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Evaluation and modification of existing methods for the quantification of exopolysaccharides in milk-based media

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Abstract

Two EPS assay procedures were evaluated using a ropy culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483) grown in a milk-based medium. Reproducible EPS measurements were unachievable because the proteins and lactose in the growth medium interfered with the separation of EPS and phenol–sulphuric acid method. Accurate determination of EPS in a complex system requires an effective regime to separate EPS from non-EPS components in fermented broths. In the development of a new EPS assay procedure, the reliability of the assay was assessed by adding a known amount of dextran to a fresh growth medium. Each processing step was then evaluated based on dextran recovery. Key improvements made to the EPS assay procedures included the use of Flavourzyme for protein hydrolysis; optimizing ethanol concentration to prevent lactose crystallization yet allowing complete EPS precipitation; and a suitable centrifugation regime to minimize EPS loss. The improved EPS assay gave reproducible results (5% coefficient of variation) with greater accuracy.

Keywords: Exopolysaccharides; *Lactobacillus delbrueckii* subsp. *bulgaricus*; Fermented milk; Milk permeate; Protein hydrolysis; EPS assay

1. Introduction

Different exopolysaccharide (EPS) assays have been developed and used by many researchers over the last two decades (Andaloussi, Talbaoui, Marczak, & Bonaly, 1995; Bouzar, Cerning, & Desmazeaud, 1996; Cerning et al., 1994; De Vuyst & Degeest, 1999; Doco, Carcano, Ramos, Loones, & Fournet, 1991; Dupont, Roy, & Lapointe, 2000; Gamar-Nourani, Blondeau, & Simonet, 1997, 1998; Gancel & Novel, 1994; Garcia-Garibay & Marshall, 1991; Gorret, Maubois, Ghoul, & Engasser, 2001; Grobben, Sikkema, Smith, & de Bont, 1995; Gruter, Leeflang, Kuiper, Kamerling, & Vliegenthart, 1993; Kimmel, Roberts, & Ziegler, 1998; Looijesteijn, van Casteren, Tuinier, Doeswijk-Voragen, & Hugenholtz, 2000; Mozzi, de Giori Graciela, Oliver, & de Valdez Graciela, 1996; Nakajima et al., 1990; Petry, Furlan, Crepeau, Cerning, & Desmazeaud, 2000; Racine, Dumont, Champagne, & Morin, 1991; Smith & Pace, 1982; Toba, Uemura, & Itoh, 1992; Torino, Taranto, & de Valdez, 2001; Urashima et al., 1999; van den Berg et al., 1995; Yang, Huttunen, Staaf, Widmalm, & Tenhu, 1999). All these methods involve a combination of techniques to isolate, purify and quantify the EPS in a culture medium. Some of the techniques used to isolate and/or purify EPS include size–exclusion chromatography, anion exchange chromatography, ultra-filtration, diafiltration, centrifugation, dialysis, ultrasonication and lyopilization. Common techniques used for precipitating

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EPS include ethanol, acetone, propanol, isopropanol, cetyltrimethylammonium bromide (CTAB) and 3,5,6-triphenyl-2,3,5,6-tetraaza bicycle-1-hexene (commercially known as Nitron. Azeredo & Oliveira, 1996). In culture media containing proteins, trichloroacetic acid (TCA) and enzyme treatments have been commonly employed to precipitate and hydrolyse the proteins, respectively. The quantification of EPS subsequent to isolation and purification commonly employs the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

Many of the assays are reliable for determining the levels of EPS for cultures grown in chemically defined and/or semi-defined media. However, for complex media containing milk, the EPS values are inaccurate due to the non-EPS components present in the media which often interfere with the assay. In milk fermentation by lactic acid bacteria, the lactic acid produced causes aggregation of casein particles leading to the formation of a weak gel. The gel in which the bacterial cells, lactose and other minor components are entrapped, is a highly complex network structure of proteins and EPS. Careful separation of the EPS from non-EPS components, particularly proteins, lactose and bacterial cells, is necessary (Cerning, 1990), as the inclusion of such components could influence the results of the chemical methods employed for total carbohydrate determination. Cerning, Bouillanne, Landon, and Desmazeaud (1992) reported that a culture medium containing yeast extract could also interfere with EPS assay due to the presence of mannans. The aim of this work was to develop a more reliable assay for quantification of EPS in a complex medium, such as milk.

2. Materials and methods

2.1. Fermentation of 2483 culture

The seed culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 (National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland), denoted as 2483, was grown in a reconstituted skim milk (RSM) and fermented at 37 °C for 24 h. The RSM was prepared by dissolving 10% (w/v) skim milk powder (SMP) (Fonterra Co-operative Ltd., New Zealand) in water. The seed culture was inoculated (1% v/v) into the following growth media: milk permeate (Fonterra Co-operative Ltd., New Zealand), milk permeate with the addition of 10% (w/v) lactose (analytical grade, BDH) and 1% (w/v) milk protein concentrate (MPC) (ALAPRO 4560, Fonterra Co-operative Ltd., New Zealand), and the RSM. All culture media were free-steamed at 100 °C for 10 min and cooled to room temperature prior to inoculation with the 2483 seed culture. Fermentation of the 2483 culture was carried out at 37 °C for 24 h in a 1 L Duran bottle.

2.2. Enzymes used for protein hydrolysis

The enzymes used in this study are as follows:

- Flavourzyme (Novo Nordisk, Bagsvaerd, Denmark).
- Mixture of Neutrase (Novo Nordisk) and Protease A Amano2 (Amano Enzyme, Nayoga, Japan) (denoted as *Enzyme A*).
- Promod 215P (Biocatalysts, Wales, UK) (denoted as *Enzyme B*).
- Promod 215P and Flavopro 192P (Biocatalysts) (denoted as *Enzyme C*).
- Flavorpro 192P (denoted as *Enzyme D*).

Each enzyme was either dissolved or diluted in Milli-Q water and then filtered through a 0.2 μm filter (Millipore) to make a 10% (w/v) enzyme preparation.

2.3. Quantification of total carbohydrates

Chemical analysis for the determination of total carbohydrate was adapted from the phenol–sulphuric acid method as described by Dubois et al. (1956). Sample (1 mL) was mixed with 1 mL phenol solution (5% w/v) followed by addition of 5 mL concentrated sulphuric acid. The sample was left at room temperature for 30 min prior to measuring absorbance at 485 nm using a spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The total amount of carbohydrate was determined based on a standard calibration curve prepared using glucose or dextran (average molecular weight: 2×10^6 Da, Sigma–Aldrich). All analyses using the phenol–sulphuric acid method were performed in duplicate.

3. Results and discussion

3.1. Evaluation of existing methods for EPS determination

Two commonly employed EPS assays were evaluated using the culture medium of 2483 grown in milk permeate supplemented with 10% (w/v) lactose and 1% (w/v) milk protein concentrate. The first method, referred to here as the *acid addition method* was carried out according to the procedure described by Kimmel et al. (1998) which was originally developed by Gancel and Novel (1994). The second method used was adapted from the procedures utilized by Cerning et al. (1994) and is referred to as the *ethanol precipitation method*. The amounts of EPS from the culture medium determined by