994; MOREIRA; PINO; VENDRUSCOLO, 2003; GÓMEZ, 2006; BENNAMA et al., 2012).

Extracellular microbial biopolymers have been widely used in several industry sectors adding value to various products, especially those food products, to improve their rheological characteristics. The production of polymers by microorganisms is still quite insignificant compared to the amount of polymers derived from plants and animals, especially by lactic acid bacteria (GÓMEZ, 2006). However, since bacteria belonging to this group are harmless to human and animal health, investing in the production of polymers for industrial use has become particularly interesting.

Therefore this paper proposes an alternative method to screen EPS-producing lactic acid bacteria for confirming the production of this biopolymer.

2 Materials and methods

Sucrose, glucose, and lactose were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA). All other chemicals used in this study were of high-quality analytical grade.

2.1 Isolation of lactic acid bacteria

This study was conducted in Feira de Santana, Bahia, Brazil, where each food was collected (cow milk and goat milk) during the milking, and the vegetable products (sugarcane, cassava, and cabbage) were acquired from farmers' markets; only one sample was used in the experiment.

All samples were collected under aseptic conditions and were immediately taken to the laboratory.

Each sample was serially diluted from 10^{-1} to 10^{-7} in phosphate buffered saline, pH 7.2 ± 0.2 . One mL of each dilution was inoculated into tubes containing 9 mL of selective medium

for exopolysaccharide (VAN DEN BERG et al., 1993). The tubes were incubated at $30 \text{ }^{\circ}\text{C} \pm 2 \text{ }^{\circ}\text{C}$ for 48 hours. After this period, isolated colonies were obtained by streaking plates containing MRS agar at each dilution (DE MAN; ROGOSA; SHARPE, 1960). Five colonies were selected, plated on MRS agar, and incubated at $35 \text{ }^{\circ}\text{C} \pm 2 \text{ }^{\circ}\text{C}$ for 24 hours. Gram staining and catalase testing were performed for each colony inoculated in MRS agar (KONEMAN, 2008). Microorganisms categorized as lactic acid bacteria (gram-positive and negative catalysis) were purified on MRS agar and preserved in LEL medium (skim milk - 10%, yeast extract - 0.5%, glucose - 1.0%) plus 10% glycerol and immediately transferred to a freezer at $-80 \text{ }^{\circ}\text{C}$.

2.2 Screening of lactic acid bacteria for the production of exopolysaccharide

Discs of sterile filter paper (5 mm Ø) were placed in Petri dishes containing the culture medium (80 mm Ø) and were inoculated with 5 µL of culture from each isolate for the production of EPS (GUIMARÃES et al., 1999). Five different isolates were tested in each Petri dish. The isolates were cultured in various medium conditions changing the type of sugar (sucrose, glucose or lactose), pH level (5.5 ± 0.2 or 7.5 ± 0.2), and temperature ($28 \pm 2 \text{ }^{\circ}\text{C}$, $35 \pm 2 \text{ }^{\circ}\text{C}$ and $45 \pm 2 \text{ }^{\circ}\text{C}$), and they were incubated for 7 days, 48 hours, and 24 hours, at the respective temperatures.

After incubation, EPS production was assessed based on the formation of a mucoid colony around the discs. The production of this biopolymer by the isolates was confirmed by mixing a portion of the mucoid substance in 2 mL of absolute alcohol as well as by viewing the saturation of the discs by the mucoid substance in scanning electron microscopy (SEM).

3 Results and discussion

In this study, 187 isolates that had the characteristics of lactic acid bacteria were obtained. All isolates were screened for the production of EPS, which depends on several factors. The parameters that were most influential were the bacterial strain, the composition of the medium (carbon and nitrogen sources and growth factors), and the conditions under which the strains were grown, i.e., temperature, pH, oxygen tension, and incubation time. (NAMPOOTHIRI et al., 2003; LEE; GILMORE, 2005; SCHIAVÃO-SOUZA et al., 2007). Table 1 shows the results of screening lactic acid bacteria in different growing conditions. The presence of EPS associated with bacterial cells can be recognized by the formation of colonies in mucous solid medium (GÓMEZ, 2006). Therefore, the presence of a translucent or creamy material involving a mucoid colony is indicative of EPS production potential (Figure 1).

The production of polymers was confirmed by mixing each colony in absolute alcohol. Precipitate formation indicated the presence of EPS (Figure 2). EPS production by colonies was also confirmed by electron microscopy analyses of matrix polysaccharides (Figure 3). Typically, screening of EPS-producing microorganisms is performed in tubes containing liquid medium or carried out in individual plates with solid

medium (CERNING et al., 1994; SARWAT et al., 2008), both of which require a great amount of medium and glassware.

Table 1 shows the result of screening lactic acid bacteria isolated under different growing conditions. The screening of 187 EPS-producing lactic acid bacteria strains resulted in 191 positive results since single strains were able to produce polymer in multiple growing conditions. The medium containing sucrose induced a higher number of isolates to produce EPS, with 99 (51.8%) positive results; most of which (42.4%) were at 28 °C and pH 7.5. The second highest number of EPS-producing

Table 1. Parameters evaluated in the screening of 187 isolates of lactic acid bacteria producing EPS.

Sugar	pH	T °C	Total isolates*
Sucrose	5.5	28	15
		35	13
		45	10
	7.5	28	42
		35	13
		45	6
Glucose	5.5	28	7
		35	9
		45	1
	7.5	28	14
		35	7
		45	1
Lactose	5.5	28	10
		35	9
		45	7
	7.5	28	15
		35	5
		45	7

*The same strain may have produced EPS under different culture conditions.

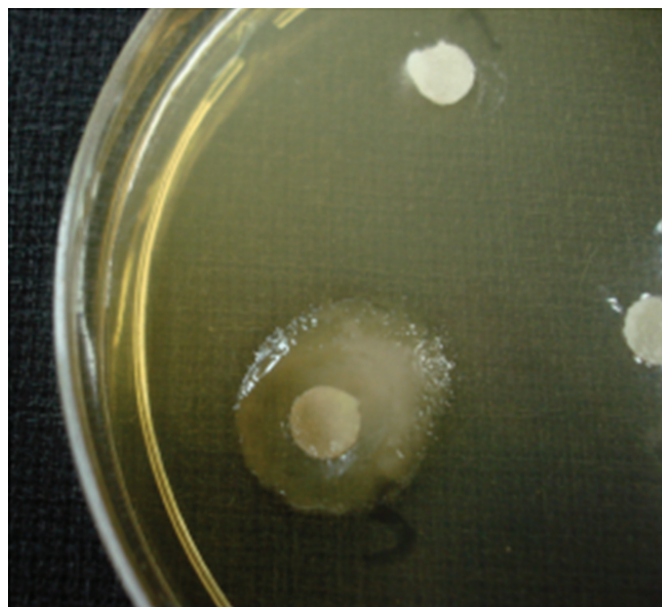


Figure 1. Translucent colony, potentially producing EPS.

isolates was found in the medium containing lactose, with 53 (27.7%) positive results, of which 28.3% were under the same conditions of pH and temperature that tested best with sucrose.

Finally, the medium containing glucose resulted in 39 (20.4%) positive results, with 35.9%, also under the same conditions of pH and temperature of other media.

In this study, the ideal environment for EPS production by the lactic acid bacteria isolates was found to be a basic medium supplemented with sucrose at pH 7.5 and a temperature of 28 °C. Many bacteria synthesize sucrose and dextran, an extracellular polymer, by the action of the dextran sucrose enzyme as the predominant source of carbohydrates. The main producer is the bacterial genus, *Leuconostoc*. Other sugars such as glucose, fructose, mannose, and lactose promote growth of bacteria that do not produce the enzyme (CAVENAGHI, 2000). The total number of EPS produced by the microorganisms is strongly influenced by the sugar present in the medium. However, there are some kinds of sugar that are better for the production of EPS because the production of the polymer depends on the strain tested and thus on the enzymatic metabolism of each strain (RUAS-MADIEDO; REYES-GAVILÁN, 2005).

In 1943, Orla-Jensen described the production of EPS by *Leuconostoc* spp., mesophilic lactobacilli, and *Pediococcus* spp.

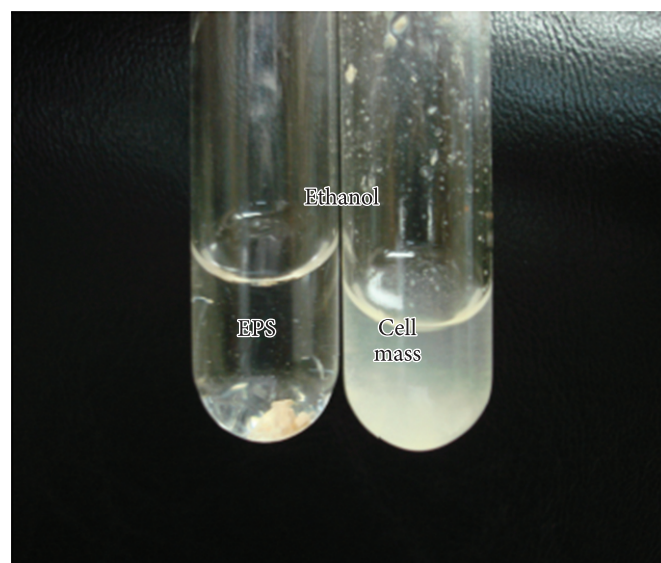


Figure 2. Confirmation of EPS production by mixture of colonies in ethanol.

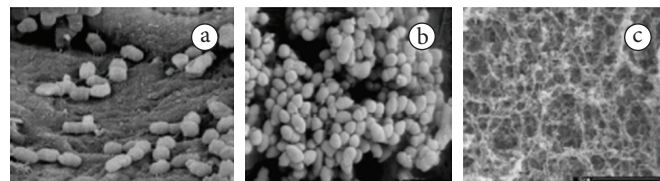


Figure 3. a) paper disc containing EPS-producing bacteria (×7000 magnification); b) paper disc with bacterial growth and without EPS production (×7000 magnification); c) negative control, paper disc without inoculation of bacteria (×1000 magnification).

by sucrose (TIEKING, 2005), leading many researchers to use this sugar to induce EPS production (OEVELEN; VERACHTERT, 2002; YAMAMOTO; YOSHIKAWA; OKADA, 1993; SUTHERLAND, 1998; LAWS; GU; MARSHALL, 2001; PADMANABHAN; KIM, 2002). Sucrose and glucose are commonly used for the isolation of EPS-producing bacteria; they provide the greatest production of biopolymers in most bacteria (SUTHERLAND, 2002; GÓMEZ, 2006; SARWAT et al., 2008). However, in this study, the second best sugar for inducing EPS production was lactose.

Temperature is a critical factor in the synthesis of polysaccharides. Higher growth and greater production of polysaccharides occurred in the range of 25 °C to 35 °C (VERMANI; KELKAR; KAMAT, 1995; PRASANNA; GRANDISON; CHARALAMPOPOULOS, 2012). Studies have shown that strains of thermophilic lactic acid bacteria such as *Streptococcus thermophilus* LY03 and *Lactobacillus delbrueckii* have a better EPS production at 42 °C (DE VUYST et al., 1998) and above 45 °C (LAWS; GU; MARSHALL, 2001), respectively. The pH also represents a significant and critical factor in the production of EPS and affects the activity of glucosyltransferases (CANILHA et al., 2006).

4 Conclusion

The technique of screening for EPS-producing lactic acid bacteria using paper discs saturated by different strains was efficient, fast, and economical.

5 Perspectives

In the future, some of these isolates will be identified, and a factorial experimental design with response surface analysis will be used to optimize the production of EPS. These exopolysaccharides will also be identified, characterized, and applied according with the physicochemical characteristics showed.

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