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Optimization of Environmental Conditions for Kefiran Production by Kefir Grain as Scaffold for Tissue Engineering

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The aim of this work was to investigate the fermentation medium and environmental requirements for the production of exopolysaccharide (EPS) Kefiran from natural culture (Kefir grains). We have found that the fermentation medium and temperature are critical for Kefiran production during the 24 h cultivation of kefir grains. The Kefiran obtained from fat cow milk was used to evaluate its potential application as scaffold for tissue engineering. Kefiran scaffold were obtained via solvent casting and direct quenching. Thermal and morphology features were evaluated by DSC and SEM, respectively.

1. Introduction

Kefir is popular fermented milk and one type of light alcoholic beverage. Kefir grains are usually used as a starter in the Caucasian regions. It is known that kefir grains consist of a gel matrix in which yeasts and lactic acid bacteria are embedded [Maeda et al., 2003].
The polysaccharide named kefiran is produced by *L. kefiranofaciens* in the centre of grain under anaerobic conditions in presence of ethanol produced by yeasts and lactic acid secreted by lactobacilli. Major role of kefiran in grains is described as a protection for the microorganisms [Badel et al., 2011]. Therefore, Kefiran is a microbial exopolysaccharide (EPS) obtained from the flora of kefir grains. It contains glucose and galactose, is water-soluble and improves the viscosity and viscoelastic properties of acid milk gels.
In addition, when compared with other polysaccharides, kefiran has several important advantages, such as antibacterial, antifungal, and antitumor properties. Several studies have reported the use of polysaccharides from different sources to prepare films and coatings with different properties, and have indicated that these carbohydrates are promising materials. According to the literature, as well as data and preliminary studies in our laboratory, kefiran can produce films with satisfactory mechanical properties and good appearance: it appears to have excellent potential as a film- forming agent.
Several studies have been conducted on biopolymer-based packaging materials originated from naturally renewable resources such as chitosan [Ojagh, 2010], kefiran [Ghasemlou, 2011 and Khodaiyan, 2011], and starch [Almasi, 2010 and Gao, 2011] polysaccharides and proteins of whey [Sothornvit, 2009], zein [Ghanbarzadeh, 2006] and gluten [Gontard, 1993 and 1994], and also agar [Rhim, 2011]. These studies have indicated that the used polysaccharides and proteins are promising materials for packaging different foods.
Some proposed applications of such films include pouches or sachets to package dry ingredients (e.g., beverage mixes), individual packaging of small portions of food, particularly products that currently are not individually packaged for practical reasons such as pears, nuts, beans and strawberries (Bourtoom, 2008).
Unfortunately, so far the use of films based on proteins or polysaccharides for food packaging has been strongly limited because of the poor barrier and weak mechanical properties. Different physical and chemical modifications including biopolymers blending [Kristo, 2007], addition of plasticizing agents [Ghanbarzadeh, 2011], and nano-fillers addition have been applied to improve the properties of biodegradable films. Despite

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several applications are proposed for Kefiran (EPS) films, no application has been proposed regarding tissue engineering.
The architecture of scaffolds used for tissue engineering is of critical importance. Scaffolds should have an interconnected pore structure and high porosity to ensure cellular penetration and adequate diffusion of nutrients to cells within the construct and to the extra-cellular matrix formed by these cells. Furthermore, a porous interconnected structure is required to allow diffusion of waste products out of the scaffold, and the products of scaffold degradation should be able to exit the body without interference with other organs and surrounding tissues [Marler, 1998 and Sung, 2004].
In this study, we test various culture conditions (temperature and medium) that might influence the production of kefiran from the Kefir grain. An isolation and purification method of kefiran was optimized and a characterization by scanning electron microscopy (SEM), and differential scanning calorimeter (DSC), in order to evaluate its potential application as biomaterial, was carried out. Indeed, dense films and porous scaffold from aqueous solutions 2% (w/v) kefiran were prepared and characterized by scanning electron microscopy (SEM), and differential scanning calorimeter (DSC), in order to evaluate its potential application as scaffold for tissue engineering.
Results not only provide new insights into the foaming methods for producing kefiran scaffolds, but also supply indications on how to optimize the fabrication parameters to design scaffolds with different morphology and mechanical proprieties, which might address new applications of bioabsorbable scaffolds in tissue regeneration.

2. Methods and materials

2.1 Determination of Kefir grain biomass increase

Kefir grains, used as a starter culture in this study, were purchased from an online shop. The grains (3%, w/v) were grown at controlled temperature in 50 ml volume Falcon tubes containing different fermentation media without stirring. The fermentation media used in this study were sheep, goat and cow milk.
The temperature was taken constant at value of 20 and 25°C by thermostatic bath. Kefir production was refreshed after 24 h. Grains were washed by sterile water, let dry on sterile paper under a flow laminar hood and weighed to measure biomass increase. Measurements were performed in triplicate every 24 h. The biomass growth rate was determined according to the Eq (1), which was used to evaluate the effects of culture conditions. All results were carried out 3 times for means.

$$\nu = \frac{W_{n+1} - W_n}{W_n} * 100\% \quad (1)$$

Where □ is growth rate, Wn is the biomass weight after n day (g) and Wn+1 is biomass weight after (n+1) day (g).

2.2 Isolation and purification of kefiran

Kefiran exopolysaccharide was extracted from the kefir grains by the described method by Piermaria et al. [1]. In brief, a weighed amount of Kefir grains were dissolved in boiling water (1:20) for 30 min with discontinuous stirring. The mixture was centrifuged at 10,000g for 20 min at 20°C (Thermo Scientific SL40R Centrifuge).
All decanted undissolved portions of grain was removed while the polysaccharide in the supernatant was precipitated by addition of two volume of cold ethanol and left at 20°C overnight. The mixture was centrifuged at 10,000g for 20 min at 4°C. Pellets were dissolved in hot water and the precipitation procedure was repeated twice. The precipitate was finally dissolved in hot distilled water and freeze-dried. The white precipitated polysaccharide is hereafter called kefiran.

2.3 Dense Film-forming solution preparation

Aqueous solutions containing 2 wt% of kefiran were prepared under continuous agitation to select the polysaccharide concentration. Dense films were obtained by casting of 0.5 g of polymeric solutions into 12 mm diameter multiwell. Filmogenic solutions were dried at room temperature in a vacuum chamber until reaching constant weight for 6 h. The obtained films were removed from the plate and stored at -20 °C at low humidity.

2.4 Kefiran scaffold via freeze drying

Aqueous solutions containing 2 wt% of kefiran were prepared under continuous agitation to select the polysaccharide concentration. Kefiran scaffold were obtained by casting of polymeric solutions into 20 mm diameter and 2 mm thick aluminum tool. Aluminum tool was submerged into thermostatic bath at -20°C for 15 minute and after freeze dried.

2.5 SEM and DSC analysis

Scanning Electron Microscopy by using Philips SEM quanta FEI, at 10 kV was employed to observe surface morphology of Kefiran dense films. Before the measurements, samples were coated by gold sputtering to make them conductive.

The crystallinity of Kefiran dense film was investigated through Differential Scanning Calorimetry (DSC), employing a DSC131 EVO (Setaram). Membrane samples were heated at 10°C/min in the range 35 ÷ 200°C.

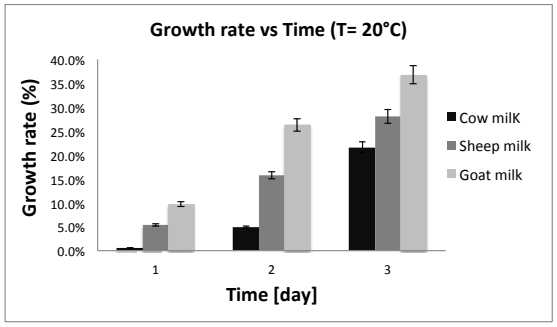
3. Results and discussion

3.1 Effect of culture medium and temperature

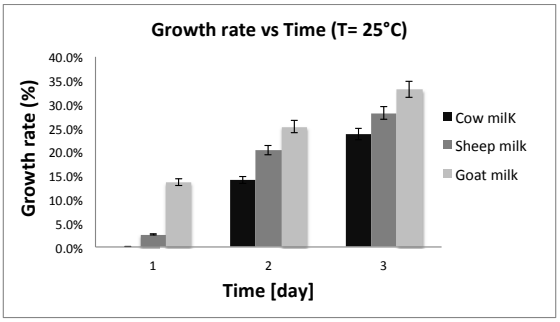
In order to investigate the effect of culture medium and growth temperature on kefir grain biomass, kefir grains were incubated in three different milk media at 20 and 25°C. Figure 1 shows that the best combination of medium/temperature for kefir grain growth and kefiran production was goat milk at 20°C. The biomass growth rate was more rapid in goat milk than sheep milk for both temperatures investigated. This finding might depend on the carbohydrate availability of the media used. Lactose is a good carbon and energy source for L. kefiranofaciens [Cheirsilp B. et al. 2001]. The capsular kefiran concentration increased with growth and decreased when cell growth stopped. If the lactose is used as a carbon source, the galactose and glucose accumulated in the medium start to decrease when the lactose concentration decreased to 20 g*l–1 [Yokoi and Watanabe 1992; Mitsue et al. 1998].

Considering that the goat milk has lower lactose content than sheep and cow milk, it can be supposed that glucose and galactose, which are monomers of the EPS, induced an increase in biomass growth rate. The results (see figure 1) indicated that the growth rate of grains is directly affected by the type of milk. Zajsek et al. observed that the optimal temperature for Kefir grain growth and kefiran production is 37°C and 25°C, respectively. At 25°C the kefiran concentration is higher mainly due to the fact that the microorganisms protect themselves against environmental influences by increasing the kefiran production.

At 37°C the kefiran content remained constant and only Kefir grain microbiota increases, which causes the increase of the Kefir grain biomass [Zajsek and Gorsek, 2011]. In this study, two different temperatures to produce kefiran were investigated. Figure1c shows the effect of the incubation temperature, at the 3th fermentation day, on grain weight variation for several fermentation medium tested.



a)



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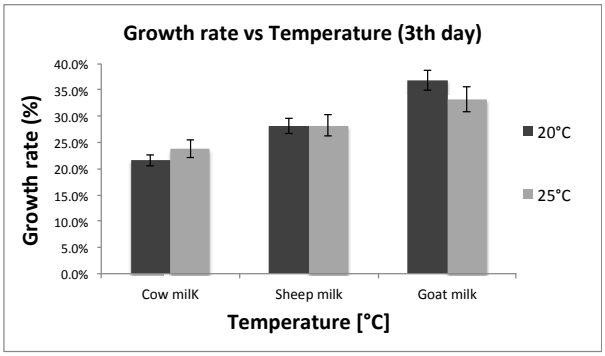


c)

Figure 1: effect of culture medium and culture temperature on kefir grains biomass: a) effect of culture medium on biomass growth rate at 20°C, b) effect of culture medium on biomass growth rate at 25°C, c) effect of the temperature on biomass growth rate.

Gao et al. [2012] reported that the biomass growth rate increased with temperature for fat cow milk. In this study, it was constant for sheep milk while it decrease for goat milk when the incubation temperature was increased from 20°C to 25°C.

3.2 SEM analysis of porous scaffold and dense films

In an attempt to study microstructural changes in the scaffold and films, scanning electron microscopy (SEM) was used to depict the surface and section topography of all prepared films and kefiran scaffolds.

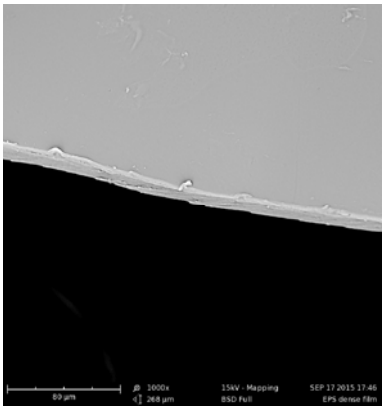
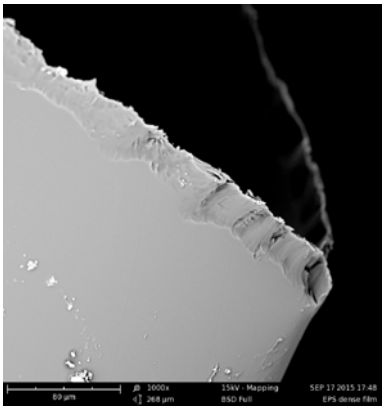
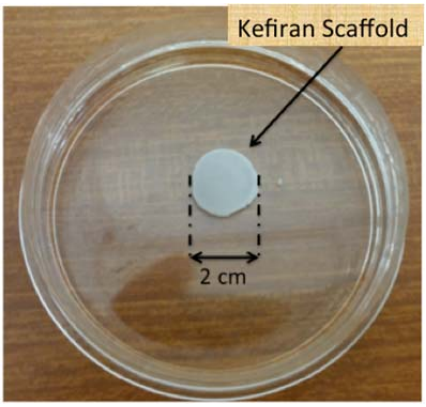
a)  b) 

Figure 2: Films obtained via solvent casting: a) surface outer, b) cross-section.

Figure 2 shows of the pictures of the outer surface (left) and cross-section (right) of films dense obtained via solvent casting. SEM observations of films did not present any cracks, breaks, or openings on the surfaces. Films obtained in this work were thick about 20 □m and transparent [Zolfi et al., 2014 and Ghasemloua et al., 2011].

a) 

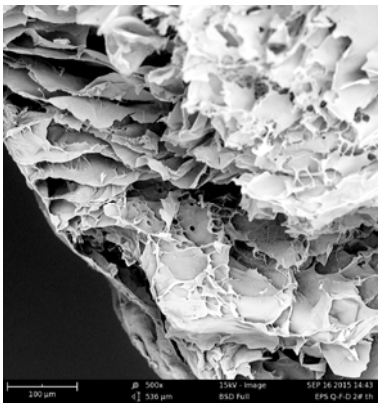
b)  c) 

Figure 3: Scaffolds porous obtained via direct quenching: a) scaffold external look, b) outer surface morphology, c) cross-section morphology.

Figure 3 shows the pictures of external look (left), outer surface (central) and cross-section (right) morphology of kefiran scaffold obtained via freeze-drying. Kefiran scaffolds, which were obtained via freeze-drying, have a stiff structure and it was opaque (see figure 3a). SEM observations of kefiran scaffolds evidence a porous structure highly interconnected with presence of open porous on external surfaces (see figure 3a and 3b).

3.3 Differential scanning calorimetry analysis

The Kefiran dense films and porous scaffolds membrane described before were characterized with Differential Scanning Calorimetry (DSC). The heat flow data were normalized with the total sample weight (figure 4): thus, the area of melting peaks is related to the crystalline fraction. Peak area and melting temperature were determined..

The melting enthalpy □Hm [J/g] were about 294.8 and 355.2 respectively for porous scaffolds and dense films. However, the melting peaks were 107.3 °C and 102.4 °C respectively for porous scaffolds and dense films for all investigated samples [Shahabi-Ghahfarrokhi et al., 2015]

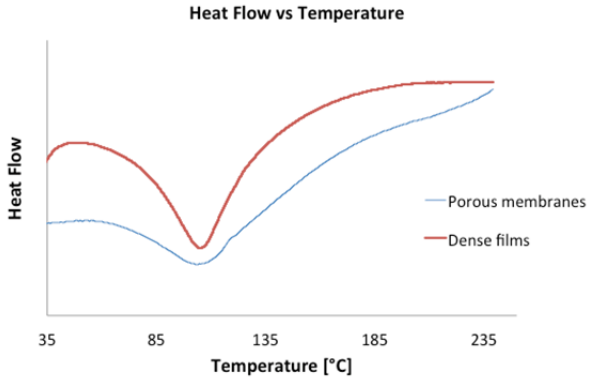


Figure 4: DSC analysis of porous and dense membranes.

A summary of collected data (melting enthalpy and melting temperature) is reported in Figure 5. The porous scaffolds were more amorphous than dense films, while the melting temperature of dense films and porous scaffolds were about identical.

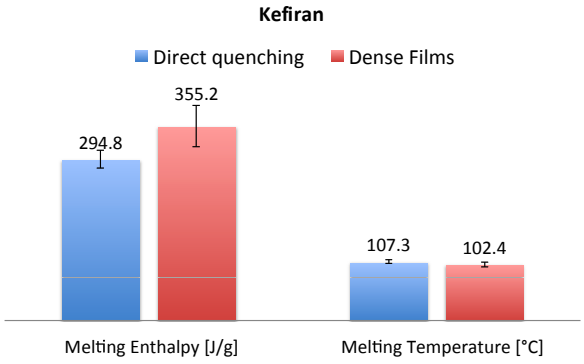


Figure 5: Thermal features of porous and dense membranes.

The thermal results show that probably the crystallinity structures in the two cases (dense vs. porous) are very similar (eg. witnessed by the almost coincident T_m) but the total crystallinity gets lower in the foamed structures, due to inhibition of crystallization related to the fast quenching protocol adopted.

4. Conclusion

The potential application of kefiran in food industry has increased the interest for the study of the production of this polymer. In this work, the authors evaluate its potential application as scaffold or/and support for tissue engineering. The optimization of the growth environment is important for achieving its maximal production from Kefir grains. In this study, the kefiran production from kefir grains was optimized after 24h at 20°C.

Based on the results presented, it was observed that porous scaffolds can be produced via freeze-drying, while as reported in literature and obtained in this work, dense films can be obtained via solvent casting. Scaffolds represent important components for tissue engineering.

However, researchers often encounter an enormous variety of choices when selecting scaffolds for tissue engineering. Scaffolds obtained in this study were with interconnected pore structure and good porosity, which allow diffusion of waste products out of the scaffold and supply of nutrient to the tissue or organ. On the other hand, dense films can be used as support for submerged culture.
Results not only provide new insights into the foaming methods for producing kefiran scaffolds, but also supply indications on how to optimize the fabrication parameters to design scaffolds with different morphology and mechanical proprieties, which might address new applications of bioabsorbable scaffolds in tissue regeneration.

Acknowledgments

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