ABSTRACT

The production of novel environmentally friendly films from kefiran, a polysaccharide produced during milk fermentation, combined with bacterial cellulose is being explored as a new application for kefiran in food packaging. Various production challenges, including generally low yields and inhibition by acid production during fermentation, have prevented large-scale, commercial production of this polysaccharide. Different conditions such as the species present, ratio of bacteria to yeasts, fermentation time, incubation temperature, storage time and other environmental conditions can affect the amount of kefiran produced. Optimizing fermentation conditions is crucial to efficiently producing a desired end product. However, the accumulation of byproducts (e.g. lactic acid in dairy fermentation) can inhibit the activity of fermentative microbes. Controlling pH is one method to optimize the fermentation reaction. Under a controlled temperature and constant agitation rate, pH can be altered to efficiently manufacture the desired product (e.g. the kefiran polysaccharide). Design of a fermentation chamber to optimize environmental conditions (temperature, agitation, pH) required for maximum kefiran production along with the implementation of a pH monitoring and control system to maintain a target pH with allow for fermentations with variable pH buffering to determine optimal pH for complete lactose metabolism and subsequent kefiran production. To monitor the efficacy of acid neutralization during fermentation rapid testing methods with be developed using enzymatic assays and spectrophotometry to define sugar and acid profiles of milk throughout fermentation. HPLC analysis will be used to further characterize and directly quantify the kefiran produced. The results obtained from this project will determine the potential for using acid neutralization during fermentation to increase kefiran yield and will help identify biomaterial production requirements for the newly created microbial cellulose-kefiran film on an industrial scale.

PROJECT DESCRIPTION

Introduction

The production and application of synthetic plastic films in food packaging has grown quickly over the past few decades, resulting in serious environmental concerns due to the intensive use of non-renewable resources as raw materials and resistance to degradation. Today's consumers are aware of resultant environmental problems and are beginning to demand new biodegradable materials that reduce plastic waste, especially from food packaging. They expect the food and packaging industries to guarantee component safety and low migration of plastic components into the food matrix. Edible and biodegradable films present an appealing alternative to synthetic packaging for materials in direct contact with food.

Polysaccharides produced by some acetic and lactic acid bacteria, such as cellulose and kefiran, have been the subject of emerging research that aims to develop packaging alternatives to off-set or replace the ubiquitous use of non-recyclable, single use plastic films and wrappers for food products. Kefiran is a polysaccharide produced and subsequently excreted by certain strains of lactic acid bacteria during the production of kefir, a fermented dairy beverage. It has been isolated, and its composition and chemical structure have been determined by acid or enzymatic hydrolysis. Numerous studies have demonstrated that the antibacterial and antimycotic activity of cells increases when exposed to kefir and kefiran[1], and the antimicrobial activity of kefir and kefiran extract has been attributed to successful and faster wound healing in rats when a topical kefiran mixture was used as an alternative to antibiotics. In addition to its positive health implications, kefiran has been explored for microencapsulation of sensitive materials, use as food ingredient, certain medical applications and the development of bioplastics[2,3]

[1] Garrote, G. L.; Delfederico, L.; Bibiloni, R.; Abraham, A. G.; Pérez, P. F.; Semorile, L.; De Antoni, G. L. Lactobacilli Isolated from Kefir Grains: Evidence of the Presence of S-Layer Proteins. J. Dairy Res. 2004, 71 (2), 222–230.

[2] Radhouani, H.; Gonçalves, C.; Maia, F. R.; Oliveira, J. M.; Reis, R. L. Biological Performance of a Promising Kefiran-Biopolymer with Potential in Regenerative Medicine Applications: A Comparative Study with Hyaluronic Acid. J. Mater. Sci. Mater. Med. 2018, 29 (8), 124. 7

[3] Hasheminya, S.-M.; Mokarram, R. R.; Ghanbarzadeh, B.; Hamishekar, H.; Kafil, H. S.; Dehghannya, J. Development and Characterization of Biocomposite.

Due to the interesting active functional properties of kefiran, it also has the potential for use as a food packaging material. However, previous studies of kefiran films reported poor mechanical and permeability properties[4,5], which has limited its use. A strategy to obtain biomaterials with improved functional properties is by combining different biopolymers. By combining kefiran with other polysaccharides, such as microbial cellulose, it is possible to obtain new biomaterials with enhanced functional properties and potential applications in the food industry.

Cellulose is the most abundant natural compound on the earth. This low-cost biopolymer is renewable, non-toxic, biocompatible, biodegradable and chemically stable[6]. It is possible to produce pure microbial cellulose by the fermentation of *Gluconoacetobacter xylinu*[7]. This bacterium synthesizes bacterial cellulose to form a three-dimensional structure with an expanded surface area and high porosity. The unique three-dimensional structure of this bacterial cellulose offers some interesting physical and mechanical properties. Previous research analyzing the reinforcing effect of several components, such as chitosan (a natural antimicrobial agent), PVOH (a biodegradable plasticizer agent) and glycerol (a natural plasticizer agent) on bacterial cellulose films showed it is feasible to obtain transparent and flexible films with important UV-barrier properties and enhanced functional properties that expand the potential applications of these composites, for example, as active packaging[6]. All these studies have shown the suitability of these materials, combined with different components, for packaging applications, and the need to continue investigating and developing new kefiran-cellulose composites.

Background and Specific Aims

Cellulose-kefiran films produced over the course of this research are expected to extend the shelf life of food without the use of non-renewable petroleum-based plastics or chemical antimicrobial and antioxidant compounds. It will be necessary to perform a complete characterization of the functional and structural properties of the individual components of these developed films to predict their behavior and suitability. This will require the ability to produce kefiran on a larger scale then our current methodology allows.

Several efforts have been made to produce kefiran on an industrial scale using *Lactobacillus* species pure culture[8] instead of kefir grains. However, all studies examining the optimization of kefiran production show that polysaccharide recovery is increased when the bacteria *Lactobacillus kefiranofaciens* grows in co-culture with the yeast *Saccharomyces cerevisiae*, or yeast extracts[9]. This symbiotic relationship naturally exists in traditional kefir cultures (i.e. kefir grains), which are composed of multiple species of bacteria, such as lactobacilli (*Lactobacillus kefir, Lactobacillus plantarum*), lactococcus (*Lactococcus lactis subsp*), acetic acid bacteria (*Acetobacter*) and yeast (*Saccharomyces*), embedded in a kefiran matrix. For this reason, the direct use of kefir grains could improve the production of kefiran exopolysaccharide. A study on optimizing the growth media (e.g. carbon and nitrogen sources) found that exopolysaccharide production in milk-based media was highest at temperatures between 32℃ and 42℃ and under a constant pH of 5.5-

[4]Ghasemlou, M.; Khodaiyan, F.; Oromiehie, A. Rheological and Structural Characterisation of Film-Forming Solutions and Biodegradable Edible Film Made from Kefiran as Affected by Various Plasticizer Types. Int. J. Biol. Macromol. 2011, 49 (4), 814–821.

[5] Piermaria, J. A.; Pinotti, A.; Garcia, M. A.; Abraham, A. G. Films Based on Kefiran, an Exopolysaccharide Obtained from Kefir Grain: Development and Characterization. Food Hydrocoll. 2009, 23 (3), 684–690.

[6] Cazon, P.; Velázquez, G.; Vázquez, M. Characterization of Bacterial Cellulose Films Combined with Chitosan and Polyvinyl Alcohol: Evaluation of Mechanical and Barrier Properties. Carbohydr. Polym. 2019, 216, 72–85.

[7] Jozala, A. F.; de Lencastre-Novaes, L. C.; Lopes, A. M.; de Carvalho Santos-Ebinuma, V.; Mazzola, P. G.; Pessoa-Jr, A.; Grotto, D.; Gerenutti, M.; Chaud, M. V. Bacterial Nanocellulose Production and Application: A 10-Year Overview. Springer Verlag 2016, pp 2063–2072.

[8] Moradi, Z.; Kalanpour, N. Kefiran, a Branched Polysaccharide: Preparation, Properties and Applications: A Review. Carbohydr. Polym. 2019, 223 (March), 115100.

[9] Cheirsilp, B.; Shimizu, H.; Shioya, S. Enhanced Kefiran Production by Mixed Culture of Lactobacillus Kefiranofaciens and Saccharomyces Cerevisiae. J. Biotechnol. 2003, 100 (1), 43–53.

6.6[10]. Concurring with these results, a similar study found that maximum exopolysaccharide production was obtained at a temperature of 42°C and pH 6.5[11]. In addition, maintaining a consistent pH by the buffering or removal of excess lactic acid can stimulate bacterial growth[11].

The literature suggests this process can be performed by two different methods: nanofiltration of organic acids[12] or buffering the solution by the addition of an alkaline reagent[10]. However, the nanofiltration method also requires a buffer solution made from 20% NaOH (w/w) making both procedures very time consuming and labor intensive. Several research projects on lipid-based drug delivery systems have used a pH stat automated titration system to maintain a constant pH during a chemical reaction[13,14]. In grain fermentation, pH maintenance was simply performed with a pH controller, keeping the fermentation pH at values of 6.5 ± 0.1 by delivering volumes of buffer solution as needed[12]. An automated system will provide continuous optimization of pH in the fermentative media and produce the maximum amount of kefiran, our desired end product. The pH controller measures and controls pH in solution, yet it is relatively smaller and more economical than other options, such as a bioreactor. The results obtained through this project will allow us to establish the potential for using acid neutralization during fermentation to increase EPS yield and determine the production requirements for the newly created microbial cellulose-kefiran film on an industrial scale, its applicability to foodstuff protection, and market acceptance**.**

Objective 1: A) Design fermentation chamber to optimize environmental conditions (temperature, agitation, pH) required for maximum kefiran production B) Implement pH monitoring and control system to maintain a target pH C) Perform fermentations with variable pH buffering to determine optimal pH for complete lactose metabolism
Objective 2: Develop rapid testing methods using enzymatic assays and spectrophotometry to define sugar and acid profiles of milk during fermentation (lactose, glucose, galactose, lactic acid)
Objective 3: Characterization of extracted and purified kefiran produced under varying environmental condition using HPLC analysis

Methodology

Implementation of a continuous pH monitoring and buffering system during fermentation to enhance kefiran production

A controlled fermentation chamber capable of maintaining specific environmental conditions (temperature, product mixing, removal/transfer of kefir grain cultures) required for kefir fermentation will be designed. The fermenter pH will be maintained at a target pH by the pH controllers, which deliver solutions of NaOH or HCl. The amounts of base or acid infused will be measured and related to the rate of acid production during fermentation. Pasteurized milk or whey (produced by rennet coagulation) will be used as the fermentative material. Samples of milk before inoculation will be collected and stored for analysis to determine total lactose. The milk or whey will be inoculated at a ratio of 1:30 w/w kefir grains to milk. The inoculated dairy will be placed in the fermentation chamber and the pH monitoring and titration system will be connected. Three pH controllers and data logging systems will be used to allow controlled data collection for multiple variables from one "batch" of milk with the same initial carbohydrate value.

10 Zhang, T. et al. Growth and Exopolysaccharide Production by Streptococcus Thermophilus ST1 in Skim Milk. 2001. Brazilian Journal of Microbiology 42(4): 1470–78.

11 Vaningelgem, F., M. Zamfir, T. Adriany, and Luc De Vuyst. Fermentation Conditions Affecting the Bacterial Growth and Exopolysaccharide Production by Streptococcus Thermophilus ST 111 in Milk-Based Medium. 2004. Journal of Applied Microbiology 97(6): 1257–73.

12 Crawford, R. J., B. J. Shriver, G. A. Varga, and W. H. Hoover. Buffer Requirements for Maintenance of PH during Fermentation of Individual Feeds in Continuous Cultures.1983. Journal of Dairy Science 66(9): 1881–90.

13 Bolko, K., Zvonar, A. and Gašperlin, M. Simulating the Digestion of Lipid-Based Drug Delivery Systems (LBDDS): Overview of in Vitro Lipolysis Models. 2004. Acta Chimica Slovenica 61(1): 1–10.

14 Kalepu, S., Manthina, M. and Padavala, V. Oral Lipid-Based Drug Delivery Systems – an Overview. 2003 Acta Pharmaceutica Sinica B 3(6): 361–72.

Analysis of kefiran production and sugar metabolism during fermentation
Throughout fermentation samples will be removed to determine the amounts of 1) mono- and disaccharides 2) organic acids and 3) kefiran. Periodic sampling will be used to create production curves for EPS that directly correlate to the utilization of lactose and its monosaccharides during fermentation. Samples will be heat treated at 95C for 10 minutes to stop fermentation and stored in the refrigerator until analysis. No other sample preparation is required prior to analysis. Sugar profile including lactose, glucose, galactose and lactic acid will be determined by rapid analysis methods (10 -15 minutes) using a Gallery Discrete Analyzer (ThermoFisher) and corresponding enzymatic assay kits. Percent total carbohydrate and EPS values will be reported as both calculated and direct measurement values. Calculated values based will be based on pre-fermentation total carbohydrates and the resulting fermentation product amounts. HPLC analysis will be used to directly quantify kefiran in the samples. We will confirm the calculated EPS value obtained from the enzymatic/spectrophotometric analysis to create a model for EPS production based on the carbohydrate fermentation curves.

Extraction and purification of kefir grains
Kefir cultures will be separated from the from whey or milk using a strainer, dissolved in hot distilled water (1:10) at 90°C and stirred vigorously until all the grain is dissolved (about 1 h). The resulting mixture will be centrifuged at 10,000 rpm for 10 min at 20 °C to remove undissolved particles. The supernatant (kefiran dissolved in water) is then removed and an equal volume of ethanol added to precipitate the polysaccharide. The mixture with the added ethanol solvent will be transferred to the freezer. After allowing the ethanol-kefiran mixture to freeze completely, the mixture will be thawed completely and centrifuged again at 10,000 rpm for 10 min at 5 °C to collect the precipitate (kefiran and other molecules undissolved in ethanol). In this case, the supernatant (ethanol and substances dissolved in ethanol) is removed. The precipitate will be re-dissolved in hot distilled water (1:10) at 90°C and stirred vigorously until complete dissolution. The mixture will again be centrifuged at 10,000 rpm for 30 min at 20 °C to remove undissolved particles. The supernatant (kefiran dissolved in water) will be collected and an equal volume of ethanol added to precipitate the polysaccharide. The mixture with ethanol will undergo a second freezing and thawing step. The kefiran sample with ethanol mixture will be centrifuged a final time at 10,000 rpm for 10 min at 5 °C to collect the precipitate (purified kefiran). All the collected precipitate will be dried in the incubator at 65C and stored for future use.

Milestones and Timeline

Study and optimization of kefiran production

Task 1. Optimization of environmental conditions for fermentation and kefiran production
- Evaluation of fermentation temperature, agitation, time for kefiran production
- Evaluation of acid removal during fermentation on kefiran production (pH control)

Task 2. Optimization of culture media
- Comparison of milk and enriched/fortified milk for kefiran production
- Evaluation of cheese whey for kefir production (an industrial by-product)
- Explore other potential media enrichments (e.g. beer wastes, wine lees)

Task 3. Study of the extraction and purification of kefiran
- Optimization of precipitation with ethanol
- Evaluation of procedures for biomass concentration (centrifugation, filtration)

Task 4. *Stabilization study of the kefiran films during drying*
- Evaluation of spray drying, oven drying and freeze drying prior to film production

Resources

Middle Tennessee State University facilities and resources devoted and available to the MTSU Fermentation Science degree program, include:

a) A newly remodeled 1640 square foot teaching laboratory with a separate 209 square foot storage area Equipment includes 2 chemical hoods, incubators, ultra-low freezer for cultures and samples, water baths, scales.
b) A 190 square foot walk-in cooler with storage racks and new refrigeration unit.
c) A separate 600 square foot research laboratory housing the following instrumentation: UHPLC with UV and RI detectors, Gallery Discrete Analyzer, centrifuge, analytical balance, alkalizer, clean hood equipped with UV light sterilization system, spectrophotometer with 96 well microplate reader, shaker tables, tissue homogenizer and Type 1 & III water purification system.

Dissemination and Potential External Funding

The success of this project will be measured by the publication and presentation of results. It is expected that at least 2 articles in high impact journals in the areas of Food Technology and Polymer Science will be published as a result of this effort. The publications obtained in this project will form an excellent background to justify funding from the USDA and FDA for the scale up and future applications of the composite films obtained based on kefiran and bacterial cellulose. In addition, this research will establish an ongoing program to continue developing and evaluating the production, by fermentation, of new biomaterials with applications in food, medicine, cosmetic and pharmaceuticals. Results from this project will be used in the application of externally funded grant opportunities to further strengthen the relationship and collaboration between MTSU and the University of Santiago de Compostela (Spain), as well as to support the application for external support for continued research from international sources. Once kefir grain production is optimized, studies modifying milk and whey with food industry by-products will be conducted. The utilization of food industry waste-streams to improve the production of kefiran will give added value to these by-products and create possibilities for industry supported funding.

Significance

The production and application of synthetic plastic films in food packaging has grown quickly over the past few decades, resulting in serious environmental concerns due to the intensive use of non-renewable resources as raw materials and resistance to degradation. Today's consumers are aware of resultant environmental problems and are beginning to demand new biodegradable materials that reduce plastic waste, especially from food packaging. They expect the food and packaging industries to guarantee component safety and low migration of plastic components into the food matrix. Edible and biodegradable films present an appealing alternative to synthetic packaging for materials in direct contact with food. Edible polysaccharide-based films have several applications as food packaging, due to their capabilities to prevent moisture loss, aroma loss, solute transport, water absorption in the food matrix and oxygen penetration. In addition, these films are an effective barrier against solvent penetration in foods, which can result in toxicity or quality loss during storage. The combination of polysaccharides and natural additives with functional properties (e.g. antimicrobial, antioxidant) has the potential to enhance the shelf-life, safety and quality of food products. The results obtained through this project will allow us to establish the potential for commercial production of the newly created microbial cellulose-kefiran film on an industrial scale, its applicability to foodstuff protection, and market acceptance.

Keely O'Brien

EDUCATION and TRAINING

Louisiana State University	Baton Rouge, LA	Animal and Dairy Sciences	Ph.D., 2015
Louisiana State University	Baton Rouge, LA	Animal Sciences	M.S., 2012
University of Tennessee at Chattanooga	Chattanooga, TN	Biological Sciences	B.S., 2007

EMPLOYMENT HISTORY

Assistant Professor of Fermentation Science, Middle Tennessee State University, Murfreesboro, TN; 2019-present

Director of Research and Development for Cultured Dairy Products, fairlife LLC, Chicago, IL; 2016-2019

Postdoctoral Researcher, Louisiana State University, Baton Rouge, LA; 2015-2016.

UNIVERSITY TEACHING EXPERIENCE

INSTITUTION	COURSES TAUGHT
Middle Tennessee State University	FERM 1000 – Introduction to Fermentation
	FERM 3860 – Wine, Beer and Spirits Industry
	AGBS 4380 – Food Quality Control
	FERM 4580/5580 – Applied Fermentation: Fruits and Vegetables
	FERM 4570/5570 – Applied Fermentation: Milk, Meat and Grains
	FERM 3710 – Brewing, Distilling and Fermentation Safety and Sanitation
	FERM 6100 – Food Contamination, Safety and Regulation
	AGBS 3810 – Milk Processing and Marketing
	FERM 4920 – Fermentation Science Research
	AGRI 4910 – Problems in Agriculture
Louisiana State University	ANSC 4020 – Cultured and Frozen Dairy Foods
	ANSC 2085 – Milk Quality Control

SELECTED PROFESSIONAL ACTIVITIES, HONORS and AWARDS

R. Gene Smith Scholarship for Dairy Foods Technology, Louisiana State University (2014)

Finalist, Graduate Student Poster Competition: Dairy Division, ADSA-ASAS Joint Annual Meeting (2014)

GRANTS RECEIVED / MANAGED

March 2020 (funded). Supporting role: collaborator. "Partnership To Develop Fermentation Science As A Curricular Enhancement To Basic Undergraduate STEM Classes." USDA NIFA Capacity Building Grant for Non-land Grant Colleges of Agriculture, **$299,999**

October 2012 – August 2013. Project Director, “Quality Attributes of Kefir and A Kefir Based Dairy Beverage”. Funded through Louisiana Agricultural Experiment Station Hatch Project, **$7500**.

REFEREED PUBLICATIONS (past four years)

L.K. Stewart, P. Smoak, D.S. Hydock, R. Hayward, **K. O'Brien,** J.K. Lisano, C. Boeneke, M. Christensen, A. Mathias. (2019) Milk and kefir maintain aspects of health during doxorubicin treatment in rats. Journal of Dairy Science 102 (3): 1910-1917.

O’Brien, K.V., Prinyawiwatkul, W., Carabante Ordonez, K.M., Stewart, L.K., and Boeneke, C.A. (2017) Short Communication: The Development and Sensory Analysis of a Kefir Product Designed for Cancer Survivors. *Journal of Dairy Science* 100(6): 4349-4353.
*JDS Editorial Office and Publisher selection for inclusion in media outreach program press release

O’Brien, K.V., Aryana, K.J., Prinyawiwatkul, W., Carabante Ordonez, K.M. and Boeneke, C.A. (2016) Short Communication: Viability of probiotic bacteria and yeasts in traditional and commercial kefir following frozen storage. *Journal of Dairy Science* 99: 7043–7048.

O’Brien, K.V., Stewart, L.K., Forney, L., Aryana, K.J., Prinyawiwatkul, W. and Boeneke, C.A. (2015) The effects of post-exercise consumption of a kefir beverage on performance and recovery during intensive endurance training. *Journal of Dairy Science* 98: 7446-7449.
*****JDS “Editor’s Choice” featured article, November 2015

RELEVANT NON-REFEREED PUBLICATIONS (past four years)