BIOPROCESS OPTIMIZATION FOR KEFIRAN PRODUCTION BY *Lactobacillus kefiranofaciens*

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To my beloved mother, father, sisters, wife and friends.

I love you all very much.

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ABSTRACT

Kefiran is water soluble heteropolysaccharides produced by *Lactobacillus kefiranofaciens*. In this work, optimization process was carried out to maximize kefiran production in both pure culture and mixed culture system using yeast strain. For pure culture studies, the cultivation medium composition was first optimized using both one factor at a time (OFAT) and statistical method. The optimal medium for kefiran production using OFAT composed of (in g L^{-1}): lactose, 50; yeast extract, 12; phosphate, 0.25; Triton X-100, 1; and medium osmotic pressure 550 mOsmol kg^{-1}. On the other hand, the optimal medium composition using statistical method composed of (in g L^{-1}): lactose, 58.74; yeast extract, 11.09; phosphate, 0.52; Triton X-100, 0.85, and medium osmotic pressure of 630 mOsmol kg^{-1}. The maximal kefiran production of 1.51 g L^{-1} was obtained in OFAT optimized medium which was approximately 8.61% higher than those obtained in the statistically optimized medium. After this step, the process was optimized in 16-L stirred tank bioreactor. The maximal kefiran production reached 2.32 g L^{-1} and 1.87 g L^{-1} in bioreactor with and without pH control, respectively. Aeration rate of 1.0 v/v/min was the best for kefiran production (2.32 g L^{-1}). Furthermore, a series of fed-batch cultivations were carried out to determine factors that limit cell growth and kefiran production. A combination of constant complete medium feeding coupled with dissolved oxygen control was found to be the best strategy for highest kefiran production (4.73 g L^{-1}). Among different yeasts tested, *S. cerevisiae* was found to be the most suitable strain for mixed culture system. The mixed culture of *L. kefiranofaciens* and *S. cerevisiae* enhanced kefiran production from 0.31 g L^{-1} in pure culture up to 0.39 g L^{-1} in the mixed culture. The cultivation medium was then optimized using OFAT method reaching a maximum kefiran production of 2.12 g L^{-1}. The optimal medium for kefiran production by the mixed culture using OFAT method composed of (in g L^{-1}): lactose, 50; yeast extract, 12; phosphate, 0.5; Triton X-100, 1.25 g L^{-1}, and osmotic pressure of 600 mOsmol kg^{-1}. Batch cultivations of mixed culture in 16-L stirred tank bioreactor with and without pH control produced kefiran with concentration of 3.01 g L^{-1} and 2.40 g L^{-1}, respectively. Aeration rate at 1.0 v/v/min produced maximal kefiran production of 4.42 g L^{-1}. Further improvement in kefiran production was achieved by using constant lactose feeding which was the best strategy to increase the production up to 5.51 g L^{-1}.

ABSTRAK

Lactobacillus kefiranofaciens menghasilkan heteropolisakarida larut air dikenali sebagai kefiran. Dalam kajian ini, proses pengoptimum dijalankan untuk memaksimumkan pengeluaran kefiran menggunakan kultur asli dan kultur campuran. Untuk kultur asli, medium kultur telah dioptimumkan menggunakan kedua-dua kaedah iaitu satu faktor semasa (OFAT) dan statistik. Keadaan optimum untuk pengeluaran kefiran dengan menggunakan kaedah OFAT ialah (dalam g L^{-1}): laktosa, 50; ekstrak yis, 12; fosfat, 0.25; tekanan osmotik, 550 mOsmol kg^{-1} dan dengan tambahan 1 g L^{-1} Triton X-100, pada masa sifar. Keadaan optimum untuk pengeluaran kefiran melalui kultur asli menggunakan kaedah statistik adalah (dalam g L^{-1}): laktosa, 58.74; ekstrak yis, 11.09; fosfat, 0.52; tekanan osmotik, 630 mOsmol kg^{-1} dan dengan tambahan 0.85 g L^{-1} Triton X-100. Penghasilan kefiran menggunakan kaedah OFAT (1.64 g L^{-1}) adalah lebih kurang 8.61% lebih tinggi daripada kaedah statistik (1.51 g L^{-1}). Proses pengoptimum seterusnya di jalankan di dalam tangki bioreaktor 16-L. Sebanyak 2.32 g L^{-1} dan 1.87 g L^{-1} kefiran dihasilkan menggunakan kultur asli masing-masing dengan dan tanpa kawalan pH. Kadar pengudaraan pada 1.0 v/v/min menghasilkan kefiran maksimum 2.32 g L^{-1}. Satu siri pengkulturan berkelompok telah dijalankan untuk menentukan faktor yang menghadkan pertumbuhan sel dan pengeluaran kefiran. Gabungan lengkap strategi pembekalan makananan secara berterusan ditambah dengan kawalan oksigen larut didapati menghasilkan pengeluaran kefiran tertinggi 4.73 g L^{-1}. Di antara semua yis yang dikaji, *S. cerevisiae* didapati terbaik untuk digunakan dalam kultur campuran. Kultur campuran *L. kefiranofaciens* dengan *S. cerevisiae* meningkatkan pengeluaran kefiran daripada 0.31 g L^{-1} dalam kultur asli sehingga 0.39 g L^{-1}. Medium kultur kemudian dioptimumkan menggunakan kaedah OFAT lalu mencapai pengeluaran kefiran maksimum sebanyak 2.12 g L^{-1}. Keadaan optimum untuk pengeluaran kefiran oleh kultur campuran menggunakan kaedah OFAT ialah (dalam g L^{-1}): laktosa, 50; ekstrak yis, 12; fosfat, 0.5; tekanan osmotik, 600 mOsmol kg^{-1} dan 1.25 g L^{-1} Triton X-100. Pengkulturan kelompok kultur campuran dalam tangki bioreaktor 16-L dengan dan tanpa kawalasn pH masing-masing menghasilkan kefiran maksimum 3.01 g L^{-1} dan 2.40 g L^{-1}. Kadar pengudaraan 1.0 v/v/min menghasilkan kefiran pada kepekatan maksimum sebanyak 4.42 g L^{-1}. Peningkatan pengeluaran kefiran seterusnya dijalankan dengan menggunakan bekalan kekal mono-laktosa didapati dapat menghasilkan kefiran pada kepekatan maksima 5.51 g L^{-1}.

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LIST OF ABBREVIATIONS

C/N	-	Carbon to nitrogen ratio
CDW	-	Cell Dry Weight
DO	-	Dissolved Oxygen
EPS	-	Exopolysaccharide
CO_2	-	Carbon Dioxide
C	-	Carbon
H^+	-	Proton
HCl	-	Hydrochloric acid
K_2HPO_4	-	dipotassium hydrogen phosphate
K	-	Potassium
kgf cm^{-2}	-	Kilogram force per square centimetre
$MgSO_4.7H_2O$	-	Magnesium sulfate heptahydrate
$MnSO_4.7\ H_2O$	-	Manganese sulphate heptahydrate
mOsmol/kg	-	MiliOsmol per kilogram of water
N	-	Nitrogen
Na	-	Sodium
NaCl	-	Sodium Chloride
NaOH	-	Sodium hydroxide
NH_4Cl	-	Ammonium Cloride
$(NH_4)_2HPO_4$	-	Ammonium phosphate
$(NH_4)_2SO_4$	-	Ammonium Sulfate
O_2	-	Oxygen
RPM	-	Revolutions per minutes
vvm	-	Volume of air per working volume of bioreaction per minute
OFAT	-	One factor at a time

RSM	-	Response surface methodology
LAB	-	Lactic acid bacteria
GRAS	-	Generally regarded as safe
FDA	-	Food and drug administration
CCD	-	Central composite design
PBD	-	Placket burman design
HPLC	-	High performance liquid chromatography
HPGFC	-	High performance gel filtration chromatography
GPC-MLLS	-	Gel permeation chromatography-multiangle laser light scattering
GFC	-	Gel filtration chromatography
EPS	-	Exopolysaccharides
ATP	-	Adenosine triphosphate
PEP	-	Phosphotransferase system
UDP	-	Uridine diphosphate
dTDP	-	Thymidine diphosphate
WPI	-	Whey protein isolate
CHAPS	-	3-((3-cholamidopropyl)dimethylammonio)-l-propanesulfonate
DCO_2	-	Dissolved carbon dioxide
OTR	-	Oxygen transfer rate
MCB	-	Master cell bank
WCB	-	Working cell bank
MRS	-	Man-Ragosa-Sharpe
YPD	-	Yeast Extract-Peptone-Dextrose
YM	-	Yeast-Mold

LIST OF SYMBOLS

%	-	Percentage
λ	-	Wavelength
°C	-	Degree Celsius
g	-	Gram
$g\ L^{-1}$	-	Gram per liter
min	-	Minutes
mL	-	Millilitre
h	-	Hour
K	-	Kelvin
L	-	Liter
$L\ h^{-1}$	-	Liter per hour
Mg	-	Magnesium
mg	-	Milligram
P	-	Pressure
μ	-	Specific growth rate (h^{-1})
μ_{max}	-	Maximum specific growth rate (h^{-1})
F	-	Feed rate ($g\ L^{-1}\ h^{-1}$)
K_S	-	Substrate utilization constant (monod)
>	-	Greater than
t	-	Time interval (h)
V	-	Bioreactor volume (L)
X	-	Biomass concentration ($g\ L^{-1}$)
$Y_{P/X}$	-	Kefiran productivity (g kefiran per g biomass)
$Y_{X/S}$	-	Substrate yield coefficient (g biomass per g substrate)

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Kefir is a traditional fermented milk beverage which is believed to be found initially from the northern Caucasian mountains of Russia (Tratnik *et al*., 2006). Commercially produced kefir and other fermented milk products have become popular around the world since it confers beneficial health effects (Farnworth *et al.*, 2005). Kefir grains is used as main component as healthy fermented milk drink with potential nutraceutical activities based on its high content of different types of probiotic bacteria and unique type of biopolymer. Different studies showed that kefir grains bioflora composed of wide range of microorganisms mainly bacteria and yeast in symbiotic relationship. However, about 90% of microbiota of grain composed of bacteria belongs to genus *Acetobacter* and lactic acid bacteria (LAB) such as *Lactobacillus, Lactococcus, Streptococcus*, and *Leuconostoc* (Chen *et al.,* 2008; Kesmen and Kacmaz, 2011). These bacteria co-exist in symbiotic relation with different types of yeasts from species of *Yarrowia, Pichia, Zygosaccharomyces, Candida, Kluyveromyces*, *Torulaspora* and *Saccharomyces* species (Latorre-Garcia *et al.,* 2007; Marsh *et al*., 2013). However, *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* were found to be the major bacterial populations in all kefir grains (Leite *et al*., 2012).

Currently, the area of study involving microbial polysaccharides such as kefiran is extensively emerging as an important source of natural biopolymer materials. Kefiran is water soluble exopolysaccharides composed of equivalent repeated units of glucose and galactose (Micheli *et al*., 1999). This carbohydrate polymer was first isolated from kefir grain and named as kefiran by La Riviére *et al.* (1967). Further studies on kefir grains shows that this polysaccharide is predominantly produced by *Lactobacillus kefir* sp. (Kandler and Kunath, 1983). Kefiran is produced either in extracellular form (broth kefiran) or capsular form (capsular kefiran) (Cheirsilp *et al*., 2001). The molecular weight and compositions of extracellular and capsular kefiran are the same (Yokoi et al., 1990). It was also reported that the average molecular weight of kefiran of about 1×10^7 Da (Piermaria *et al*., 2008).

Kefiran is currently applied in food, nutraceutical and cosmeceutical industries based on its GRAS (Generally Regarded As Safe) status (Guzel-Seydim, 2011; Ahmed *et al*., 2013). Moreover, it was also proven to have many functional propeties in pharmaceutical applications as antimicrobial and immunostimulant polysaccharides (Medrano *et al*., 2011). In addition, recent studies also showed that it has many applications in nanotechnology research and application and used in the preparation of UV-protective kefiran/ZnO nanocomposite and in fabrication of nanofibers as well (Esnaashari *et al*., 2014; Shahabi-Ghafgarrokhi *et al*., 2015). The industrial kefiran production is carried out by fermentation using submerged cultivation system using *L. kefiranofaciens* pure culture or in mixed culture system with other bacteria such as *L. acidophilus, L. parakefir, L. kefirgranum* and/or some yeasts like *Saccharomyces cerevisiae*, and *Candida kefir* (Badel *et al*., 2011).

The present study was focused on the bioprocess optimization of kefiran production using *L. kefiranofaciens*. The area of study involved in optimizing production of kefiran was by designing the production medium. Therefore, many studies were focuses on the development of industrial medium for this process (Wang and Bi, 2008; Cheirsilp and Radchabut, 2011; Zajšek et *al*., 2013). However, the current data in literatures clearly demonstrate that there is no standard medium developed so far for this process EPS production mainly depends on the type of strain used, physical conditions maintained during fermentation, and type of medium components applied for the production. The present work is focused on the development of suitable medium for kefiran production using the standard strain *L. kefiranofaciens* in pure culture and in mixed culture system with *S. cerevisiae* type of yeast in submerged cultivation system. Two methods of optimization process that were OFAT and statistical experimental design were used in shake flask level. The OFAT optimization was carried out in shake flask by optimizing one factor after individually. For statistical method, Minitab 16 software was used to design the experiment in order to optimize many factors at the same time and to understand the factors interaction. The results obtained for OFAT and statistical method were compared to obtain the most suitable medium for maximal EPS production.

Furthermore, kefiran production was improved by using mixed culture technique to minimize the inhibition effect of lactic acid during kefiran production. By applying mixed culture, new medium formulation is needed for the cell growth and kefiran production. Therefore, production medium was optimized to maximize kefiran production. In addition, the effect of additives was also studied in this work. Sodium chloride introduced into the production medium in order to create osmotic stress on the cultivated cells. It is well known that polysaccharide microbial produce polysaccharide to react with the extreme environment (Sheng *et al*., 2006). However, for such a microbial that lives in non-extreme conditions will have tolerance towards osmotic stress from their surroundings. On the other hand, surfactant was also introduced into the production medium to support the kefiran production. Surfactant effect is through increasing cell membrane permeability (Wu *et al*., 2008). Up to date, no report was shown for the effect of osmotic stress and surfactant on kefiran production. Further investigation was carried out to improve kefiran production in bioreactor. However, it is important to understand that moving for shake flask to bioreactor is not just a simply a matter of increasing culture and vessel volume, but also dominate by a number of engineering considerations (Hewitt and Nienow, 2010). Therefore, different bioprocessing parameters such pH, aeration rate and various feeding strategies were tested during this study.

1.2 Problem Statement

In industrial fermentation, medium and process conditions plays critical role as they lead to formation and yield of desirable product thus effecting process economy. Industrial kefiran production is needed in enormous amount but at a lower cost. This is important for it to be reliable and marketable in many business sectors. However, developing a fermentation process from laboratory scale to a commercial one involve many challenges. This is due to the complication in evaluating the factors affecting the scale-up process during the cultivation process. Some other challenges related to process optimization are the expensive, laborious and time consuming process implying many experiments. Apart from this, with the new strains that are being continuously found, there is always a need to carry out optimization experiments. Developments of new chemically defined medium or semi defined medium are required to fulfill the need for lower cost medium but at the same time supply significant kefiran production.

The inhibition effect on kefiran production through pH reduction in medium caused by lactic acid production during fermentation yet is another problem during fermentation. Hence, a mixed culture system of *L. kefiranofaciens* and yeast was applied to minimize the lactic acid inhibition effect in the medium. With the existence of yeast, there will be need to optimized the production medium to avoid nutrients competition between both organisms. The yeast selected must be able to consume lactic acid efficiently and avoid utilizing lactose in the production medium.

New alternative to increase kefiran production is needed by using cheap and simple process. Osmotic pressure can be easily manipulated in the production medium by using sodium chloride. However, to increase the kefiran production, an optimum osmotic pressure needed to be investigating at different level to avoid inhibition on cell growth. Another method that can be applied to increase the kefiran production is by the addition of surfactant. Suitable type and concentration of surfactant is needed to increase the membrane permeability of cell. Increasing the membrane permeability of cell is important to ease the nutrient intake and excretion of kefiran outside the cell. In addition, surfactant is added at different addition time to study their effect on cell growth and kefiran production.

The performance of a mixed culture system which composed of *L. kefiranofaciens* and yeast may differ at the shake flask level and bioreactor. Process optimization from shake flask level to 16-L bioreactor may affect the performance of *L. kefiranofaciens* toward kefiran production. Further optimization of bioprocessing parameters is needed to increase the kefiran production by using the most suitable medium. This process takes place in the bioreactor to overcome the limitations of shake flask. Parameters such as pH, aeration rate and substrate feeding were monitored. During cultivation in bioreactor, pH of cultivation may change due to acid production that inhibit cell growth and consequently affected the kefiran production. Appropriate aeration rate is needed to overcome the air limitation in the bioreactor which terminates cell growth. Nutrient limitation is also another problem during cultivation. Therefore, different types of feeding strategies are needed to be studied to deliver the appropriate nutrient for cells growth and kefiran production. In conclusion, investigation of optimization process utilizing different methods, medium components synergy and process conditions were required in order to determine the optimal cultivation condition in conjunction with the physiological state best contributed for maximal kefiran production.

1.3 Objective

The objective of this study is to maximize the production of kefiran using *L. kefiranofaciens* pure culture or in mixed culture system. This include optimization of medium composition, development of mixed culture system, culture supplementation with different additives and bioprocess optimization in batch and fed-batch cultivation systems.

1.4 Scope of Study

The scopes of this thesis are:

1. To optimize the semi-defined medium design in shake flask level by using classical method and statistical experimental design.

2. To study the kefiran production in pure and mixed culture systems (cultivation of *Lactobacillus kefiranofaciens* with/without yeast strain in same culture).

3. To study the effect of osmotic pressure and surfactants on the kefiran production.

4. To study the kefiran production by using *L. kefiranofaciens* in pure culture or mixed culture with yeast in 16-L bioreactor.

5. To study the effect of some bioprocessing parameters on kefiran production (pH control and aeration rate) in 16-L bioreactor.

6. To design proper fed-batch cultivation strategy for kefiran production.

1.5 Benefits of the Study

Kefiran is a unique type of polysaccharides with various beneficial applications in the chemical, food, neutraceutical, cosmeceutical and pharmaceutical industries. Optimization for kefiran production is a need to fulfill the increase market demand. Each medium component such as carbon, nitrogen and other trace elements has a specific concentration effect the kefiran biosynthesis. By studying the key factors that influence the productivity of *L. kefiranofaciens,* a maximal amount of kefiran is able to be produced by using the optimal concentration of medium. Lactic acid produced during cultivation inhibited kefiran production. The use of membrane separation to remove lactic acid that accumulates in the bioreactor is expansive and tedious. Therefore, using mixed culture system of *L. kefiranofaciens* with non-lactose utilizing yeast which are able to assimilate lactate will help to reduce the cost of operations and at the same time stimulate kefiran production. By applying osmotic pressure, it shows stimulatory effect on kefiran production. Moreover, introducing surfactant in the production medium in low quantities stimulate the kefiran production. Studying the process in 16-L bioreactor is important where limitations in shake flask such as maintaining pH of cultivation medium, limitation of air supply and nutrients deficiency can be overcome. Understanding the challenges with process monitoring and controlling will help to improve the kefiran production. Optimizing and monitoring the process parameters such as pH, aeration rate and substrate feeding mode significantly enhanced the kefiran production. Hence, this will help to reduce the operation cost and at the same time maximize kefiran production.

1.6 Outline of Thesis

This thesis begins with Chapter 1 describing the research background, problem statement, objective, scopes and benefits of this study. Chapter 2 reviewed the literatures related to kefiran and current works about the optimization process. Chapter 3 described the experimental optimization of kefiran production and chapter 4 concerned with data processing and discussing the effects of medium compositions, medium osmolality, addition of surfactant and bioprocessing parameters on kefiran production. The conclusion and recommendations for future studies were represented in Chapter 5.

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