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Separation of PCR-ready DNA from dairy products using magnetic hydrophilic microspheres and poly(ethylene glycol)–NaCl water solutions

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ABSTRACT

Carboxyl group-containing magnetic nonporous poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) (P(HEMA-*co*-GMA)) and magnetic glass microspheres were used for the isolation of bacterial DNA. P(HEMA-*co*-GMA) microspheres were prepared by the dispersion polymerization in toluene/2-methylpropan-1-ol mixture in the presence of magnetite nanoparticles obtained by coprecipitation of Fe(II) and Fe(III) salts with ammonium hydroxide. Carboxyl groups were then introduced by oxidation of the microspheres with potassium permanganate. The most extensive DNA recovery was achieved at PEG 6000 concentrations of 12% or 16% and 2 M NaCl. The method proposed was used for bacterial DNA isolation from different dairy products containing *Bifidobacterium* and *Lactobacillus* cells. The presence of target DNA and the quality of isolated DNA were checked by polymerase chain reaction (PCR) amplification with specific primers.

1. Introduction

Polymerase chain reaction (PCR) has become a powerful diagnostic tool for the analysis of microorganisms in food samples. However, the sensitivity of PCR may be reduced when this method is applied to complex biological samples. Reduced sensitivity or false-negative results can occur in PCR identification due to the presence of extracellular PCR inhibitors in tested samples [1]. The identification of bacteria in real samples implies the following steps: preparation of PCR-ready DNA and identification of bacterial species by PCR. Pre-PCR processing procedures have been developed to reduce the effects of PCR inhibitors. The problem of pure DNA preparation can be solved by means of various isolation and purification methods:

- phenol/chloroform extraction and DNA precipitation in ethanol [2];
- chromatographic methods [3,4];
- solid phase systems non-selectively or selectively adsorbing DNA or target cells [5,6].

Solid phase systems based on silica carriers have become popular for DNA isolation [5] and are used in many available kits. Further progress was achieved by the application of magnetically responsive particles [7–9]. Magnetic particles carrying a specific antibody can be used for the capture of target cells and their separation from the environment containing extracellular inhibitors or competitive microflora. Magnetic particles need not be detached from the target microbial cells, and the immunomagnetic separation (IMS) technique can be effectively combined with PCR (IMS–PCR) [6]. The use of this technique is dependent on the availability of antibodies against target microorganisms.

A reversible DNA adsorption on the surface of particles containing carboxyl groups was used primarily for the isolation of short DNA fragments [10]. Magnetic hydrophilic 2-hydroxyethyl methacrylate (HEMA)-based microspheres were used with success for the isolation of high-molecular-weight bacterial DNA [8,9] from different real samples in the presence of poly(ethylene glycol) (PEG) 6000 and NaCl water solutions. The authors [11] recently published a paper on the PEG–KF-water system. An equilibrium diagram for NaCl has not been reported yet.

The aim of this work was the isolation of whole PCR-ready DNA from dairy products containing *Bifidobacterium* and *Lactobacillus* cells by magnetic hydrophilic poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) (P(HEMA-*co*-GMA)) microspheres in the

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presence of water solutions composed of 2 M NaCl and PEG 600 or PEG 6000.

2. Methods

2.1. Chemicals, microorganisms, samples, and equipment

Agarose was purchased from Serva (Heidelberg, Germany), ethidium bromide from Sigma (St. Louis, USA). Magnetite (ferrous–ferric oxide Fe_3O_4, 10 nm) was precipitated from an aqueous solution of $FeCl_3$ and $FeCl_2$ with ammonium hydroxide and stabilized by oleic acid [12]. Monomers, 2-hydroxyethyl and glycidyl methacrylate (HEMA and GMA), both from Röhm (Darmstadt, Germany), were purified by distillation under reduced pressure in nitrogen atmosphere. The stabiliser, cellulose acetate butyrate, was a kind gift of Eastman (Kingsport, USA). The primers for PCR were synthesised by Generi-Biotech (Hradec Králové, Czech Republic); TaqI DNA polymerase was from Bio-Tech (Prague, Czech Republic); DNA marker 100 bp ladder (100–1500 bp long DNA fragments) for gel electrophoresis was from Malamité (Moravské Prusy, Czech Republic); PEG 600 and PEG 6000 were purchased from Sigma (St. Louis, USA). Microbial DNA was isolated from cell lysates. Other chemicals and solvents were of analytical grade and obtained from commercial sources. Magnetic glass particles (5 μm, CPG, New York, USA) were used for the comparison of DNA isolation under the same experimental conditions.

The strain of *Bifidobacterium longum* CCM 3764 (obtained from the Czech Collection of Microorganisms, CCM, Brno, Czech Republic) was used for DNA isolation (positive control). Dairy products (yoghurt, kefir, and probiotic milk drinks) were obtained from the market.

UV measurements were carried out on a DMS 100 spectrophotometer (Varian, Mulgrave, Australia). Magnetic microspheres were separated on an MPC-M magnetic particle concentrator, Dynal (Oslo, Norway). The PCR reaction mixture was amplified on a MJ Research Programme Cycler PTC-100 (Watertown, USA). Agarose gel electrophoreses were carried out using a 3000 Xi power supply (Bio-Rad Laboratories, Richmond, USA). The PCR products were visualised on an UV transilluminator EB-20E from UltraLum (Paramount, USA), and photographed with a CD 34 Polaroid Camera (Polaroid, Cambridge, USA).

2.2. Preparation of microspheres

Magnetic nonporous P(HEMA-*co*-GMA) (50/50 w/w) microspheres were prepared by cellulose acetate butyrate-stabilised and dibenzoyl peroxide-initiated dispersion copolymerisation of 2-hydroxyethyl and glycidyl methacrylate in toluene/2-methylpropan-1-ol mixture in the presence of colloidal oleic acid-coated magnetite (Fe_3O_4) particles as described earlier [12]. Subsequently, the hydroxy groups of the microspheres were oxidised with a 2% aqueous solution of potassium permanganate under acidic conditions (2 M sulphuric acid) yielding 0.85 mM COOH/g [13]. The content of carboxyl groups in the microspheres was determined by titration using 0.1 M NaOH on a 799 GPT Titrino (Metrohm, Herrisau, Switzerland) after ion exchange with a 10% aqueous solution of $BaCl_2$ [14]. The microspheres were of 2.2 μm size with a rather narrow size distribution characterised by a polydispersity index, PDI = 1.1 (PDI = D_w/D_n, where D_w and D_n is the weight- and number-average particle diameter, respectively, determined from the measurement of at least 500 microspheres on scanning electron micrographs).

2.3. Cell cultivation, DNA isolation, and PCR amplification

Bacterial cells of *Bifidobacterium longum* were cultivated anaerobically on MRS medium (Oxoid, Great Britain) with cysteine (0.5 g/l) overnight (18 h) at 37 °C. Altogether 1 ml of the cells was washed and resuspended in 500 μl lysis buffer (10 mM Tris–HCl, 5 mM EDTA (pH 7.8) containing lysozyme (3 mg/ml) and incubated at 22 °C for 1 h; 10 μl proteinase K (1 mg/ml) and 2.5 μl SDS (20%) was then added and the mixture was incubated at 55 °C for 18 h. DNA was extracted from crude cell lysates with phenol [2] or separated using the magnetic microspheres. The integrity of nucleic acid was confirmed by gel electrophoresis and the purity of DNA was determined by UV spectrophotometry. The ratio $A_{260\,nm}/A_{280\,nm}$ was used as a test of nucleic acid purity [15].

Dairy products (1 g) were dispersed in 1 ml of water. Bacterial cells were sedimented (10,000 g/5 min), dispersed in lysis buffer, and treated as *Bifidobacterium longum* cells (see above). A total of 50 μl of crude cell lysate, 10 μl of microsphere suspension (2 mg/ml), and the appropriate amount of 40% PEG and 5 M NaCl (end concentration 4%, 8%, 12%, or 16% PEG, 2 M NaCl) were mixed and incubated for 10 min at laboratory temperature. The DNA captured on the microspheres was washed using 500 μl of 70% ethanol and eluted in 50 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA; pH 7.8).

DNA purified by phenol extraction [2] or DNA isolated using the magnetic microspheres were used as DNA matrix in PCR. PCR was performed with PbiF1 and PbiR2 primers specific to the *Bifidobacterium* genus [16], which enabled the amplification of 914 bp long amplicons, and LBLMA 1 and R16 primers specific to the *Lactobacillus* genus [17], which enabled the amplification of 250 bp long amplicons. Briefly, the PCR mixture contained 0.5 μl of each 10 mM dNTP, 0.5 μl (10 pmol/μl) of each primer, 0.5 μl of Taq 1.1 polymerase (1 U/μl), 2.5 μl of buffer (1.5 mM), 1–2 μl of DNA matrix, and PCR water was added up to a 25 μl volume. The amplification reactions were carried out using the following cycle parameters: 5 min of the initial denaturation period at 94 °C (hot start), 60 s of denaturation at 94 °C, 60 s of primer annealing at 50 °C, and 120 s of extension at 72 °C for the *Bifidobacterium* genus; 5 min of the initial denaturation period at 94 °C (hot start), 60 s of denaturation at 94 °C, 60 s of primer annealing at 55 °C, and 60 s of extension at 72 °C for the *Lactobacillus* genus, respectively. The final polymerization step was prolonged to 10 min, the number of cycles was 30. PCR products were detected using electrophoresis with 1.5% agarose in TBE buffer (45 mM boric acid, 45 mM Tris–base, 1 mM EDTA; pH 8.0). The DNA was stained with ethidium bromide (0.5 μg/ml), and photographed at 305 nm UV light on a TT667 film.

3. Results and discussion

The PEG–NaCl system (8% PEG 6000 and 2 M NaCl) was used in our previously published papers [8,9,13]. Condensation of DNA occurred [18] under the given experimental conditions and condensed DNA was adsorbed on the surface of solid magnetic microspheres. In this work, newly designed carboxyl group-containing magnetic hydrophilic poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) P(HEMA-*co*-GMA) were prepared by dispersion polymerization of both monomers in a toluene/2-methylpropan-1-ol mixture in the presence of oleic acid-coated magnetite nanoparticles. Dispersion copolymerisation was stabilized by cellulose acetate butyrate and initiated by dibenzoyl peroxide [12]. Advantage of dispersion polymerization consists in that almost monodisperse magnetic microspheres in the micrometer-size range can be obtained in a single step. At the same time, the particles are nonporous, as the dispersion polymerization involves precipitation of oligomeric chains from a solution of