Universidade de Aveiro Departamento de Química

Ano 2020

JOÃO DIOGO MENDES D'ASCENSÃO

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, no Ramo de Biotecnologia Industrial e Ambiental, realizada sob a orientação científica da Doutora Ana Maria Rebelo Barreto Xavier, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Luísa Alexandra Seuanes Serafim Martins Leal, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho à minha mãe, ao meu pai e ao meu irmão, por toda a paciência e apoio demonstrados.

o júri

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palavras-chave

Kefirano, grãos de kefir, soro de leite, exopolissacarídeo, *Lactobacillus kefiranofaciens*, fermentação microbiana, otimização do processo, processamento downstream, caracterização do EPS.

resumo

O soro de leite é o subproduto principal da indústria dos lacticínios, e é massivamente produzido a nível mundial. O seu descarte pode representar um sério problema ambiental devido à sua elevada carga orgânica, portanto, é de interesse procurar mais soluções para a sua valorização. Uma alternativa interessante é a conversão da sua lactose em produtos de valor acrescentado, através da fermentação microbiana. O kefirano é um exopolissacarídeo com aproximadamente as mesmas quantidades de D-glucose e D-galactose, cujas notáveis características tornam possível o seu uso numa ampla gama de aplicações na medicina e na indústria farmacêutica e alimentar. Este polímero pode ser produzido durante a fermentação com grãos de kefir, uma complexa comunidade microbiana embebida numa matriz de kefirano, contendo várias espécies de bactérias do ácido lático (LAB), bactérias do ácido acético (AAB) e leveduras, que vivem em simbiose. Contudo, a bactéria *Lactobacillus kefiranofaciens* é considerada como sendo a principal responsável pela produção de kefirano.

O objetivo deste trabalho foi o estudo da produção de kefirano, utilizando grãos de kefir, em soro de leite de vaca (CWC), soro de leite de vaca diluído (CWCD), soro de leite de ovelha (CWS) e em meio sintético (MRSL broth). Neste estudo, foram realizadas fermentações em modo batch e fed-batch.

Inicialmente, o meio MRSL foi utilizado para a otimização do processo. O tempo de fermentação de 100 h foi considerado como ótimo, porque resultou no valor de produtividade de kefirano mais elevado. A agitação ideal foi de 80 rpm, uma vez que demonstrou o melhor balanço entre a produção de kefirano e a conservação dos grãos de kefir. Relativamente à ótima concentração inicial de lactose, os resultados foram dependentes da agitação, onde não foi observada uma inibição por substrato significativa. Os melhores resultados em termos de concentração de kefirano, 1,640 g/L, e produtividade, 0,390 g/L.dia, foram ambos alcançados após 100 h de fermentação, usando um meio MRSL broth contendo 40 g/L de lactose, a 160 rpm.

Em relação aos ensaios com soro de leite, os valores de produção de kefirano foram promissores. A concentração mais alta de kefirano, 1,190 g/L, foi atingida na fermentação em fed-batch da amostra de soro de leite CWC, que durou 510 h, a 80 rpm, enquanto que o valor mais alto de produtividade de kefirano, 0,224 g/L.dia, foi alcançado na fermentação em batch da amostra de soro de leite CWCD, depois de 50 h, a 80 rpm.

Ao caracterizar as amostras de kefirano produzidas com os diferentes meios, uma hidrólise ácida confirmou o rácio de glucose para galactose de aproximadamente 1:1 e, juntamente com a análise por espectroscopia de infravermelhos com transformadas de Fourier (FTIR), também confirmou que o kefirano foi produzido com sucesso neste estudo. Adicionalmente, a análise por microscopia eletrónica de varrimento (SEM) revelou uma estrutura tipo espuma com um elevado nível de porosidade, o que constitui uma característica muito desejável para a produção de scaffolds de kefirano.

Este estudo demonstrou que é possível produzir kefirano a partir de fontes de carbono de baixo custo, como o soro de leite, com resultados bastante sólidos, apesar de ainda ser necessário estudar o aumento de escala do processo, de modo a tornar possível uma eventual implementação industrial.

keywords

Kefiran, kefir grains, cheese whey, exopolysaccharide, *Lactobacillus kefiranofaciens*, microbial fermentation, process optimisation, downstream processing, EPS characterisation.

abstract

Cheese whey is the main by-product of dairy industries, which is massively produced worldwide. Its disposal may represent a serious environmental issue due to its high organic content, therefore, it is of interest to find more solutions for its valorisation. An interesting approach is the conversion of its lactose into value-added products through microbial fermentation. Kefiran is an exopolysaccharide with approximately equal amounts of D-glucose and D-galactose, whose remarkable properties make it possible to be of use in a wide range of applications in the medicine, pharmaceutical and food industries. This polymer can be produced during fermentation, with the use of kefir grains, a complex microbial community embedded within a kefiran matrix, containing several species of lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeasts, living in symbiose. Nevertheless, the bacterium *Lactobacillus kefiranofaciens* is considered to be the main responsible for kefiran production.

The objective of this work was the study of kefiran production, by kefir grains, in cow's cheese whey (CWC), diluted cow's cheese whey (CWCD), sheep's cheese whey (CWS) and in synthetic medium (MRSL broth). In this study, fermentations in batch and fed-batch mode were performed.

Initially, MRSL broth medium was used to optimise the process. A fermentation time of 100 h was found to be optimal, since it led to the highest kefiran productivity value. The ideal stirring speed was concluded to be 80 rpm, as it provided the best balance between kefiran production and kefir grains' conservation. Relatively to the optimal initial lactose concentration, results were dependant on the stirring speed, with no noticeable inhibition by substrate occurring. The best results in terms of kefiran concentration, 1.640 g/L, and productivity, 0.390 g/L.day, were both reached after 100 h of fermentation, using a MRSL broth medium containing 40 g/L of lactose, at 160 rpm.

Regarding cheese whey, kefiran production values were promising. The highest concentration of kefiran, 1.190 g/L, was attained in the fed-batch fermentation of the cheese whey sample CWC, which lasted for 510 h, at 80 rpm, while the highest kefiran productivity value, 0.224 g/L.day, was achieved in the batch fermentation of the cheese whey sample CWCD, after 50 h, at 80 rpm.

When characterising the kefiran samples produced with the different media, an acid hydrolysis confirmed a glucose to galactose ratio of approximately 1:1 and, together with the analysis by Fourier-transform infrared (FTIR) spectroscopy, also confirmed that kefiran was successfully produced in this study. Additionally, the analysis by scanning electron microscopy (SEM) revealed a foam-like structure with a high porosity level, which is a very desirable characteristic for the production of kefiran scaffolds.

This study showed that it is possible to produce kefiran with the use of a low-cost carbon source, such as cheese whey, with fairly solid results, even though the process scale-up still needs to be addressed to enable an eventual industrial implementation.

Index

List of Figures

List of Tables

List of Abbreviations

AAB	Acetic acid bacteria
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
CWC	Cheese whey sample originated from cow milk
CWCD	Diluted cheese whey sample originated from cow milk
CWS	Cheese whey sample originated from sheep milk
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
EPS	Exopolysaccharide
FTIR	Fourier-transform infrared
GPC-SEC	Gel permeation chromatography – Size exclusion chromatography
GRAS	Generally Recognised As Safe
h	Hours
HPLC	High performance liquid chromatography
IEA	International Energy Agency
LAB	Lactic acid bacteria
MRS	Man-Rogosa-Sharpe
MRSL	Man-Rogosa-Sharpe with Lactose
N/A	Not applicable
PCR-DGGE	Polymerase chain reaction – Denaturing gradient gel electrophoresis
RAPD-PCR	Random amplified polymorphic DNA – Polymerase chain reaction
NMR	Nuclear magnetic resonance
$r_{kefiran}$	Kefiran volumetric production rate
$r_{lactic\ acid}$	Lactic acid volumetric production rate
rpm	Rotations per minute
$r_{substrate}$	Substrate volumetric consumption rate
SEM	Scanning electron microscopy
STA	Simultaneous thermal analyser

TGA	Thermogravimetric analysis
UHT	Ultra-high temperature
WPC	Whey protein concentrate
XPS	X-ray photoelectron spectroscopy
$Y_{kefiran/substrate}$	Kefiran yield on substrate
$Y_{lactic\ acid/substrate}$	Lactic acid yield on substrate
YM	Yeast extract-Malt extract

1. Introduction

1.1 Background

In the current days, environmental issues attract a lot of attention from the general population, which prevents industries from functioning without ecological concerns. However, one major environmental problem still resides in the disposal of the industrial wastes produced by some manufacturing processes. These by-products are inevitably generated during the production of the intended main product and are frequently viewed as waste with a low range of applications. However, they cannot be simply discarded to the environment, due to elevated organic matter contents, which are shown by the high COD (chemical oxygen demand) and BOD (biochemical oxygen demand) levels, making them important pollutants (Guimarães et al., 2010).

Some examples of these industrial by-products are the molasses produced during the processing of sugar cane and sugar beet into refined sugar; the paper sludge that is produced by papermaking industries; the wastewater that originates from fruit processing in food manufacturing industries; and cheese whey, which is the main by-product from the production of cheese and is produced in very high quantities (Sze and Lin, 2014).

Cheese whey results from the precipitation and removal of milk casein during the cheesemaking process and is a major pollutant, since it retains a very high concentration of lactose (Guimarães et al., 2010), therefore requiring new industrial applications, in order to reduce its disposal problem. One interesting approach is the conversion of lactose from cheese whey into value-added products, like kefiran, trough bacterial or yeast fermentation (Yadav et al., 2015).

Kefiran is an exopolysaccharide (EPS), with approximately equal amounts of D-glucose and D-galactose, that shows incredible features, such as antibacterial and antifungal properties, granting it a wide range of applications in the food and medical fields. The production of kefiran is mainly associated to the bacteria *Lactobacillus kefiranofaciens* and its presence in kefir grains (Radhouani et al., 2018a).

1.2 Objectives

The main objective of this work was to study the use of cheese whey as the substrate to produce kefiran by fermentation with kefir grains. Several cheese whey samples were tested, such as two versions of cow's cheese whey and one of sheep's cheese whey, in both batch and fed-batch fermentation modes. In order to achieve this main goal, several specific objectives were considered, as described below.

Initially, a stage of process optimisation was carried out in batch, using MRSL broth (a modified MRS (Man-Rogosa-Sharpe) broth, with lactose instead of glucose in its composition) as the synthetic medium. In this stage, the effect of different operational conditions, such as fermentation time, initial concentration of lactose and stirring speed, were tested.

After fermentation processes, downstream processing operations were performed and, afterwards, the obtained samples were examined with the use of analytical techniques such as Fourier-transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM), in order to check the identity of kefiran and to characterise this exopolysaccharide. An effort to produce kefiran films was also carried out, with the addition of glycerol as plasticiser.

Cheese whey is already used in some applications. A common practice is to apply filtration and drying processes, in order to produce whey powders and whey protein concentrates that are widely used in the food and pharmaceutical industries. With the production of kefiran, this work aimed to give cheese whey a different, but equally relevant, application.

2. State of the Art

This chapter intends to review a literature focused on kefiran production, use of cheese whey as substrate, and related topics.

2.1 Cheese whey

Cheese whey is the main by-product originated in the dairy industries, and results from the transformation of milk into cheese, more precisely from the process of agglomeration of casein micelles (Lappa et al., 2019). This by-product represents 85% to 90% of the milk volume, and while it is frequently seen as a waste product, it still retains around 55% of the milk nutrients, like lactose, soluble proteins, lipids, salts and minerals (Londero et al., 2011).

Dairy and cheese processing plants continuously produce several types of cheese, generating huge volumes of cheese whey. In 2013, the worldwide production of this by-product was estimated to be between 180 and 190 million tons per year and only about 50% of it was processed afterwards (Mollea et al., 2013). In order to produce 1 kg of cheese, 9 kg of cheese whey are simultaneously generated (Siso, 1996). These enormous volumes combined with its elevated COD and BOD, which are associated with its high organic matter content, make cheese whey a major pollutant (Pescuma et al., 2015).

Cheese whey is a liquid with a greenish-yellow colour, and can be classified according to the procedure used for casein precipitation: acid cheese whey (pH<5), which is the one obtained after fermentation or from addition of organic or mineral acids, or sweet cheese whey (6<pH<7), which results from the addition of proteases, such as chymosin (Carvalho et al., 2013).

In the recent years, and due to the rising environmental concerns, as well as to governmental regulations, cheese manufacturing plants must include a step of waste recovery and treatment in the process. In 2011, Londero *et al*. reported that, at larger plants, cheese whey was dried to produce whey powder or fractioned into different components by membrane processes. At smaller plants, however, due to the expensive equipment required to carry out these procedures, cheese whey was generally used as animal feed or fertiliser. Although, in some cases, it was still discarded without any treatment (Londero et al., 2011).

2.1.1 Cheese whey composition

Cheese whey is mostly composed of water, with its organic portion being constituted by lactose, proteins, minerals, lactic acid and lipids (Ramos, 2016). However, the composition varies significantly depending on the milk origin and quality and on the applied cheesemaking technologies (Matijević et al., 2011). For example, differences can be observed in the concentration of salts, lactic acid and lactose between bovine and ovine cheese whey (Londero et al., 2011). Cheese whey composition also depends on the feed supplied to the milk-producing animals, the season of the year, the time of lactation and the type of cheese produced (Carvalho et al., 2013; Panesar et al., 2007; Pescuma et al., 2015). Differences are also to be expected between sweet and acid whey, namely in respect of lactate, which affects the pH of the respective whey, as observed in **Table 1** (Kosseva et al., 2009; Yadav et al., 2015)**.**

In addition, some cheese making plants choose to concentrate the whey in order to minimise storage issues, namely bovine cheese whey, which is produced in very high quantities. Usually, cheese whey is concentrated around three times its initial solid concentration (Ferrari et al., 1993).

Table 1. Typical composition of sweet and acid cheese whey (Kosseva et al., 2009; Yadav et al., 2015).

Component	Sweet cheese whey (g/L)	Acid cheese whey (g/L)
Total solids	63.0-70.0	63.0-70.0
Lactose	46.0-52.0	44.0-46.0
Proteins	6.0-10.0	6.0-8.0
Lipids	5.0	0.4
Lactate	2.0	6.4
Ash	5.0	8.0
Calcium	0.4-0.6	1.2-1.6
Phosphate	1.0-3.0	2.0-4.5
Chloride	1.1	1.1

2.1.2 Cheese whey pre-treatment

Lactose present in cheese whey can be used as a carbon source for the fermentation with microorganisms that are able to metabolise it. Although, previously, it is recommended to do a pre-treatment step in order to remove the proteins and lipids that are also found in the whey (Bosco et al., 2018). After this step, whey is frequently referred to as deproteinised cheese whey (Roukas and Kotzekidou, 1998).

Since proteins represent a valuable component of whey, it is of interest to recover them during the pre-treatment step, which can be achieved by means of thermo-calcic precipitation, followed by microfiltration. These proteins can be used to produce whey protein concentrate (WPC), as one of several applications, contributing to its further valorisation (Bosco et al., 2018).

2.1.3 Cheese whey applications

Cheese whey has already been used in a wide range of applications. For example, it can go through a tangential ultrafiltration, retaining proteins but not lactose and salts, essentially turning the whey into a lactose solution rich in salts (Bosco et al., 2018). Afterwards, whey permeate can be used, whether concentrated or not, as a substrate for fermentation, to produce several value-added products, like ethanol (Koushki et al., 2012), butanol, through ABE fermentation (Qureshi et al., 2014), lactic acid (Panesar et al., 2007), citric acid (El-Holi and Al-Delaimy, 2004), succinic acid (Wan et al., 2008), hydrogen (Davila-Vazquez et al., 2009), methane (Yadav et al., 2015), biogas (Gelegenis et al., 2007), single-cell protein (Paraskevopoulou et al., 2003), biosurfactant (Joshi et al., 2008) and polymers, such as kefiran (Blandón et al., 2018; Cheirsilp and Radchabut, 2011; Ghasemlou et al., 2012) or polyhydroxyalkanoates (Colombo et al., 2019).

The production of a whey powder rich in proteins is another economically relevant application. With the development of membrane filtration methods, it is possible to separate and concentrate the whey protein fraction while retaining its integrity. By doing successive filtrations, protein fractions of different compositions and degrees of purity can be achieved. Then, by applying a spray-drying step, these protein fractions result in a dry product, known as WPC. The protein content of a WPC varies, depending on the cheese whey used in its production. Bovine whey is the most relevant in terms of production volume, and a WPC

from this origin generally contains β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin, lactoferrin and lactoperoxidase, with an approximate concentration of 50%, 20%, 10%, 10%, 3%, and 0.3%, respectively. WPC are becoming increasingly used in pharmaceutical and dietary supplement food markets (Ramos, 2016).

2.2 Biorefinery

Currently, the massive dependence on fossil fuels and the strong demand for products that originate from them, still represents a very serious environmental and socio-political problem. Fossil fuels are limited, since they require a large amount of time to be replenished, so a more sustainable option is needed to answer the population needs (Yuan and Macquarrie, 2015).

The concept of biorefinery has been gaining relevance over the years and its usage in scientific publications keeps increasing. According to the International Energy Agency (IEA), a biorefinery consists in the processing of biomass, by sustainable means, resulting in a range of marketable products and energy (González-Delgado and Kafarov, 2011). This concept is vital for the development of alternative routes for the production of important materials using renewable resources (Yuan and Macquarrie, 2015). Thus, the main goal of a biorefinery is to convert feedstock's sugars, proteins, lipids and lignins, into the value-added products, such as biopolymers like kefiran, through biotechnological and chemical processes. A biorefinery differs substantially from a conventional petroleum refinery, starting with the fact that it uses biomass instead of petroleum, both having a complex composition. Whereas a biorefinery can surely present more advantages than disadvantages when compared with a petrochemical refinery, in some cases it is still harder to implement, due to the limited technology and infrastructures available (Kamm and Kamm, 2007).

Biorefineries may face a serious problematic if using edible biomass, entering in the issue of "feed vs fuel". The use of expensive edible biomass is not only costly to begin with, but it also leads to the depletion of available food, thus increasing its price even more. This is a severe problem, mainly in poorer countries and densely populated regions, where it could lead to some harsh social problems. Therefore, a biorefinery should ideally focus on the use of non-edible biomass, such as industrial by-products (Maity, 2015).

2.2.1 Integrating a biorefinery in a cheesemaking factory plant

Since cheese whey is one of the most produced and polluting by-products of the food industry, it is of interest for a cheesemaking factory to implement a biorefinery into its infrastructure, reducing its impact on the environment. It is of high importance for the integrated biorefinery to focus on a wide range of viable end-products, that can stand as functional foods or components on their own, in order to maximise its cost-effectiveness (Lappa et al., 2019).

When designing an integrated biorefinery, it is crucial to take some points into consideration. The ideal scenario would be for the biomass source to be as close as possible to the biorefinery, to ease the logistic aspect and to avoid a high transportation cost (Abdullah et al., 2016; Maity, 2015). Following the same logic of enhancing the overall process, it is also important for the biorefinery to maximise the energy and material recovery (Goh and Ng, 2015). For example, as water is used intensively in cottage cheese production (Carvalho et al., 2013), a water recovery system should be considered, in order to recycle it and, therefore, to reduce its overall consumption (Goh and Ng, 2015).

As an example of a cheese whey-based integrated biorefinery, Chandra *et al*. 2018 proposed a model for its valorisation, together with a second dairy waste, cattle dung. Cheese whey can be used in two distinct ways: by direct conversion of lactose to added-value products, e.g. lactic acid; or after a first hydrolysis into glucose and galactose. These monosaccharides can be used via alcoholic fermentation, to produce ethanol or in an anaerobic digestion, to produce hydrogen or methane. On the other hand, cattle dung may be used directly as a biofertilizer or it can also go to an anaerobic digester in order to produce biomethane and biocompost (Chandra et al., 2018).

2.2.2 Circular economy

The notion of linear economy refers to the production, use and discard of a product, repeating this process indefinitely. By other words, it follows a mentality of "buy-and-throw-away", which exploits natural resources indiscriminately and, since nor recycling nor reusing concepts are present here, it accumulates residues and wastes in the environment until worrying levels are reached (Sørensen, 2018).

Circular economy is a somewhat recent concept, that opposes linear economy, as seen in **Figure 1** (Sauvé et al., 2016). Although the definitions found in the literature are rather ambiguous (García-Barragán et al., 2019), the idea behind a circular economy, as the name suggests, is about promoting a circular flow of materials in order to reduce environmental impacts and maximise resource efficiency. Thus, it is essential to promote material recycling and product reuse (Moreau et al., 2017).

The traditional 3R's of a sustainable economy (reuse, recycle and reduce) were enlarged to ten with the circular economy ideal: recycle, reuse, recover, reduce, repurpose, refurbish, refuse, repair, remanufacture and rethink (Morseletto, 2020). Such approaches should be considered when designing an integrated biorefinery in a cheesemaking factory plant, for example, the reuse of industrial wastes to manufacture new value-added products and the recycling of water used in the process, making it possible for the biorefinery to follow the circular economy concept (Chandra et al., 2018).

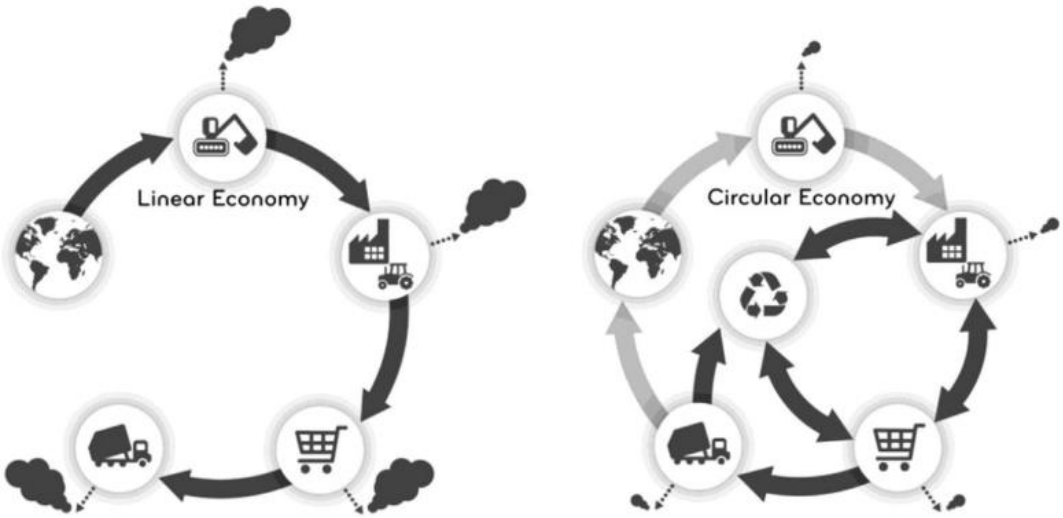

Figure 1. The concept of linear and circular economy (Sauvé et al., 2016).

2.3 Kefir grains

Milk kefir grains consist of white or yellowish irregular grains that resemble small cauliflowers and vary in size between several mm to a few cm (**Figure 2.A**). These contain a complex microbial community composed of many different species of lactic acid bacteria

(LAB), acetic acid bacteria (AAB), and yeasts, living in symbiose, embedded within a polysaccharide matrix known as kefiran (Nielsen and Gu, 2014). Bacteria from the *Lactobacillus* genus are predominant in kefir grains and are easily recognisable in a scanning electron micrograph, due to its long and rod-shaped structure, as observed in **Figure 2.B** (Schoevers and Britz, 2003).

If the grains are kept healthy and under favourable conditions, like when inoculated into milk that is changed daily, a significant increase in their biomass is expected (Garrote et al., 2001).

While milk kefir grains are the ones of interest for kefiran production, there is another type of kefir grain known as water kefir grains. These, while much less popular and studied than their milk counterpart, can also be used in the fermentation of sugary water (water with sucrose), resulting in a beverage known as water kefir. Water kefir grains are similar in appearance to milk kefir grains, except that they are much more translucent (Marsh et al., 2013). Another major difference between the two types of grains results from the different EPS where microorganisms are embedded. While milk kefir grains are embedded in kefiran, in water kefir grains this function is carried out by dextran, a polysaccharide composed of linked glucose units (Fels et al., 2018). Considering the main objective of the present study, which is the production of kefiran, only milk kefir grains will be considered. From now on, milk kefir grains will be designated as kefir grains.

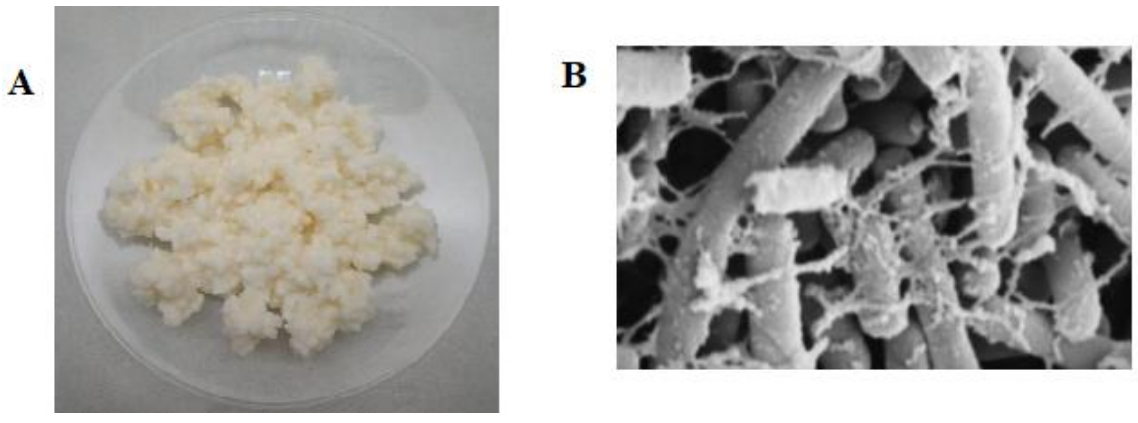

Figure 2. Usual appearance of milk kefir grains. Exterior and macroscopic photograph (**A**); Scanning electron micrograph (22000×) (**B**) (Schoevers and Britz, 2003).

2.3.1 Microbial composition

The microbial community composition, as well as other factors such as kefiran content, can vary significantly depending on the origin, culture conditions or storage processes of the kefir grains (Garrote et al., 2001). Even so, it is widely accepted that the most commonly found microorganisms in grains are bacteria from the *Lactobacillus* genus, such as *L. kefiranofaciens* and *L. kefiri*. Relatively to the fungi species, yeasts like *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* are frequently reported as present in kefir grains (Plessas et al., 2017; Vardjan et al., 2018).

In **Table 2**, the most common species of bacteria and yeasts are shown, although, the number of different microbial species living inside kefir grains is estimated to be more than 300 (Reis et al., 2017).

Table 2. Most common species of bacteria and yeasts found in kefir grains.

	Species	**Reference**
Bacteria	*Lactobacillus kefiranofaciens*	(Machado et al., 2013); (Vardjan et al., 2013); (Walsh et al., 2016); (Reis et al., 2017)
	Lactobacillus kefiri	(Machado et al., 2013); (Vardjan et al., 2013); (Reis et al., 2017)
	Lactobacillus parakefiri	(Machado et al., 2013); (Vardjan et al., 2013)
	Lactobacillus delbrueckii	(Machado et al., 2013); (Vardjan et al., 2013)
	Lactobacillus acidophilus	(Machado et al., 2013); (Vardjan et al., 2013)
	Lactobacillus fermentum	(Machado et al., 2013); (Vardjan et al., 2013)
	Lactobacillus brevis	(Machado et al., 2013)
	Lactobacillus helveticus	(Vardjan et al., 2013); (Walsh et al., 2016)
	Lactobacillus casei	(Vardjan et al., 2013)
	Lactobacillus paracasei	(Vardjan et al., 2013)
	Lactobacillus plantarum	(Vardjan et al., 2013)
	Lactobacillus gasseri	(Vardjan et al., 2013)

	Lactococcus lactis	(Machado et al., 2013)
	Streptococcus thermophilus	(Machado et al., 2013)
	Leuconostoc mesenteroides	(Machado et al., 2013); (Walsh et al., 2016)
	Leuconostoc lactis	(Gao et al., 2012)
	Bifidobacterium bifidum	(Taş et al., 2012)
	Acetobacter fabarum	(Gao et al., 2012)
	Acetobacter pasteurianus	(Walsh et al., 2016)
	Bacillus subtilis	(Gao et al., 2012)
Yeasts	*Saccharomyces cerevisiae*	(Machado et al., 2013); (Diosma et al., 2014); (Gao et al., 2012)
	Saccharomyces unisporus	(Diosma et al., 2014)
	Saccharomyces turicensis	(Machado et al., 2013)
	Kluyveromyces marxianus	(Machado et al., 2013); (Diosma et al., 2014); (Vardjan et al., 2013)
	Kluyveromyces lactis	(Machado et al., 2013); (Vardjan et al., 2013)
	Rhodosporidium kratochvilovae	(Vardjan et al., 2013)
	Debaryomyces hansenii	(Machado et al., 2013)
	Debaryomyces occidentalis	(Machado et al., 2013)
	Dekkera anomala	(Machado et al., 2013)
	Torulaspora delbrueckii	(Machado et al., 2013); (Vardjan et al., 2013)
	Pichia fermentans	(Machado et al., 2013); (Vardjan et al., 2013)
	Pichia kudriavzevii	(Gao et al., 2012)
	Kazachstania unispora	(Machado et al., 2013); (Vardjan et al., 2013)
	Kazachstania exigua	(Vardjan et al., 2013)
	Issatchenkia occidentalis	(Diosma et al., 2014)

2.3.2 *Lactobacillus kefiranofaciens*

L. kefiranofaciens is one of the main constituents of the microflora that constitute kefir grains, forming colonies (Wang et al., 2011), and is the major responsible for kefiran production (Cheirsilp et al., 2018; Irigoyen et al., 2005; Zajšek et al., 2013).

This species is described as a gram-positive, homofermentative, capsulated, non-spore-forming, non-motile, catalase-negative, long rod-shaped lactic acid bacterium. It can produce lactic acid from lactose, glucose, galactose, fructose, sucrose, maltose, mannose, raffinose and melibiose, but not from xylose, arabinose, ribose, rhamnose, melezitose, cellobiose, trehalose, sorbitol, mannitol, salicin, esculin or amygdalin (Arihara et al., 1990; Fujisawa et al., 1988).

2.3.3 Microbial Fermentation

Both types of fermentation of lactose occur when inoculating kefir grains into the medium, namely lactic acid fermentation, carried out by lactic acid bacteria and alcohol fermentation, performed by yeasts (Altay et al., 2013; Machado et al., 2013). Lactic acid is massively produced during the fermentation of lactose, but the production of acetic acid by AAB can also be observed (Matijević et al., 2011). Consequently, due to the production of these acids, the medium suffers acidification, with the pH decreasing from around 6.5 (average initial cheese whey pH), to 3.5, at the end of fermentation (Londero et al., 2011).

Using the enzyme β-galactosidase, LAB initiate the fermentation process with the hydrolysis of the lactose present in cheese whey, breaking it down into glucose and galactose, which are both hexoses (Matijević et al., 2011). Then, the galactose that results from this hydrolysis is employed by the kefir grains microflora in the production of kefiran, in order to allow both grain growth and the formation of new grains (Irigoyen et al., 2005).

The production and consumption rates of glucose and galactose by *L. kefiranofaciens* are deeply related to the concentration of lactose in the medium: the production rates are directly proportional, while the consumption rates are inversely proportional to the lactose concentration (Cheirsilp et al., 2001).

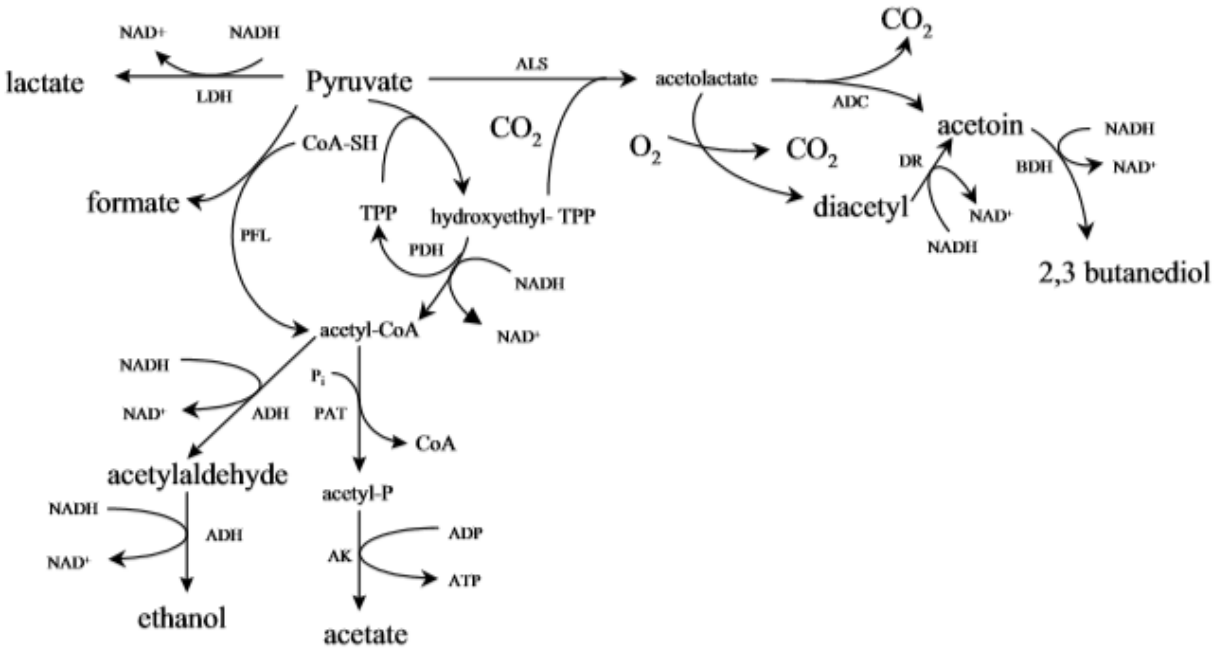

Figure 3. Metabolic pathways involved in pyruvate catabolism (Tamime et al., 2006).

Lactose fermentation by LAB can be rather time-consuming, and some studies tested a previous step of hydrolysis, successfully reducing the duration of this process (Matijević et al., 2011). This step also provides substrate for lactose-negative microorganisms present in kefir grains (Guimarães et al., 2010).

As the microflora living in kefir grains can produce several components through the fermentation process, the metabolic pathways are shown in **Figure 3** (Tamime et al., 2006). After lactose hydrolysis, LAB metabolise glucose and galactose through the glycolysis process, producing pyruvate, that is later catalysed by lactate dehydrogenase to lactic acid (Basso et al., 2014). In contrast, yeasts dissimilate the hexoses, also via glycolysis, originating pyruvate, but in this case it will be decarboxylated by pyruvate decarboxylase into CO_2 and acetaldehyde, which is catalysed to ethanol by alcohol dehydrogenase (Wszolek et al., 2006).

2.3.4 Kefir

Kefir is a dairy beverage similar to yoghurt, produced through milk fermentation (Nielsen and Gu, 2014). This is carried out by kefir grains that are inoculated into fresh milk,

converting the lactose into lactic acid and ethanol (Kourkoutas et al., 2002; Matijević et al., 2011).

Kefir is believed to have its origin in the Caucasus mountains, located in central Asia, and currently shows a growing worldwide acceptance (Nielsen and Gu, 2014; Reis et al., 2017). The name “kefir” most likely derives from the Turkish word “keyif”, which can be translated to “good feeling”. Due to the acidification that occurs during fermentation, kefir is an acidic, viscous, slightly alcoholic and effervescent beverage. Kefir can be made using any type of milk, with cow’s milk being the most frequently used. Nonetheless, milks with a higher amount of fat usually lead to a kefir with better organoleptic properties (Nielsen and Gu, 2014).

One of the major points that raised the interest in kefir was the fact that it is considered a probiotic beverage. Some of the microflora living in the grains is also present in kefir, and these are correlated with improvements in human health, as some studies suggest (Prado et al., 2015). These may include an improvement to lactose tolerance and digestion, an anti-inflammatory effect and an enhanced antioxidant activity (Reis et al., 2017).

After fermentation, the grains are filtered out of the finished kefir and are ready to be used again in another batch (Nielsen and Gu, 2014). Following successive fermentations, kefir grains can propagate by dividing into new grains, which will grow and have the same characteristics as the grains they originated from (Prado et al., 2015).

2.4 Kefiran

Polysaccharides are macromolecular compounds commonly found among living organisms. Microbial polysaccharides are produced by bacteria and fungi during metabolic processes and are often associated with structural and defensive functions, such as antiviral properties (Chen and Huang, 2018). Some polysaccharides are secreted by the cells to the environment medium and are, therefore, known as extracellular polymeric substances or exopolysaccharides (Staudt et al., 2004).

In recent years, polysaccharides produced by LAB have received great attention, mainly due to being classified as Generally Recognised As Safe (GRAS). This makes them very attractive regarding their application in food and medical industries. Moreover, the

growing demand for natural products and health-related concerns has pushed the interest in these compounds even further (Gradova et al., 2015).

Kefiran is a water-soluble exopolysaccharide produced by the LAB present in kefir grains, namely by homofermentative lactobacillus species such as *L. kefiranofaciens* and *L. kefir* (Irigoyen et al., 2005). Almost half of the dry kefir grain weight usually correspond to kefiran (Exarhopoulos et al., 2018a), and it consists of a resilient matrix that embeds the bacteria and fungi living inside kefir grains (Nielsen and Gu, 2014). This polymer is composed of approximately equal amounts of D-glucose and D-galactose (Zajšek et al., 2013), as can be seen in its chemical structure, illustrated in **Figure 4**. Its backbone chain is composed of (1 → 6)-linked Glc, (1 → 3)-linked Gal, (1 → 4)-linked Gal, (1 → 4)-linked Glc and (1 → 2, 6)-linked Gal, with a branch attached to O-2 of Gal residues, finishing with Glc residues (Ghasemlou et al., 2012).

During the fermentation process, a part of the produced kefiran is excreted to the environmental medium and, therefore, it is known as broth kefiran. On the other hand, kefiran is also found within the cells, where it is applied to maintain their structure (Cheirsilp, 2006; Cheirsilp et al., 2001). In kefir grains, kefiran is responsible for the microflora's embedment (Nielsen and Gu, 2014) and for the development of new grains (Irigoyen et al., 2005). In the case of *L. kefiranofaciens*, this polymer forms a capsule that surrounds the cells, known as capsular kefiran. If the objective is to quantify the total kefiran produced during fermentation, or to maximise its production, it is necessary to take into consideration both broth and capsular kefiran (Cheirsilp, 2006).

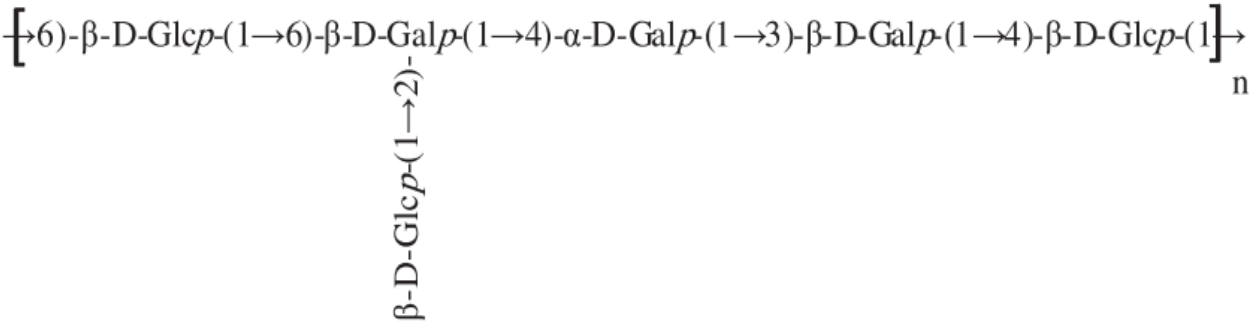

Figure 4. Chemical structure of kefiran (Ghasemlou et al., 2012).

2.4.1 Kefiran production

Kefiran was first described by La Rivière *et al*. in 1967. However, they could not find the main responsible for the EPS production, since the various studies with isolated microorganisms failed to produce kefiran. Therefore, they concluded that, while the stronger candidate was thought to be *L. brevis*, none of the isolated microorganisms from the kefir grains population could be considered, with certainty, to be the responsible for the production of the EPS (la Riviére et al., 1967). Nowadays, and for some time now, kefiran production has been mainly associated to the bacterium *L. kefiranofaciens*, present in kefir grains (Cheirsilp et al., 2018; Irigoyen et al., 2005; Zajšek et al., 2013).

Kefiran can be produced by fermentation with different microbial cultures, such as kefir grains (mixed culture) (Rimada and Abraham, 2001), *L. kefiranofaciens* (pure culture) (Dailin et al., 2014) and *L. kefiranofaciens* with *S. cerevisiae* (co-culture) (Cheirsilp and Radchabut, 2011), and by using different substrates, like milk (Chen et al., 2015), cheese whey (Blandón et al., 2018), starch (Yeesang et al., 2008), and synthetic media such as modified MRS broth, where glucose is replaced by lactose (Cheirsilp et al., 2018; Cheirsilp and Radchabut, 2011; Taniguchi et al., 2001).

2.4.2 Kefiran production from cheese whey

The production of kefiran from industrial by-products, such as cheese whey, can contribute to reduce the disposal problem of the huge quantities of cheese whey produced. It can also decrease the production cost due to the lower price of cheese whey when compared to other available substrates (Guimarães et al., 2010). Although cheese whey can be used, to this end, without further supplementation, some studies also supplement the fermentation medium with additional carbon sources, such as glucose, galactose or lactose (Blandón et al., 2018).

There are several works in the literature referring the production of kefiran from cheese whey, either using a mixed, a pure or a co-culture, as summarised in **Table 3**. A study of kefiran production by kefir grains, published in 2003, by Rimada and Abraham, used both UHT skim milk that suffered a heat treatment, and deproteinised cheese whey, provided the best results of 0.225 g/L and 0.266 g/L, respectively. These lower results may be explained by the loss of kefiran during the isolation steps (Rimada and Abraham, 2003). Another study

published in 2011, by Cheirsilp and Radchabut, used a pure culture of *L. kefiranofaciens* to ferment a modified MRS broth medium, containing whey lactose, attained a kefiran concentration of 0.568 g/L. However, a higher concentration, 0.938 g/L, was reached with a co-culture of *L. kefiranofaciens* with *S. cerevisiae*, under microaerobic conditions, which can be explained by the yeast's capacity of consuming lactic acid, reducing its inhibiting potential. Moreover, in this study, the maximum kefiran concentration, 3.250 g/L, was reached when scaling up the co-culture in a 2 L bioreactor, in fed-batch mode, while maintaining dissolved oxygen at 5% and pH at 5.5 (Cheirsilp and Radchabut, 2011). A different study, published by Cheirsilp *et al*., in 2018, used a pure culture of *L. kefiranofaciens* in the fermentation of a modified MRS broth medium, containing whey lactose, to co-produce kefiran and lactic acid. With pH control, to reduce the inhibition by lactic acid, authors attained a kefiran concentration of 1.693 g/L. In fed-batch mode, with an intermittent addition of whey lactose, this value reached 2.514 g/L of kefiran, while also producing 135 g/L of lactic acid (Cheirsilp et al., 2018). Finally, the studies published by Blandón *et al*., in 2018, and by Ghasemlou *et al*., in 2012, are discussed in **Chapter 2.4.3**.

Table 3. Kefiran production values, using several cultures and media, in different conditions.

Culture	Medium	pH control	Kefiran production	Reference
Kefir grains	UHT skim milk	No	0.225 g/L	(Rimada and Abraham, 2003)
Kefir grains	Deproteinised cheese whey	No	0.266 g/L	(Rimada and Abraham, 2003)
Kefir grains	Supplemented cheese whey	No	0.370 g/L	(Blandón et al., 2018)
Kefir grains	Supplemented cheese whey	Yes	0.700 g/L	(Ghasemlou et al., 2012)
L. kefiranofaciens	MRS broth with whey lactose	No	0.568 g/L	(Cheirsilp and Radchabut, 2011)
L. kefiranofaciens	MRS broth with whey lactose	Yes	1.693 g/L	(Cheirsilp et al., 2018)
L. kefiranofaciens and *S. cerevisiae*	MRS broth with whey lactose	Yes	3.250 g/L	(Cheirsilp and Radchabut, 2011)

2.4.3 Process optimisation

When optimising kefiran production from cheese whey, the main goals are finding the ideal medium composition, as well as the optimal physicochemical conditions, in order to reach the maximum kefiran productivity and yield values, while maintaining its properties (Blandón et al., 2018).

An optimisation study published in 2012, by Ghasemlou *et al*., determined that the best conditions for this process were a medium with 67 g/L of lactose, supplemented with 13 g/L of yeast extract, pH 5.7 and a temperature of 24 °C. Under the optimal conditions, the model predicted a maximum response of kefiran production of 0.678 g/L, which was slightly lower than the best observed result of 0.700 g/L (Ghasemlou et al., 2012).

A similar study, published by Blandón *et al*., in 2018, found the optimal conditions using cheese whey medium supplemented with 15% (w/w) glucose, a temperature of 30 °C, a fermentation time of 10 h, with no stirring. In these conditions, the process produced 0.20 g/L.h of kefir grains composed of 18.57% (w/w) kefiran, which is the equivalent of a kefiran productivity of 0.037 g/L.h, or a concentration of 0.370 g/L after 10 h. This can be observed in the respective response-surface plot, which relates kefiran productivity with temperature and fermentation time, as seen in **Figure 5** (Blandón et al., 2018).

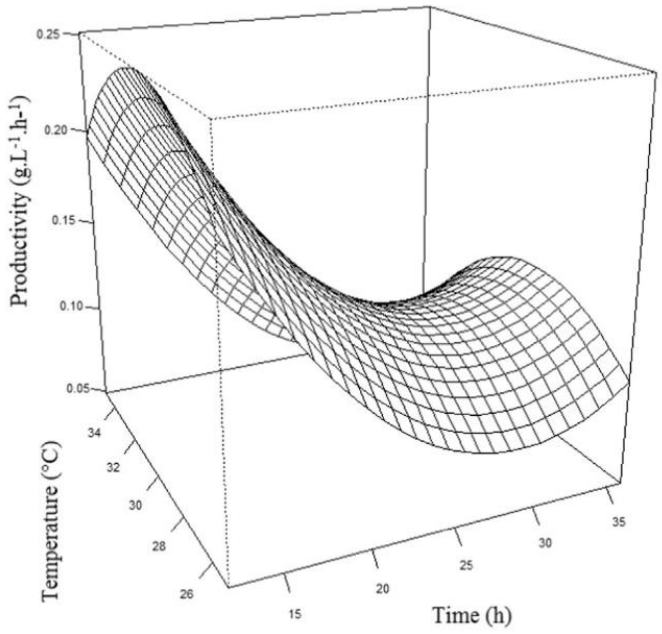

Figure 5. Response-surface plot for kefiran productivity, relating it with temperature and fermentation time (Blandón et al., 2018).

Both studies used a culture of kefir grains to ferment the lactose present in a supplemented cheese whey, in Erlenmeyer flasks, and applied a response-surface model in the process optimisation (Blandón et al., 2018; Ghasemlou et al., 2012).

When studying the production of kefiran using isolated microorganisms instead of kefir grains, one of the best ways to optimise the process was found to use a co-culture of *L. kefiranofaciens* and *S. cerevisiae* (Cheirsilp and Radchabut, 2011). Lactose fermentation produces high amounts of lactic acid, which greatly inhibit the growth of kefiran producing bacteria, leading to a serious drawback. To remove this organic acid, fermentations in continuous mode with separation membranes have been carried out, however, this meaningfully increases the complexity and cost of the process. Therefore, by adding a lactic-acid-consuming yeast, such as *S. cerevisiae*, the lactic acid accumulation issue is significantly reduced, since it will be utilised by the yeast as an energy and carbon source (Cheirsilp and Radchabut, 2011).

2.4.4 Kefiran precipitation, purification and quantification

After fermentation, it is necessary to collect the produced kefiran from the medium and the grains. This is a rather complex process, which include various distinct steps, such as several centrifugations, a dialysis (Rimada and Abraham, 2001) and a lyophilisation (Montesanto et al., 2016). Depending on whether or not the kefir grains are to be reused in the future, there are two main methods to obtain the kefiran produced: one that destroys the grains and, therefore, makes it possible to collect both capsular (intracellular) and broth (extracellular) kefiran, and one that only allows to collect the kefiran released into the medium (extracellular), while allowing to keep the grains intact (Blandón et al., 2018; Ghasemlou et al., 2012; Montesanto et al., 2016; Rimada and Abraham, 2001).

The first method mentioned above, begins with a weighed amount of kefir grains being boiled in distilled water for 15 min, with discontinuous stirring (Blandón et al., 2018) or 30 min (Montesanto et al., 2016). Then, to remove biomass residues, the mixture is centrifuged at 10000g and 20 °C, for 20 min. After this step, in order to precipitate kefiran, two (Montesanto et al., 2016) or three (Blandón et al., 2018) volumes of cold ethanol (-20 °C) are added, and left at 20 °C overnight. At this point, the samples are centrifuged again at 10000g, for 15 mins at 4 °C, and the resulting precipitate is then dissolved in hot distilled

water. The precipitation procedure is repeated twice and, at the end, the samples are freeze-dried (Blandón et al., 2018; Montesanto et al., 2016).

On the other hand, the first step of the second method is the removal of kefir grains from the medium by filtration. Only then the fermented medium is heated in a boiling water bath for 15 minutes, to help dissolving the kefiran and to inactivate the enzymes that could otherwise hydrolyse it. Next, the samples are centrifuged at 10000g, for 15 min at 20 °C, to remove the cells. Then, to precipitate kefiran, two volumes of cold ethanol are added, and the samples are maintained at 4 °C for 24 h. Afterwards, samples are centrifuged at 10000g and 4 °C, for 15 min, and the resulting precipitate is dissolved in hot distilled water. Finally, the samples are dialysed against distilled water for 24 h, at 4 °C, for further purification (Rimada and Abraham, 2001).

There are several variations to these two methods, such as using cold acetone instead of cold ethanol to precipitate kefiran (Zajšek et al., 2013, 2011), or simply a different boiling duration or stirring (Ghasemlou et al., 2012).

After precipitation and purification, a quantification step is required to quantify the produced kefiran. The most obvious and direct one is by means of gravimetrics, where the dry mass of the lyophilised kefiran (**Figure 6.A**) can be measured after 48 h of drying at 37 °C (Zamfir and Grosu-Tudor, 2014). Several methods make use of colorimetric analysis, which is cheap and relatively easy to perform, but can present serious interference issues. Additionally, there are other procedures that quantify total sugars, such as the anthrone method, but these are not ideal, since they do not discriminate contaminating carbohydrates, leading to imprecise quantifications (Enikeev, 2012; Leroy and De Vuyst, 2016).

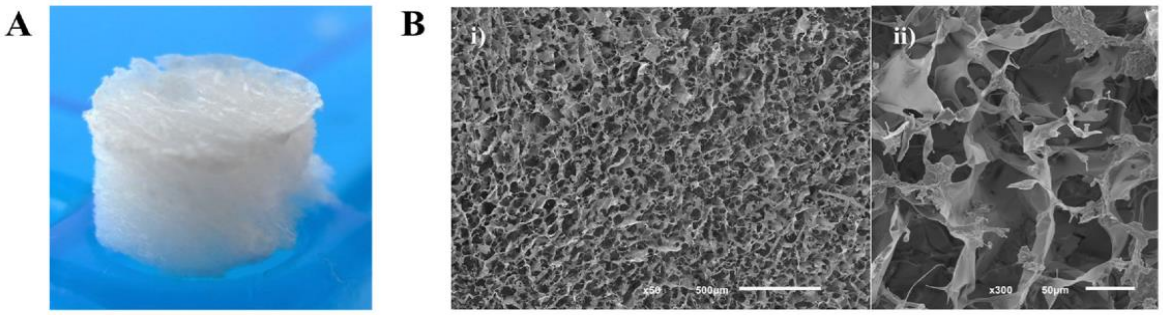

Figure 6. Typical appearance of kefiran after freeze-drying. Macroscopic photograph (**A**); Scanning electron micrograph: i) x50 and ii) x500 (**B**) (Radhouani et al., 2019).

2.4.5 Kefiran characterisation

In order to better understand the characteristics of kefiran and its possible applications, it is vital to do a broad characterisation.

Kefiran is composed of a branched heterosaccharide repeating unit with approximately equal amounts of D-glucose and D-galactose (Zajšek et al., 2013). However, reported studies are not entirely in agreement whether this branching structure contains a hexasaccharide or heptasaccharide repeating unit (Maeda et al., 2004).

Regarding analytical methods, there are several used to evaluate kefiran biological and physicochemical properties, for example, FTIR, gel permeation chromatography - size exclusion chromatography (GPC-SEC), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), nuclear magnetic resonance spectroscopy (NMR), X-ray photoelectron spectroscopy (XPS), simultaneous thermal analyser (STA) analysis, and other procedures, such as rheological, rotational and oscillatory analyses (Radhouani et al., 2018a).

Some reported values for the main characteristics of kefiran are shown in **Table 4**, namely its D-glucose to D-galactose ratio, molecular weight and optical rotation. As expected, the values vary depending on the carbon source used for kefiran production (Maeda et al., 2004).

Table 4. Main characteristics of kefiran, namely D-glucose to D-galactose ratio, molecular weight and optical rotation.

Characteristic		Reference
D-glucose : D-galactose	0.90 : 1.10	(Mukai et al., 1990)
	1.00 : 1.05	(Maeda et al., 2004)
	1.00 : 0.70	(Zajšek et al., 2011)
	1.21 : 1.00	(Piermaría et al., 2016)
	1.00 : 0.90	(Exarhopoulos et al., 2018a)
Molecular weight	10^6 Da	(Mukai et al., 1990)

	10^7 Da	(Piermaria et al., 2008)
	1.35×10^6 Da	(Ghasemlou et al., 2012)
	5.5×10^4 Da	(Ahmed et al., 2013)
	6.71×10^5 Da	(Exarhopoulos et al., 2018a)
Optical rotation	$[\alpha]_D = +68°$	(Kooiman, 1968; la Riviére et al., 1967)
	$[\alpha]_D = +54°$	(Mukai et al., 1990)
	$[\alpha]_D = +64°$	(Ghasemlou et al., 2012; Micheli et al., 1999)

2.4.6 Kefiran applications

Kefiran is a biodegradable and non-toxic polymer, possibly leading to a reduction in the use and discard of synthetic ones, which frequently bring harmful effects to the environment and human health (Moradi and Kalanpour, 2019).

Due to being a biological and GRAS polymer, and as a result of its remarkable features, such as antimicrobial activity, antitumour, antioxidant and anti-inflammatory properties, to name a few (**Table 5**), kefiran has a wide range of applications in the food, pharmaceutical and medicine fields (Moradi and Kalanpour, 2019; Zajšek et al., 2013).

In the food industry, kefiran can serve a very relevant purpose in the form of a functional, food-grade additive, since it has health promoting properties, such as helping to modulate the gut immune-system and protecting cells against the effects of pathogenic extracellular factors (Medrano et al., 2008; Piermaria et al., 2008). Moreover, it also improves rheological properties of the final product, due to the fact that it can act as a thickening and gelling agent or even as a stabiliser for dispersions and solutions, like ice cream and other frozen desserts (Exarhopoulos et al., 2018b; Zavala et al., 2015). There are other polysaccharides that have been widely used as a food addictive, namely xanthan and gellan gum, but these are not suited for all the different applications, thus increasing the need of research for novel polymers (Piermaria et al., 2008).

Furthermore, kefiran can also be used in the production of films for food packaging, with good mechanical and barrier properties, but presenting high permeability to water,

which is undesirable in this type of application. This sensitivity to moisture can limit its usage as a food packaging material, although, the modification of kefiran films with hydrophobic compounds, such as fatty acids, can solve the problem (Moradi and Kalanpour, 2019). Another issue of kefiran films can be their brittleness, however this can be somewhat minimised by adding a plasticiser, like glycerol (15-35% w/w), to the composition, which enhances the flexibility of the films (Ghasemlou et al., 2011; Piermaria et al., 2009).

Nanotechnology is a growing field in many scientific areas, and nanocomposites are used to improve the properties of several materials, very often applied in industrial packaging, electronic, automotive and aerospace industries (Goudarzi and Shahabi-Ghahfarrokhi, 2018; Moradi and Kalanpour, 2019). There are numerous reports of kefiran being included in the production of bio-nanocomposites and nanofibers. In 2018, a study successfully produced kefiran-carboxymethyl cellulose bio-nanocomposite films containing different amounts of copper oxide nanoparticles (0, 1, 1.5 and 2%). An increase in nanoparticles concentration increased the film thickness and improved the barrier property against visible and ultraviolet light transmission, and also enhanced the water vapour barrier and mechanical properties (Hasheminya et al., 2018). Jenab *et al.* 2017 produced kefiran-polyethylene oxide nanofibers, using the electrospinning method. The antimicrobial activity of these nanofibers was tested against different species of microorganisms, such as *Rhizoctonia* sp., *Pseudomonas* sp. and *Staphylococcus aureus*. All assays exhibited promising results, which shows that these nanofibers can be used in food packaging applications to inhibit the growth of microorganisms and reduce food spoilage (Jenab et al., 2017).

Another interesting application of kefiran is its potential use in the medicine field, more precisely in tissue engineering scaffoldings and drug delivering systems (Moradi and Kalanpour, 2019; Radhouani et al., 2019). Polymeric scaffolds are three-dimensional, providing an optimal environment for cell adhesion, growth and differentiation, in addition to allowing an efficient drug delivery to a specific site and, simultaneously, reducing drug concentration at non-target locations. Three-dimensional cryogels can be made by applying a freeze gelation technique to the scaffolds and these consist of a sponge-like matrix filled with interconnected macropores which, thanks to this structure, may be used as a carrier to cells and molecules. Due to the high level of biocompatibility, several polysaccharides have been recently used to this end, including chitosan, dextran and gellan gum. While still not

being so frequently applied in this field as the ones mentioned before, kefiran cryogels seem to be an appealing biomaterial to help regenerate damaged tissues (Radhouani et al., 2019). In the pharmaceutical area, kefiran can find another application when combined with other polysaccharides, like alginate, forming kefiran-alginate gel microspheres, used for the oral delivery of pharmaceuticals, such as ciprofloxacin, one of the most used antibiotics worldwide (Blandón et al., 2016).

Table 5. Main reported properties of kefiran.

Property	Reference
Antibacterial	(Dadashi et al., 2019; Zajšek et al., 2011)
Antifungal	(Moradi and Kalanpour, 2019; Zajšek et al., 2011)
Antitumour	(Toscano et al., 2018; Zajšek et al., 2011)
Antiallergenic	(Jeong et al., 2017)
Anti-inflammatory	(Moradi and Kalanpour, 2019; Radhouani et al., 2018b)
Biodegradable	(Dadashi et al., 2019; Jenab et al., 2017)
Biocompatible	(Dadashi et al., 2019; Toscano et al., 2018)
Water soluble	(Maeda et al., 2004; Wang et al., 2008)
Non-toxic	(Dadashi et al., 2019)
Cholesterol-lower	(Jeong et al., 2017)
Gut immune-system modulator	(Jenab et al., 2017; Moradi and Kalanpour, 2019)
Viscous	(Exarhopoulos et al., 2018b; Piermaria et al., 2008)
Viscoelastic	(Piermaría et al., 2016)
Resistant to enzymatic hydrolysis	(Maeda et al., 2004)
Newtonian fluid in diluted solutions	(Piermaria et al., 2008; Radhouani et al., 2018a)
Able to form cryogels	(Zavala et al., 2015)
Able to form scaffolds	(Montesanto et al., 2016; Toscano et al., 2018)
Able to form films	(Ghasemlou et al., 2011; Piermaria et al., 2009)

3. Materials and Methods

3.1 Microorganisms

3.1.1 Kefir grains

In this study, commercial kefir grains obtained from Kefiralia (Burumart Commerce S.L., Spain), were used to produce kefiran, as a mixed culture. In order to activate the grains, they were inoculated into UHT (ultra-high temperature) whole fat cow milk (Mimosa – Lactogal Produtos Alimentares S.A.), in closed 250 mL glass jars (previously autoclaved for 20 minutes at 121 °C), at room temperature without stirring. To promote the growth, the medium was changed daily. When no further growth was required, grains were maintained at 4 °C, in the same medium, which was changed once per week. The nutritional composition of the UHT milk is shown in **Table 6**.

Table 6. Nutritional composition of the UHT milk as shown in the package.

Component	Concentration (g/L)
Total lipids	36
Saturated lipids	23
Total carbohydrates	49
Sugars	49
Proteins	33
Salt	1

3.2 Substrates

3.2.1 Cheese whey

All cheese whey samples used in this study were provided by the company Lacto Serra – Comercialização e Fabrico de Lacticínios, Lda., located in Aguiar da Beira, Portugal.

Two different cheese whey samples were supplied, one derived from cow milk (CWC), which was concentrated by an ultrafiltration plant, and another from sheep milk (CWS).

Both cheese whey samples were pre-treated, to ensure protein and lipid removal and to minimise the possibility of potential contaminations. This was done according to the procedure of Ahn *et al*. (2000), with some modifications. Initially, cheese whey was autoclaved for 20 minutes at 121 °C (AJC® Uniclave 88). After cooled, the samples were decanted to remove most of the protein aggregates, followed by a centrifugation at 5000 rpm, for 1 h, at 4 °C (Megafuge 16R centrifuge, Thermo Scientific), in sterilised 400 mL centrifuge flasks (Thermo Scientific). Finally, the supernatant was decanted to remove the remaining precipitated fraction and samples were then stored at -20 °C.

Additionally, since CWC was concentrated, a portion of this whey was diluted 50% (CWCD), in order to attain a similar lactose concentration (determined by HPLC) as in CWS. Therefore, three different cheese whey samples were tested, as presented in **Table 7**.

Table 7. Cheese whey samples used in this study, origin, lactose concentration, pH and conductivity values after pre-treatment.

Cheese whey	Origin	Lactose (g/L)	pH	Conductivity
CWC	Cow milk	≈ 80 g/L	5.18	6.92 mS
CWCD	Cow milk	≈ 30 g/L	5.22	2.43 mS
CWS	Sheep milk	≈ 35 g/L	5.67	15.30 mS

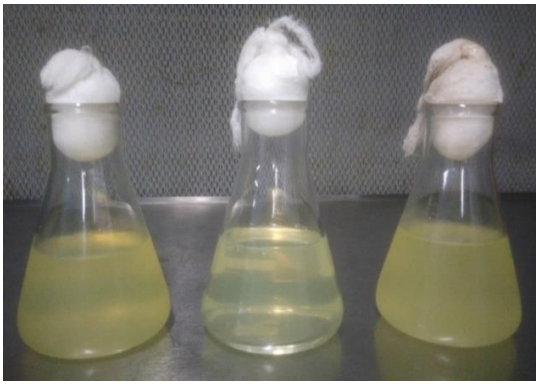

Figure 7. Cheese whey samples used in this study: CWC, CWCD and CWS, respectively.

3.3 Culture media

3.3.1 MRSL broth medium

MRSL broth was used in this study as the synthetic medium, to determine the optimal conditions for kefiran production. Its composition is basically the same as the commercially available MRS broth medium, with the exception of containing lactose instead of glucose. All reagents used and their respective concentration are shown in **Table 8**, with the addition of 0.1% (v/v) of Tween 80 (Sigma-Aldrich) and adjusting the pH to 5.5 ± 0.2. The medium was then sterilised by autoclaving at 121 °C for 20 minutes (AJC® Uniclave 88).

Table 8. MRSL broth medium composition.

Reagent	Chemical formula	Concentration (g/L)
Lactose monohydrate	$C_{12}H_{22}O_{11}.H_2O$	40, 80, 120
Peptone	N/A *	10
Meat extract	N/A *	8
Yeast extract	N/A *	4
Dipotassium hydrogen phosphate	K_2HPO_4	2
Sodium acetate trihydrate	$CH_3COONa.3H_2O$	5
Diammonium citrate **	$C_6H_{14}N_2O_7$	2
Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	0.2
Manganous sulfate tetrahydrate	$MnSO_4.4H_2O$	0.05

* Not applicable

** Diammonium citrate was used instead of triammonium citrate, as indicated for the MRS broth medium.

3.3.2 MRSL, MRS and YM agar media

MRSL agar medium composition was the same as the version of MRSL broth medium containing 40 g/L of lactose, as seen in **Table 8**, including 0.1% (v/v) of Tween 80,

but with the addition of 1% (w/v) of agar. The composition of MRS agar medium can be observed in **Table 9**, with the addition of 0.1% (v/v) of Tween 80. YM agar medium composition is shown in **Table 10**. All culture media had their pH adjusted to 5.5 ± 0.2 and were then sterilised by autoclaving at 121 °C for 20 minutes.

Table 9. MRS agar medium composition.

Reagent	Chemical formula	Concentration (g/L)
Glucose	$C_6H_{12}O_6$	20
Peptone	N/A	10
Meat extract	N/A	8
Yeast extract	N/A	4
Dipotassium hydrogen phosphate	K_2HPO_4	2
Sodium acetate trihydrate	$CH_3COONa.3H_2O$	5
Triammonium citrate	$C_6H_{17}N_3O_7$	2
Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	0.2
Manganous sulfate tetrahydrate	$MnSO_4.4H_2O$	0.05
Agar	N/A	10

Table 10. YM agar medium composition.

Reagent	Chemical formula	Concentration (g/L)
Glucose	$C_6H_{12}O_6$	10
Peptone	N/A	5
Yeast extract	N/A	3
Malt extract	N/A	3
Agar	N/A	20

3.4 Kefir grains – Microbial community assessment

Several agar plates were prepared in order to assess the dominant species of bacteria and yeasts living in kefir grains. The different media used were CWC with 1% (w/v) agar, CWS with 1% (w/v) agar, MRS agar, MRSL agar and YM agar. Kefir grains were then inoculated into the agar plates, with the exterior region of the grain being inoculated into the right half of the plate, while the interior part of the grain was inoculated into the left half, to assess for additional differences. Each agar plate was done in duplicate and was left incubating at 28 °C, for 100 h.

3.5 Kefiran production by kefir grains

Each kefiran production assay was carried out in duplicate in 250 mL glass jars, previously autoclaved at 121 °C for 20 minutes. Some experimental conditions were kept constant between all assays, namely temperature (28 °C), medium volume (100 mL) and initial kefir grains biomass (10 g), which corresponds to 10% (w/v) inoculum. Medium samples of 2 mL were taken periodically to measure pH, and then centrifuged in order to remove biomass residues. Afterwards, samples were frozen until HPLC analysis. At the end of fermentation, kefir grains were removed from the medium using a metal sieve, washed with sterile distilled water and their biomass was determined by gravimetry (Sartorius BP 3100 S). The fermented medium was frozen and stored at -20 °C until the kefiran downstream processing operations. In order to ensure a sterile environment, all the procedures were conducted in a laminar flow chamber (BBH4 Braun Horizontal).

3.5.1 Kefiran production – MRSL broth medium

In these assays, three different variables were tested: fermentation time (100 h, 220 h, 340 h), lactose concentration (40 g/L, 80 g/L, 120 g/L) and stirring speed (0 rpm, 80 rpm, 160 rpm).

3.5.2 Kefiran production – Cheese whey: batch and fed-batch fermentations

Cheese whey samples CWC, CWCD and CWS, containing approximately 80 g/L, 30 g/L and 35 g/L of lactose, respectively, were used in both batch and fed-batch mode

fermentations, at 80 rpm. The fermentation time varied according to the objective of the assay. Fed-batch fermentations initiated with a medium volume of 100 mL, and two substrate pulses of 50 mL were added over the course of the assay.

3.5.2.1 Batch fermentation – Kefiran concentration over time

In this particular assay, CWS medium, containing around 35 g/L of lactose, was used. The fermentations were performed at 80 rpm, for the following durations: 3.05 h, 27.03 h, 51.17 h, 75.08 h and 99.15 h.

3.6 Analytical methods

3.6.1 pH

The pH was measured in samples using a pH meter (Hach sensION^{TM}+ MM340), which was periodically calibrated using three buffer solutions of different pH values: 4.01, 7.00 and 10.00 (at 25 °C).

3.6.2 Conductivity

The conductivity in cheese whey samples was assessed with a conductivity meter (Randell Model RL105), equipped with a Sentek electrode.

3.6.3 Determination of substrates and metabolites concentration by HPLC

Lactose, glucose, galactose, lactic acid, acetic acid and ethanol concentrations were determined by high performance liquid chromatography (HPLC), for all samples taken during fermentation. Samples were centrifuged at 13000 rpm for 10 minutes, for the removal of biomass (Eppendorf MiniSpin). The solid was discarded and the supernatant was collected and stored at -20 °C. Then, samples from assays using cheese whey, containing lipids that could interfere with HPLC analysis, required a pre-treatment step, where 500 μL of supernatant was mixed with 25 μL of Carrez Reagent 1 and 25 μL of Carrez Reagent 2, as described by Indyk *et al*. (1996). Before injection into the HPLC equipment, samples were diluted in order to obtain a lactose concentration lower than 5 g/L, and centrifuged at 8000

rpm, using specific Eppendorf tubes equipped with a nylon filter consisting of a membrane with a 0.2 μm pore (VWR), for 20 minutes. Finally, samples were transferred into glass vials and 10 μL of sample were injected into a LaChrom Elite HPLC chromatograph (Hitachi), equipped with a L-2130 pump (Hitachi), a L-2200 autosampler injector (Hitachi), a Gecko 2000 oven operating at 65 °C (Cluzeau Info Labo), a Rezex^{TM} ROA – Organic Acid H^+ (8%) column (Phenomenex), and a L-2490 Refractive Index (RI) Detector (Hitachi), using H_2SO_4 0.005 N as eluent, with a flow rate of 0.5 mL/min. To determine the concentration of each analysed compound, a standard calibration curve was done (**Appendix**), using standard solutions of lactose, glucose, galactose, lactic acid, acetic acid and ethanol, with concentrations ranging from 0 g/L to 5 g/L.

3.7 Kefiran downstream processing and quantification

Kefiran downstream processing was based on the procedures published by Rimada and Abraham (2003), with some modifications. The methodology initiated with a pre-treatment step, in order to inactivate any enzymes that could degrade kefiran. A sample volume of 10 mL was heated in a boiling water bath for 15 minutes. Samples were then acidified with the slow addition of trichloroacetic acid 20% (w/v), under agitation, and kept at 4 °C for 24 h. Afterwards, samples were centrifuged at 5000 rpm for 20 minutes at 4 °C (Megafuge 16R centrifuge, Thermo Scientific) and the precipitated fraction, containing the inactivated enzymes, was discarded.

Then, kefiran was precipitated with cold (-20 °C) absolute ethanol: one volume of the supernatant was mixed with three volumes of ethanol and kept at -20 °C for 24 h. Later, samples were centrifuged at 5000 rpm for 15 minutes at 4 °C and the supernatant was discarded. The precipitate was resuspended in 5 mL of hot distilled water, 20 mL of cold absolute ethanol were added and samples were kept at -20 °C for another 24 h. Afterwards, samples were centrifuged again, in the same conditions, the supernatant discarded, the precipitate resuspended in 10 mL of hot distilled water and, in the end, samples were stored at -20 °C until the next step.

A purification stage was required for the removal of residual carbohydrates, such as lactose, which might have co-precipitated with kefiran during the previous step. Samples were dialysed for 48 h, at 4 °C, against 1 L of distilled water, which was changed twice a

day, in order to promote diffusion. The dialysis membranes (Spectra/Por) used in this procedure had a molecular weight cut-off between 6000 and 8000 Da, to prevent the loss of kefiran fractions of low molecular mass.

Following dialysis, a lyophilisation step was performed: samples were frozen and stored at -20 °C, and then moved to -80 °C (Kaltis) for at least 30 minutes before being transferred to the freeze-drying equipment (VirTis) for lyophilisation, which lasted for approximately three days.

The final step consisted of kefiran quantification and was performed by gravimetry, by determining the dry mass of the lyophilised kefiran samples using an analytical balance (Kern).

3.8 Kefiran characterisation

3.8.1 Glucose to galactose ratio quantification

Acid hydrolysis of kefiran was performed after the downstream processing steps in order to assess the glucose to galactose ratio of the obtained kefiran samples. In this method, 200 μL of a H_2SO_4 (72%) solution were added to 3 mg of each sample and incubated for 3 h, at room temperature. Afterwards, 1 mL of distilled water was added, and samples were left incubating for 1 h, at 120 °C. Lastly, after hydrolysis, pH was adjusted to 1 and samples were analysed by HPLC.

3.8.2 Fourier-transform infrared spectroscopy

Approximately 2 mg of each lyophilised kefiran sample was pressed into a thin pellet in order to be analysed through FTIR, to determine the presence of specific functional groups. The transmission spectrum was obtained on the spectrometer (Perkin Elmer), at a wavelength range between 4400 and 400 cm^{-1}, with 64 scans at a resolution of 4 cm^{-1}. Initially, the raw signal was pre-processed by a baseline correction technique and then vector normalised using the Spectra software.

3.8.3 Scanning electron microscopy

Kefiran samples were prepared by direct fixation onto the carbon tape and then coated by carbon evaporation (Emitech K950X), in order to get conductive samples. Scanning electron microscopy observations were performed using a Field Emission Gun - SEM Hitachi S4100 microscope (Japan), operated at 25 kV, with magnifications of ×100 and ×500.

3.9 Kefiran-based films

The production of films based on kefiran was also attempted, based on the method published by Piermaria *et al*. (2009), with some modifications. As reported, films based solely on kefiran were very brittle and hard to handle, therefore, glycerol was included in their composition as a plasticiser. As kefiran was recovered in very low concentrations, this procedure was only attempted with samples produced from MRSL broth medium. Initially, to prepare 1 g of the film-forming solution, 10 mg of kefiran were dissolved in 987.5 μL of distilled water containing 1.98 μL of glycerol. Due to these low quantities, this procedure was carried out in a 25 mL beaker (with a diameter of 1,74 cm), and the filmogenic solution was dried inside an incubator, at 40 °C, for 6 h.

3.10 Calculation methods

3.10.1 Volumetric rates

The volumetric production rate of kefiran, $r_{kefiran}$ (g/L.day), was determined at the end of fermentation, using **Equation 1**:

$$r_{kefiran} = \frac{Kefiran\ concentration_{final}}{Time_{final} - Time_{inicial}} \times 24 \qquad \textbf{(Equation 1)}$$

The volumetric production rate of lactic acid, $r_{lactic\ acid}$ (g/L.h), as well as the volumetric consumption rate of lactose, $r_{lactose}$ (g/L.h), were determined by adjusting linear

functions to the experimental data for the concentration of each variable over time, and calculating the first derivative at time zero.

3.10.2 Yields

The yield of kefiran on substrate, $Y_{\text{kefiran/substrate}}$ (g/g), was determined at the end of fermentation, and the yield of lactic acid on substrate, $Y_{\text{lactic acid/substrate}}$ (g/g), was determined from the beginning of fermentation until the maximum concentration of lactic acid in the medium was reached. Both yields were calculated using **Equation 2**:

$$Y_{p/s} = \frac{Product\ concentration_{final} - Product\ concentration_{inicial}}{Substrate\ concentration_{inicial} - Substrate\ concentration_{final}} \qquad \textbf{(Equation 2)}$$

3.10.3 Kefir grains biomass variation

Kefir grains biomass was measured at the beginning and end of fermentation, and this biomass variation, Δm (%), was calculated using **Equation 3**:

$$\Delta m\ (\%) = \frac{Kefir\ grains\ biomass_{final} - Kefir\ grains\ biomass_{initial}}{Kefir\ grains\ biomass_{initial}} \times 100 \qquad \textbf{(Equation 3)}$$

4. Results and Discussion

4.1 Kefir grains – Microbial community assessment

After inoculating several agar plates with microorganisms living in kefir grains, the growth of colonies was observed, as shown in **Figures 8**, **9**, **10**, **11** and **12**.

No substantial differences were visually detected relatively to the microbial colonies' morphology when comparing the microbiota from the exterior (right side of each plate) and interior region (left side) of kefir grains. However, in general, it is extremely difficult to distinguish the diverse species, or even genus, just by analysing the colonies' morphology. More advance techniques are required, such as sequencing of 16S ribosomal DNA for bacteria, random amplified polymorphic DNA – polymerase chain reaction (RAPD-PCR) and polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), for a more complete and reliable study of the microflora within kefir grains (Vardjan et al., 2013).

Nevertheless, the development speed of the colonies growing in synthetic media (MRS, MRSL and YM) was notoriously higher than in CWC and CWS plates. This was expected, since synthetic media provided the required nutrients for the growth of LAB and yeasts. Agar plates with cheese whey samples (CWC and CWS), while not as evident as the ones mentioned before, still display a pretty relevant number of visible colonies. The punctiform and circular colonies observed were very likely to consist of dominant species of LAB, such as *Lactobacillus kefiranofaciens* (Dertli and Çon, 2017), while the ones seen in **Figure 10** are probably colonies of *Saccharomyces cerevisiae* or *Kluyveromyces marxianus*, both dominant yeast species in kefir grains (Diosma et al., 2014), since YM medium is selective for the growth of yeasts.

In all cases, and as expected, the differences relatively to the development and size of the colonies, when comparing the incubation time of 50 and 100 h, were very clear, especially in the plate containing the MRSL agar medium, as observed in **Figure 9**.

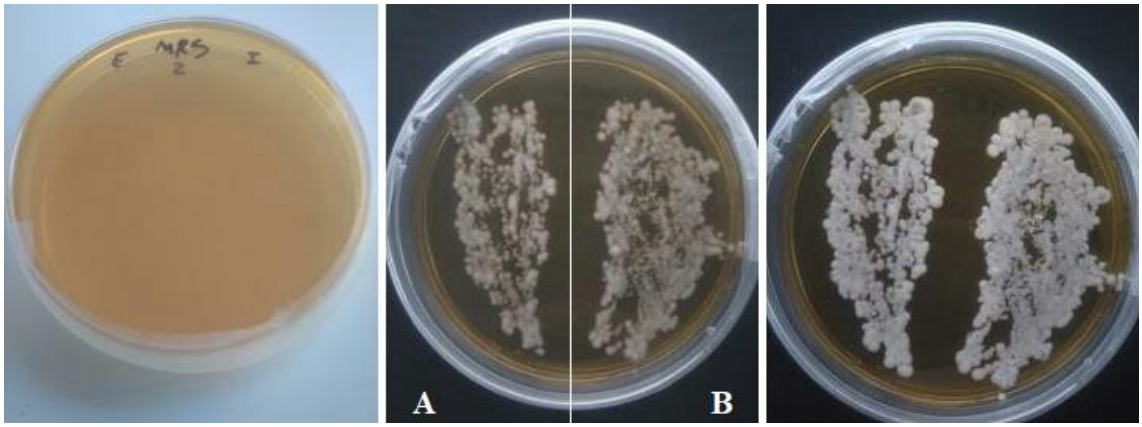

Figure 8. Kefir grains' microbial community growing on MRS agar medium, after incubating at 28 °C for 0, 50 and 100 h, respectively. Interior (**A**) and exterior (**B**) region of the kefir grain.

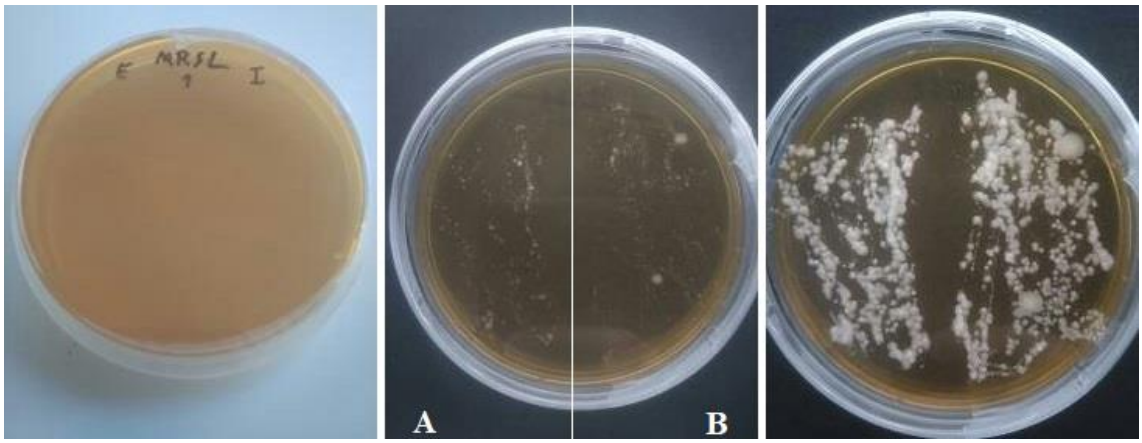

Figure 9. Kefir grains' microbial community growing on MRSL agar medium, after incubating at 28 °C for 0, 50 and 100 h, respectively. Interior (**A**) and exterior (**B**) region of the kefir grain.

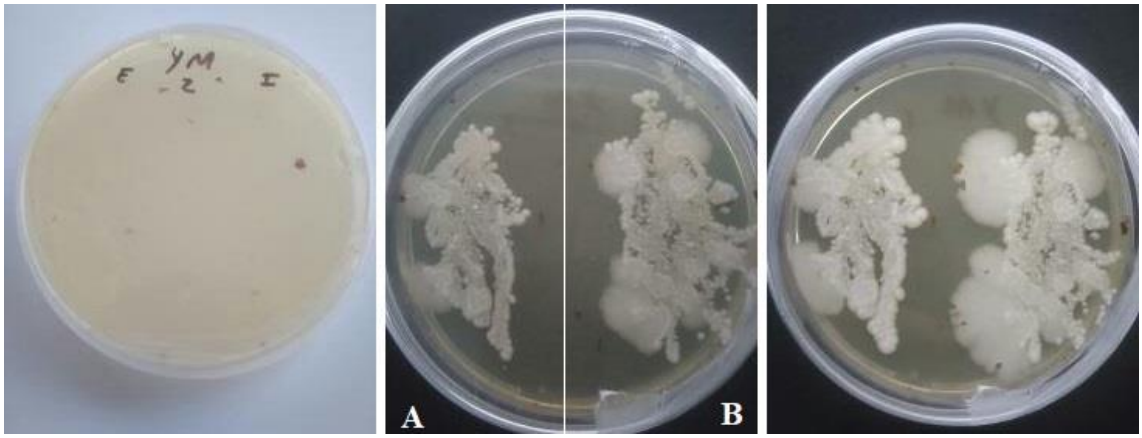

Figure 10. Kefir grains' microbial community growing on YM agar medium, after incubating at 28 °C for 0, 50 and 100 h, respectively. Interior (**A**) and exterior (**B**) region of the kefir grain.

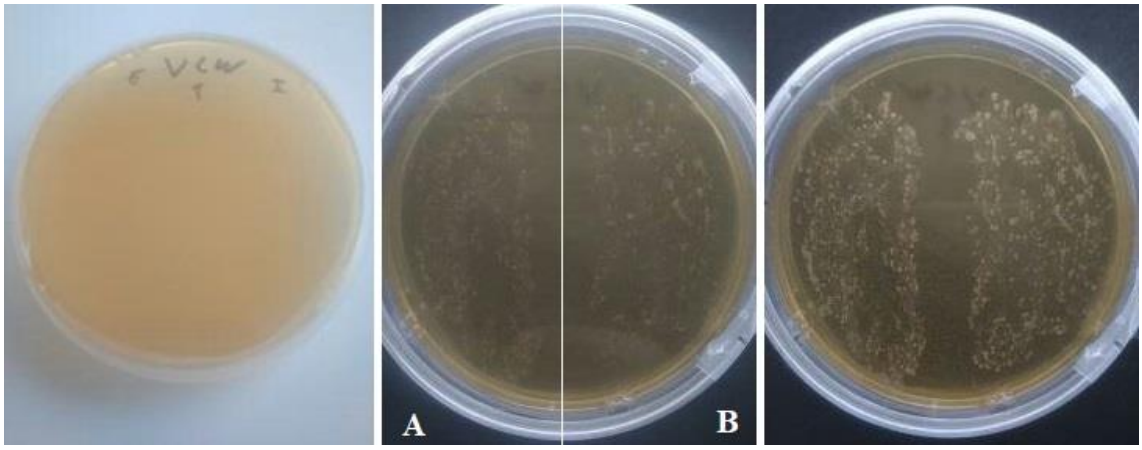

Figure 11. Kefir grains' microbial community growing on CWC agar medium, after incubating at 28 °C for 0, 50 and 100 h, respectively. Interior (**A**) and exterior (**B**) region of the kefir grain.

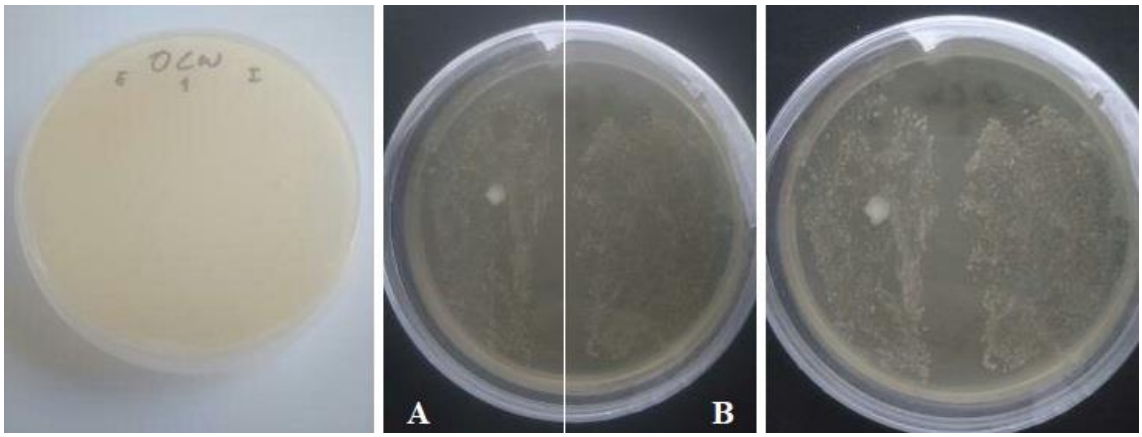

Figure 12. Kefir grains' microbial community growing on CWS agar medium, after incubating at 28 °C for 0, 50 and 100 h, respectively. Interior (**A**) and exterior (**B**) region of the kefir grain.

4.2 Kefiran production – MRSL broth

The study of kefiran production by kefir grains initiated with the assessment of the optimal fermentation conditions, using MRSL broth medium. The effect of three different variables was tested: fermentation time (100 h, 220 h, 340 h), lactose concentration (40 g/L, 80 g/L, 120 g/L) and stirring speed (0 rpm, 80 rpm, 160 rpm).

4.2.1 Effect of fermentation time

The first assays were performed with the objective of determining the optimal fermentation time (100 h, 220 h and 340 h), while maintaining the same initial lactose concentration (80 g/L) and stirring speed (80 rpm).

As can be observed in **Figure 13**, 100 h of fermentation were not enough for the complete consumption of lactose. Glucose, one of the monomers of lactose, was immediately consumed, since its concentration was extremely low during the entire assay duration. Lactic acid was produced in very high concentrations (39.17 g/L) and was the main responsible for the decrease in pH (from 5.09 to 3.39), which inhibits the LAB, producers of kefiran. Ethanol and acetic acid were also produced, in lower quantities (11.79 g/L and 1.31 g/L, respectively), probably due to the species of yeasts and acetic acid bacteria in kefir grains.

When the fermentation time was extended to 220 h, lactose was completely consumed. As shown in **Figure 14**, this was detected after 174 h of fermentation. However, when analysing the lactose present in the previous samples, it can be expected that it was already depleted long before that. Glucose was quickly consumed, and the maximum concentrations of lactic and acetic acid were very close to those obtained after 100 h of fermentation. From there, both concentrations remained constant until the end of the assay. In this assay, ethanol was produced in higher concentrations (18.14 g/L), probably due to an unusually high activity of the yeast species mentioned before. Lactic acid bacteria are known to be inhibited by high concentrations of lactic acid (Cheirsilp et al., 2018), which means that in longer fermentations, when exposed to a low pH for a large period of time, they may get harshly inhibited, leaving more room for the yeasts to thrive. In these assays, without pH control, pH decreased very quickly at the beginning of fermentation, and then kept slowly decreasing. After only 30 h of fermentation, pH went from 5.10 to 3.49 and, by the end of the assay, it was at 3.35, which was even lower than 3.90, as reported by Cheirsilp *et al*. (2019) with a pure culture of *L. kefiranofaciens*. The author stated that when pH was controlled, an increase in sugar consumption and in kefiran production, were observed.

After extending the fermentation for a longer period of time (340 h), no considerable changes were noticed after 220 h. As can be seen in **Figure 15**, all concentrations remained constant after that and, until then, this fermentation showed a similar behaviour compared to the 100 h and 220 h assays. In this longer assay, pH value ranged from 5.15 to 3.39.

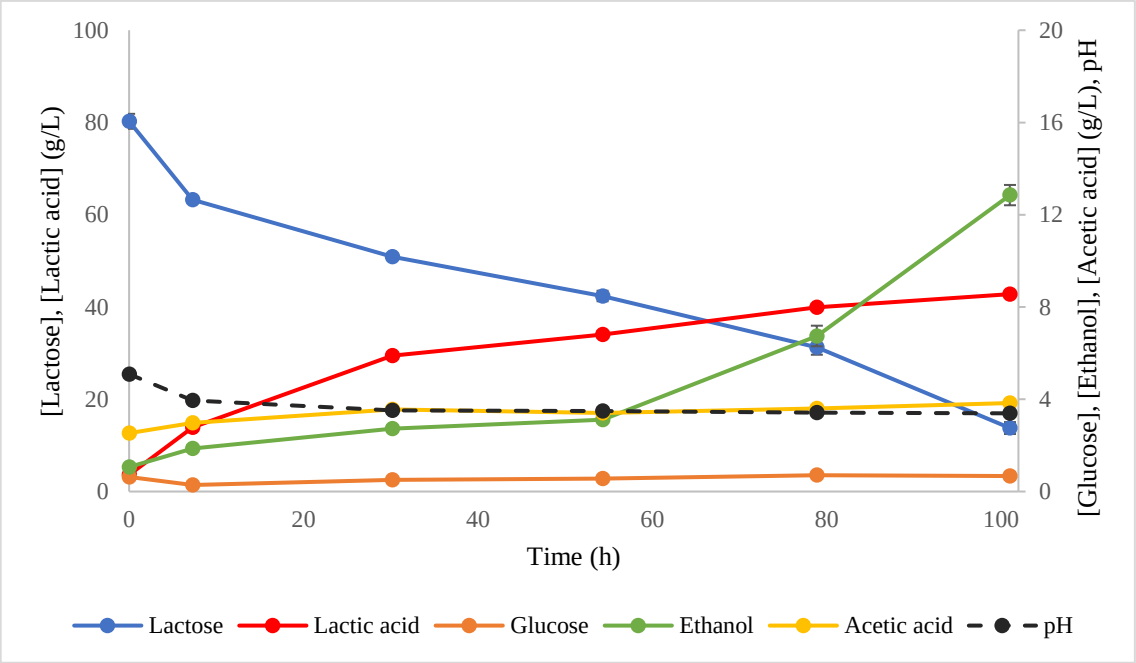

Figure 13. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of MRSL broth medium with 80 g/L of lactose, at 80 rpm.

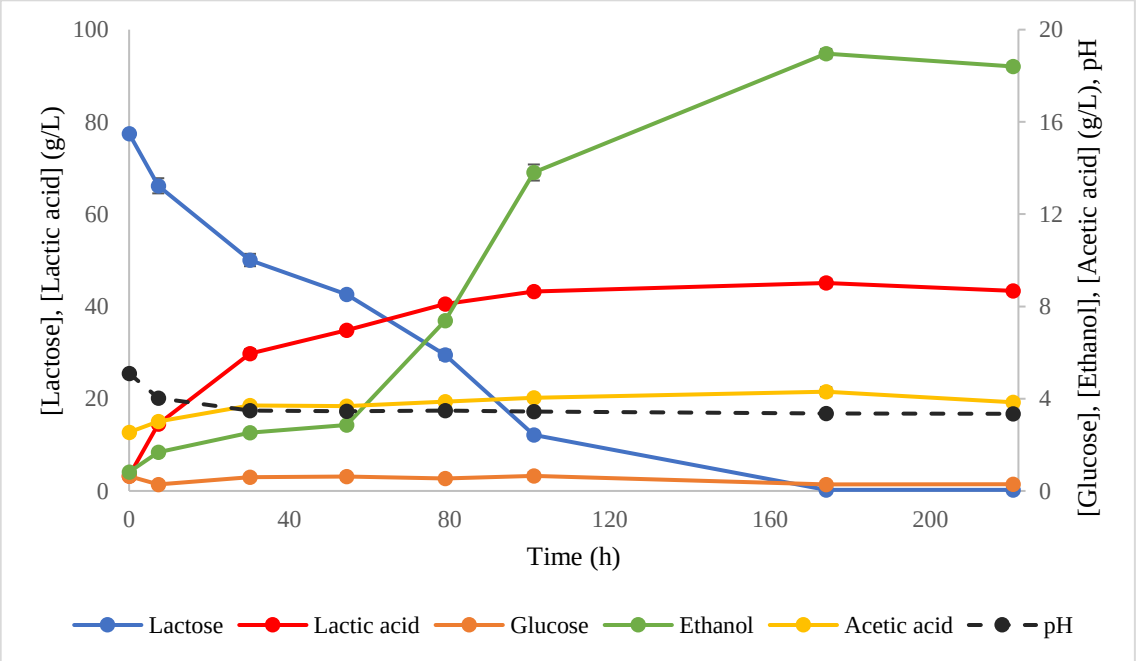

Figure 14. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 220 h of batch fermentation of MRSL broth medium with 80 g/L of lactose, at 80 rpm.

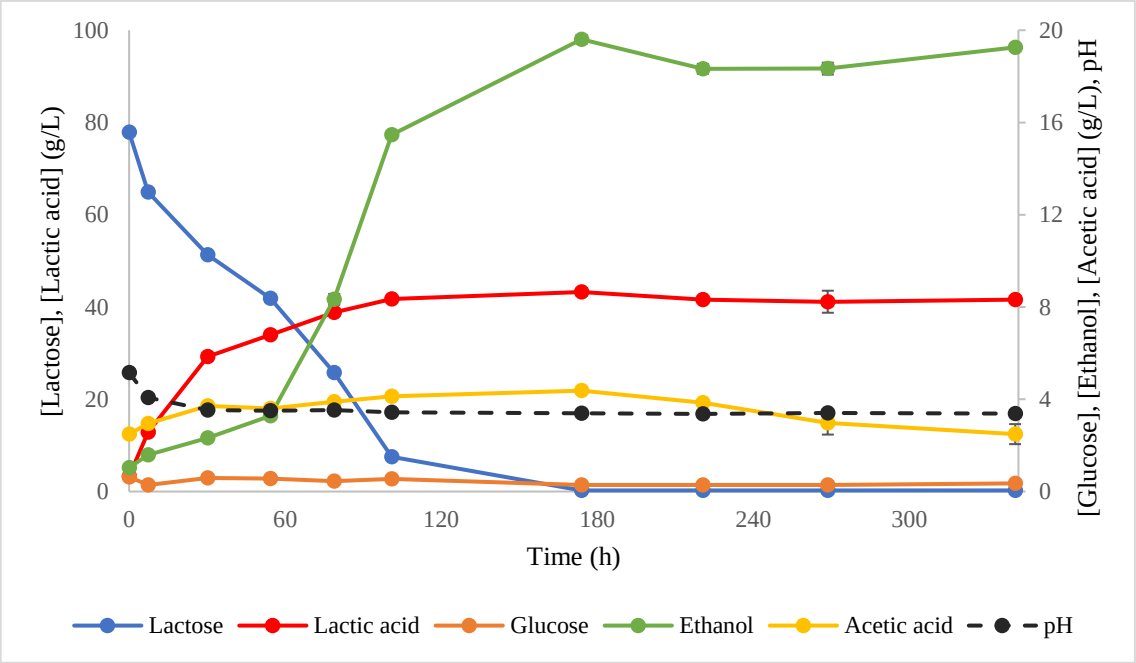

Figure 15. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 340 h of batch fermentation of MRSL broth medium with 80 g/L of lactose, at 80 rpm.

The maximum concentration of lactic acid was very similar in the three assays (42.79 g/L, 45.08 g/L and 43.25 g/L, in the 100 h, 220 h and 340 h fermentations, respectively), which shows that it was not affected by the fermentation duration. Lactic acid productivity in the assay of 100 h was significantly higher, since it took a shorter time to reach the maximum, while in the longer assays, the maximum concentration was only reached later in the fermentation.

Relatively to kefiran production, a considerable difference can be observed in kefiran final concentration (0.760 g/L, 1.065 g/L and 1.420 g/L, in the 100 h, 220 h and 340 h fermentations, respectively) and yield on substrate (0.0114 g/g, 0.0138 g/g and 0.0183 g/g, respectively), which were directly proportional to the length of fermentation, while, on the other hand, kefiran productivity (0.1805 g/L.day, 0.1159 g/L.day and 0.1000 g/L.day, respectively) is inversely proportional to fermentation time, as shown in **Table 11**. Therefore, from an industrial point of view, shorter fermentations are preferable, since the main objective would be to produce as much kefiran as possible. From this study, the fermentation time of 100 h was chosen for all future assays with MRSL broth medium.

Kefir grains biomass was also severely affected by fermentation time. After 100 h of fermentation, the grains gained a little biomass, while after longer periods of fermentation, the grains began to lose significant biomass, most probably due to the depletion of lactose. This is not favourable, since it negatively affects the grains stability, limiting their reuse in future assays.

Table 11. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, with different times of batch fermentation of MRSL broth medium with 80 g/L of lactose, at 80 rpm.

Parameters	**Fermentation time**		
	100 h	**220 h**	**340 h**
Kefiran concentration (g/L)	0.760 ± 0.050	1.065 ± 0.025	1.420 ± 0.020
$r_{kefiran}$ (g/L.day)	0.181 ± 0.0119	0.116 ± 0.0027	0.100 ± 0.0014
$Y_{kefiran/substrate}$ (g/g)	0.011 ± 0.0007	0.014 ± 0.0002	0.018 ± 0.0003
Lactic acid production (g/L)	39.17 ± 0.076	41.79 ± 0.950	39.94 ± 0.526
$r_{lactic\ acid}$ (g/L.h)	0.360 ± 0.0027	0.218 ± 0.0067	0.211 ± 0.0016
$Y_{lactic\ acid/substrate}$ (g/g)	0.589 ± 0.0018	0.541 ± 0.0092	0.515 ± 0.0057
Lactose consumption (g/L)	66.49 ± 0.329	77.22 ± 0.445	77.61 ± 0.170
$r_{substrate}$ (g/L.h)	0.573 ± 0.0094	0.441 ± 0.0014	0.451 ± 0.0013
Kefir grains biomass variation (%)	+ 4.07 ± 1.49	- 17.85 ± 0.68	- 37.59 ± 4.82

4.2.2 Effect of initial lactose concentration and stirring speed

The effect of different initial concentrations of lactose (120 g/L, 80 g/L, 40 g/L) and different stirring speeds (0 rpm, 80 rpm, 160 rpm) were studied simultaneously, and every fermentation lasted for 100 h. These lactose concentrations were chosen to approximately match the concentrations of lactose in the cheese whey samples, 40 g/L and 80 g/L, while the highest concentration, 120 g/L, was also included since it is common to find this amount of lactose in whey, due to the common industrial procedure of concentrating cheese whey three times (Ferrari et al., 1993).

Initially, a MRSL broth medium containing 120 g/L of lactose was used as the substrate for a static fermentation by kefir grains (0 rpm). As can be observed in **Figure 16**, since the initial concentration of lactose was very high, only near a third was consumed (38.68 g/L). The concentration of glucose during the assay was almost negligible, since it was immediately consumed after the hydrolysis of lactose. Lactic acid was also produced in a considerable amount (30.31 g/L), contributing to the fast decrease in pH, which ranged from 5.04 to 3.27 at the end of the fermentation, while acetic acid and ethanol were produced in much lower quantities (1.56 g/L and 1.70 g/L, respectively).

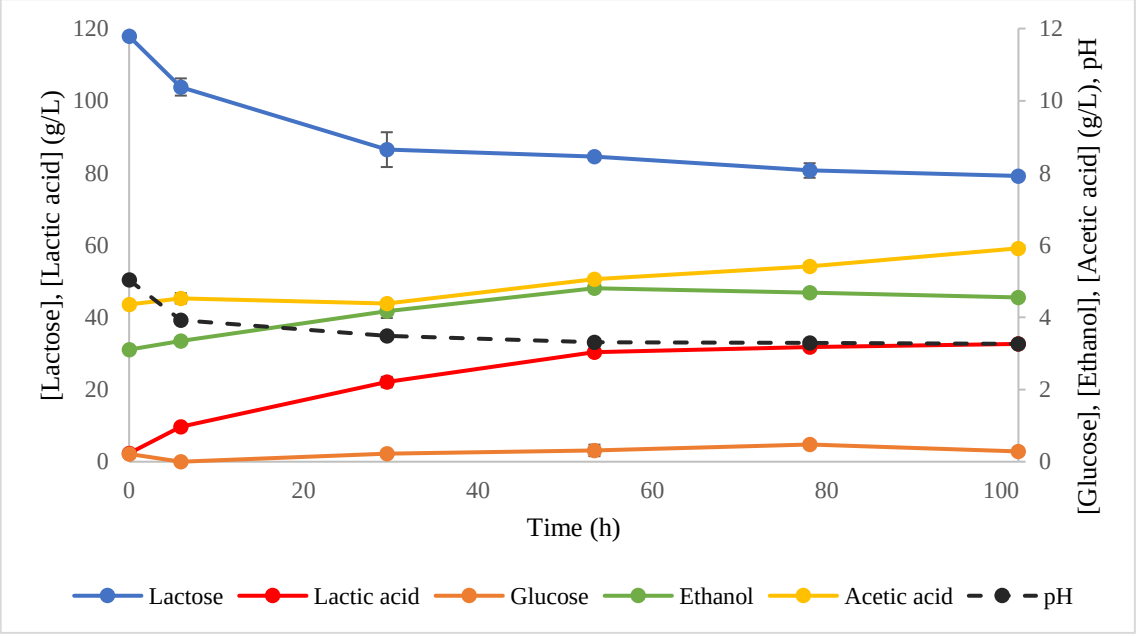

Figure 16. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of MRSL broth medium with 120 g/L of lactose, at 0 rpm.

When the stirring speed was increased to 80 rpm, using the same MRSL broth medium with 120 g/L of lactose (**Figure 17**), the consumption of lactose was slightly higher (40.77 g/L), while the production of lactic acid was somewhat lower (28.93 g/L) and the value of pH went from 5.04 to 3.26. Glucose, ethanol and acetic acid concentrations were very similar to the values observed without stirring.

Finally, when fermenting using a stirring speed of 160 rpm (**Figure 18**), the consumption of lactose was even higher (43.68 g/L). This can be explained by the enhanced mass transfer provided by higher stirring speeds, which leaves the carbon source more accessible to microorganisms. The production of lactic acid (25.00 g/L) was lower than in the experiments with lower stirring speeds, which led to a slightly higher pH value (3.40) at the end of the assay. Additionally, the concentrations of glucose, ethanol (1.69 g/L) and acetic acid (2.38 g/L) were identical to the ones obtained with the lower stirring speeds.

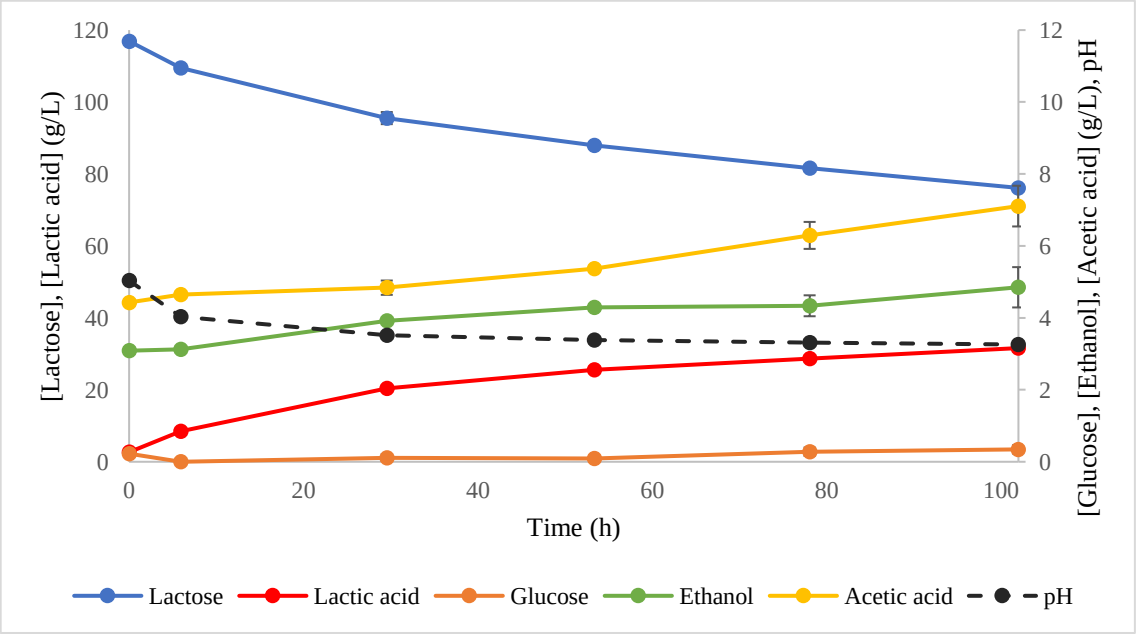

Figure 17. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of MRSL broth medium with 120 g/L of lactose, at 80 rpm.

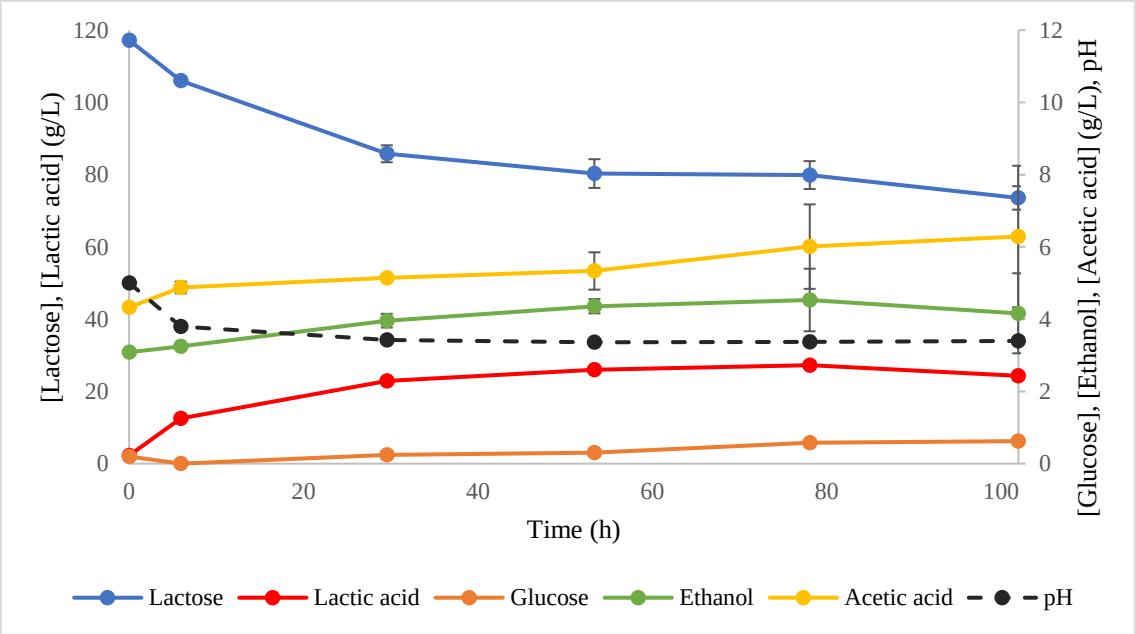

Figure 18. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of MRSL broth medium with 120 g/L of lactose, at 160 rpm.

Regarding kefiran production, the best result in this assay, 1.475 g/L, was obtained with a stirring speed of 160 rpm (**Table 12**). This probably resulted from the improved mass transfer phenomenon, provided by higher agitations, which allowed for a better nutrient distribution in the medium, as well as a more uniform pH and temperature. Also, an intensive stirring was observed to promote the release of kefiran bounded to the surface of kefir grains, therefore increasing its excretion into the medium, while simultaneously fragmenting the grains into smaller pieces, increasing their surface (Zajšek et al., 2013). Nevertheless, even when a milder stirring speed (80 rpm) was applied in this assay, mass transfer could be enhanced, accelerating kefir grains growth, as microorganisms are reproducing and growing optimally, however, the lowest concentration of kefiran was also obtained with this stirring speed (0.640 g/L). Regarding kefiran productivity and yield on substrate, the values did not change significantly between 0 and 80 rpm, but were notably higher with 160 rpm, due to the higher final kefiran concentration.

When analysing the kefir grains at the end of the three assays, it can be concluded that a stirring speed of 160 rpm was too extreme, since the grains lost 10.83% of their initial biomass during the fermentation. This was also observed by Zajšek *et al*. (2013), but it is not

a desired situation, since kefir grains should be reused in the following assays. When 80 rpm or no stirring were applied, the grains grew considerably (22.88% and 10.12%, respectively), as observed in **Table 12**.

Table 12. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, during 100 h of batch fermentation of MRSL broth medium with 120 g/L of lactose, at different stirring speeds.

Parameters	Stirring speed		
	0 rpm	**80 rpm**	**160 rpm**
Kefiran concentration (g/L)	0.775 ± 0.025	0.640 ± 0.020	1.475 ± 0.145
$r_{kefiran}$ (g/L.day)	0.182 ± 0.0059	0.151 ± 0.0047	0.347 ± 0.0341
$Y_{kefiran/substrate}$ (g/g)	0.020 ± 0.0004	0.016 ± 0.0007	0.034 ± 0.0006
Lactic acid production (g/L)	30.31 ± 0.283	28.93 ± 0.939	25.00 ± 0.604
$r_{lactic\ acid}$ (g/L.h)	0.288 ± 0.0014	0.271 ± 0.0061	0.287 ± 0.0076
$Y_{lactic\ acid/substrate}$ (g/g)	0.784 ± 0.0036	0.709 ± 0.0155	0.575 ± 0.0331
Lactose consumption (g/L)	38.68 ± 0.539	40.77 ± 0.435	43.68 ± 3.562
$r_{substrate}$ (g/L.h)	0.330 ± 0.0099	0.383 ± 0.0141	0.383 ± 0.0382
Kefir grains biomass variation (%)	+ 10.12 ± 0.65	+ 22.88 ± 0.49	- 10.83 ± 0.36

When 80 g/L of lactose were tested, without stirring (**Figure 19**), more than half of the lactose was consumed after 100 h of fermentation (53.42 g/L). This value was substantially higher than in the assay with 120 g/L. This might indicate that substrate inhibition probably occurred with a higher concentration, and a lower concentration of lactose could be preferable for the production of kefiran. Lactic acid produced was also

significantly higher (43.56 g/L) when compared to the assay with 120 g/L of lactose, without agitation, which can be explained by the higher lactose consumption, contributing to the decrease of pH, from 5.11 to 3.28. As in previous assays, glucose concentration always remained very low, since it was quickly consumed, while acetic acid and ethanol were produced in small quantities (2.60 g/L and 1.26 g/L, respectively).

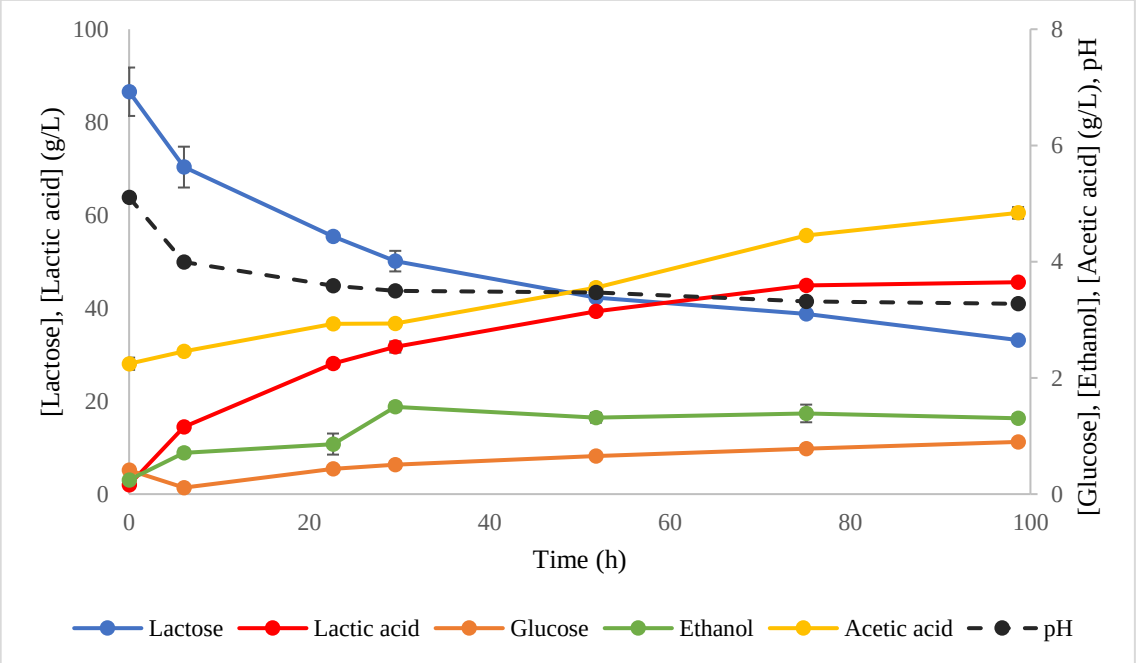

Figure 19. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of MRSL broth medium with 80 g/L of lactose, at 0 rpm.

With a stirring speed of 80 rpm, lactose consumption increased slightly (55.96 g/L), while the production of lactic also increased (47.09 g/L), when compared to the assay without stirring (**Figure 20**), and pH ranged from 5.20 to 3.28. Once more, the concentration of glucose was very low during the entire fermentation. The production of acetic acid (0.71 g/L) was lower than with 0 rpm, while ethanol was produced in a higher quantity (3.70 g/L).

Lastly, when 160 rpm were applied, no significant changes related to lactose consumption (55.56 g/L), lactic acid production (47.92 g/L) and pH decrease (from 5.14 to 3.26) were observed when compared to 80 rpm, being higher than with no stirring (**Figure 21**).

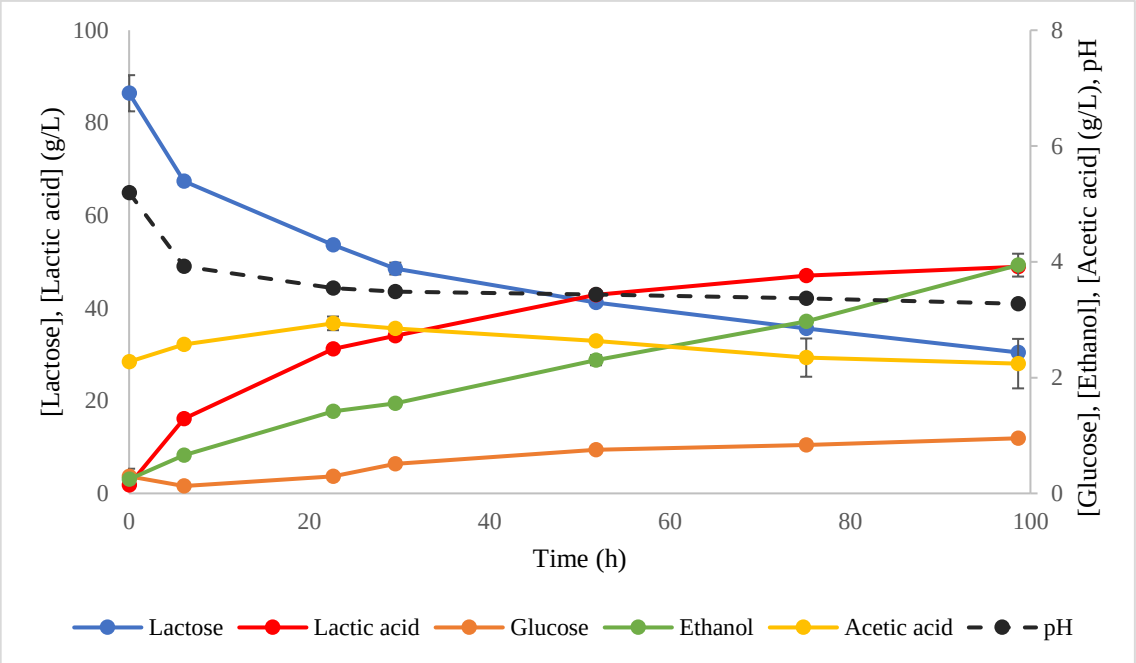

Figure 20. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of MRSL broth medium with 80 g/L of lactose, at 80 rpm.

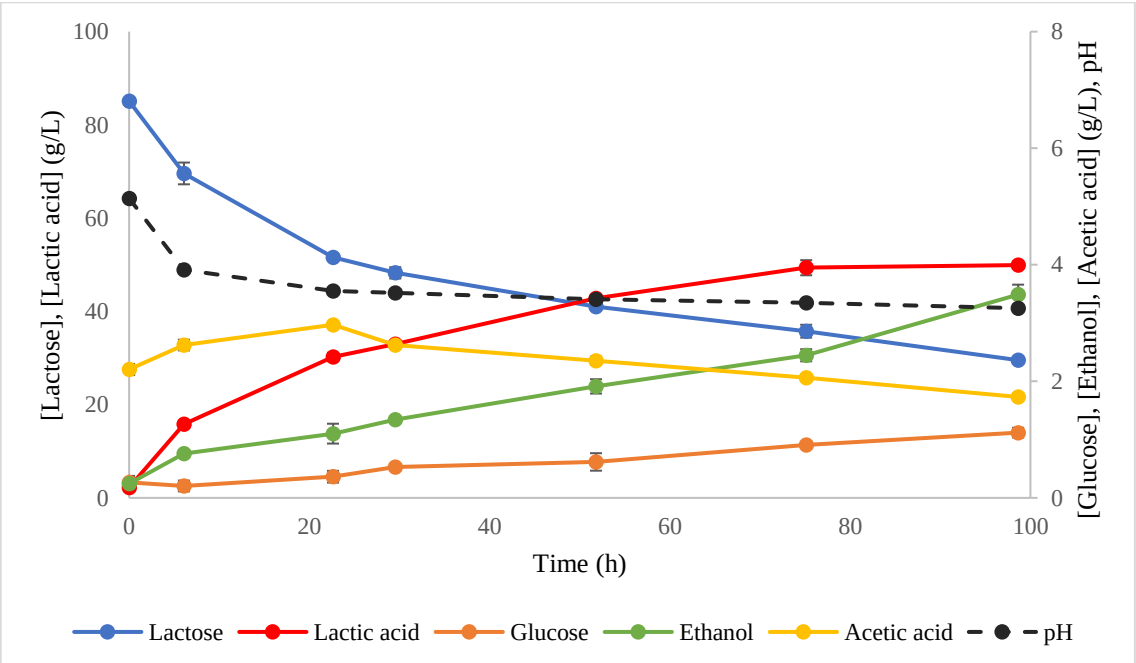

Figure 21. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values during 100 h of batch fermentation of MRSL broth medium with 80 g/L of lactose, at 160 rpm.

Table 13. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, during 100 h of batch fermentation of MRSL broth medium with 80 g/L of lactose, at different stirring speeds.

Parameters	Stirring speed		
	0 rpm	**80 rpm**	**160 rpm**
Kefiran concentration (g/L)	0.545 ± 0.015	0.670 ± 0.100	1.050 ± 0.030
$r_{kefiran}$ (g/L.day)	0.133 ± 0.0037	0.163 ± 0.0243	0.256 ± 0.0073
$Y_{kefiran/substrate}$ (g/g)	0.010 ± 0.0008	0.012 ± 0.0009	0.019 ± 0.0003
Lactic acid production (g/L)	43.56 ± 0.314	47.09 ± 0.476	47.92 ± 0.660
$r_{lactic\ acid}$ (g/L.h)	0.403 ± 0.0071	0.425 ± 0.0050	0.514 ± 0.0811
$Y_{lactic\ acid/substrate}$ (g/g)	0.825 ± 0.0899	0.846 ± 0.0680	0.863 ± 0.0239
Lactose consumption (g/L)	53.42 ± 5.446	55.96 ± 3.934	55.56 ± 0.777
$r_{substrate}$ (g/L.h)	0.469 ± 0.0512	0.484 ± 0.0183	0.488 ± 0.0015
Kefir grains biomass variation (%)	+ 23.13 ± 2.77	+ 8.22 ± 1.55	- 6.26 ± 1.09

Similar to what was observed with an initial lactose concentration of 120 g/L, the highest kefiran concentration, 1.050 g/L, was achieved at 160 rpm. On the other hand, the lowest kefiran concentration, 0.545 g/L, was attained without agitation. Additionally, the difference in terms of kefiran concentration between 80 and 160 rpm (56.7 % increase) was considerably superior than between 0 and 80 rpm (22.9 % increase). Therefore, in this assay, both kefiran productivity and yield on substrate were directly proportional to the stirring speed.

Relatively to kefir grains, once more, a stirring speed of 160 rpm had a negative impact on their biomass, due to the visible grain fragmentation, while an agitation of 80 and 0 rpm allowed grains to grow substantially (8.22 % and 23.13 %, respectively), as observed in **Table 13**, which is in accordance to what was seen previously, when 120 g/L of lactose were used.

The different stirring speeds were also tested with an initial lactose concentration of 40 g/L. When fermenting at 0 rpm, due to its lower concentration, lactose was depleted before 80 h of fermentation, as is shown in **Figure 22**. Lactic acid was, again, produced in very high quantities (43.13 g/L), leading to a very fast decrease in pH, from 5.08 to 3.35. As usual, glucose concentration was extremely low during the entire assay, as it is quickly consumed, and both ethanol and acetic acid production values were also quite similar to previous assays (3.05 g/L and 1.38 g/L, respectively).

With a stirring speed of 80 rpm, no substantial changes were detected during fermentation. However, when analysing the rate of lactose consumption, in **Figure 23**, it can be seen that it was being consumed considerably faster than without stirring, which is corroborated by the different lactose consumption rates between the two agitations (0.5126 g/L.h at 80 rpm and 0.3821 g/L.h at 0 rpm), presented in **Table 14**. The amount of lactic acid produced was slightly lower than before (39.96 g/L), but still represents a very high concentration, contributing to the reduction of pH, from 5.10 to 3.41 at the end of the assay. Ethanol was produced in a higher quantity than with 0 rpm, while the concentration of acetic acid was lower, which also happened in the MRSL broth medium containing 80 g/L of lactose.

The fermentation at 160 rpm provided almost identical results than the one at 80 rpm (**Figure 24**), where lactose was depleted at the same point into the assay, and with a lactic acid production of 41.79 g/L. At this stirring speed, pH ranged from 5.11 to 3.38.

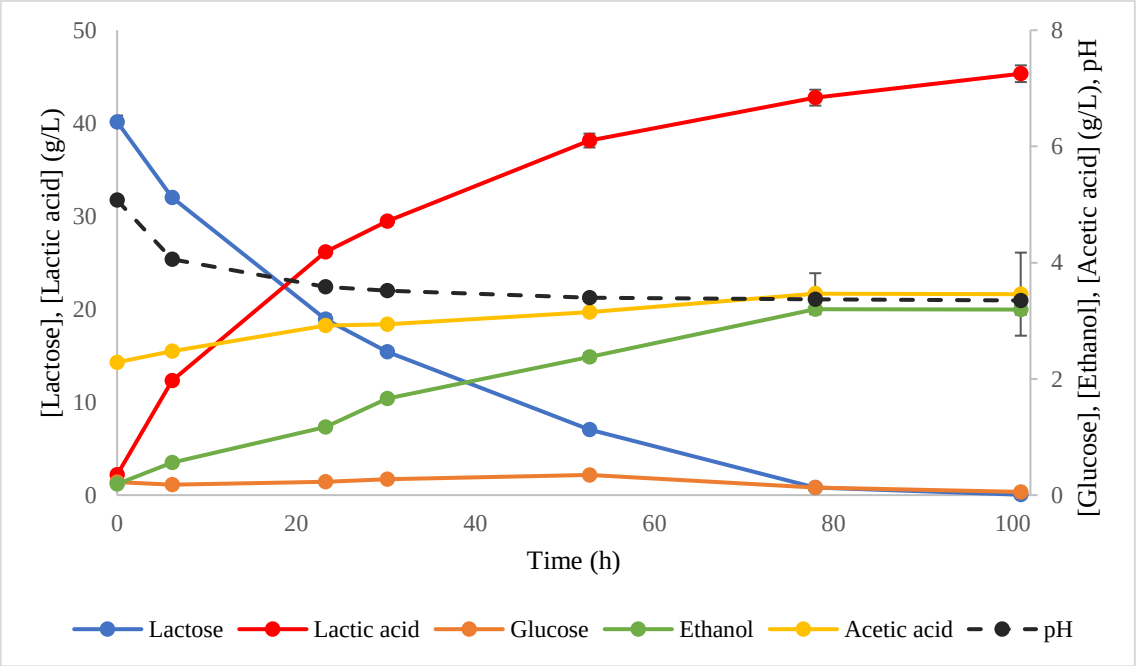

Figure 22. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of MRSL broth medium with 40 g/L of lactose, at 0 rpm.

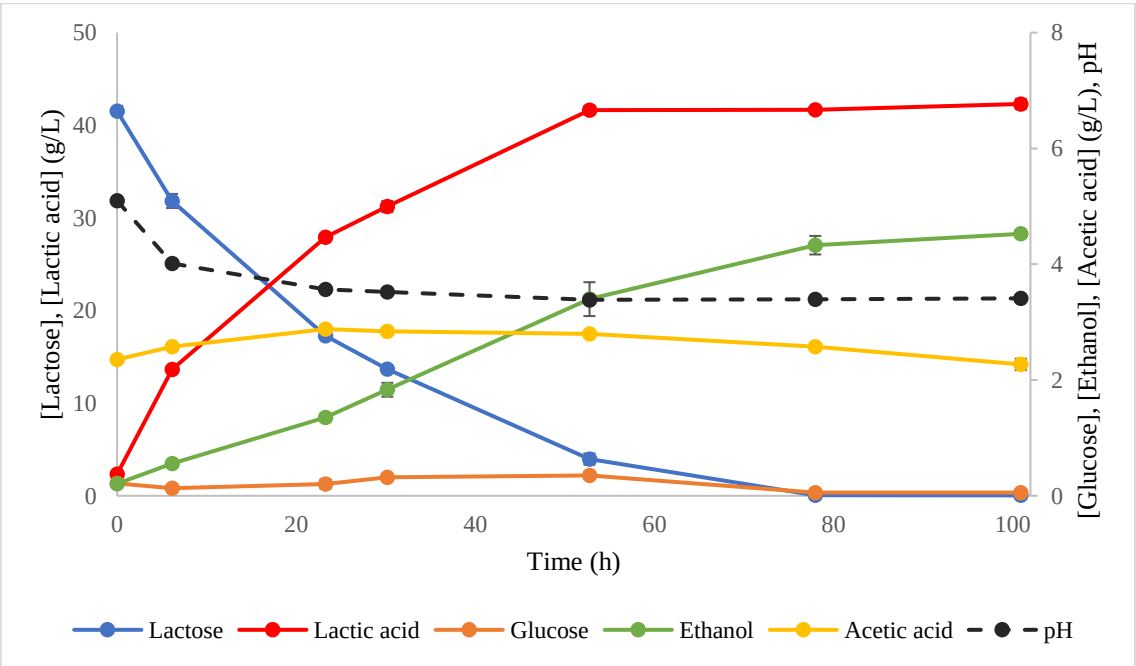

Figure 23. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of MRSL broth medium with 40 g/L of lactose, at 80 rpm.

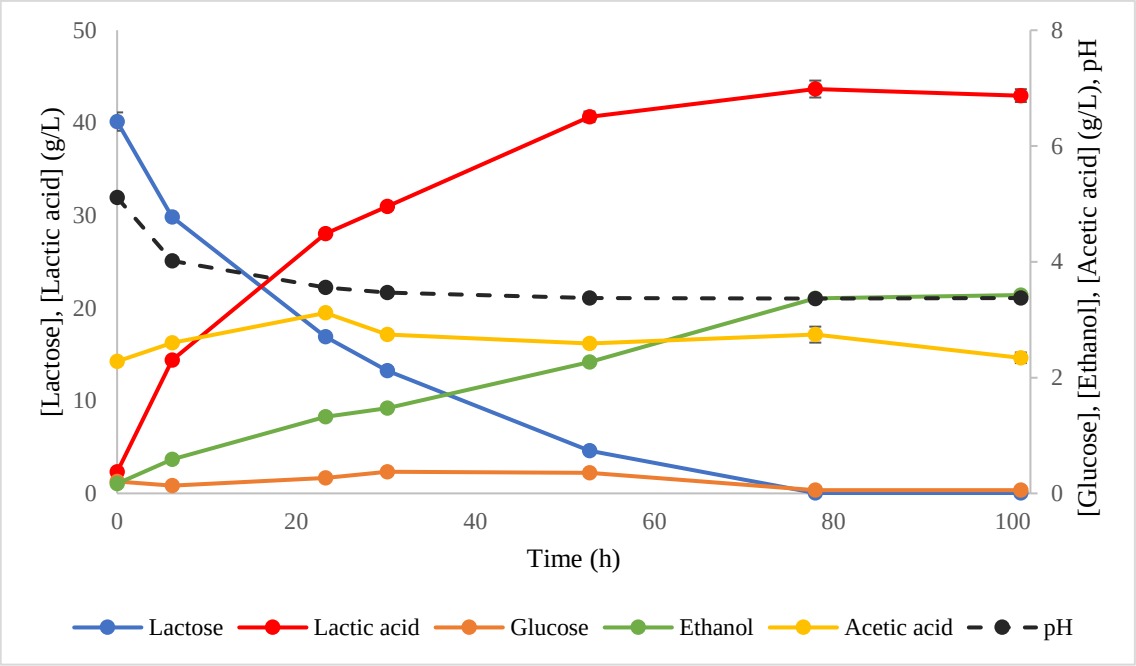

Figure 24. Lactose, lactic acid, glucose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of MRSL broth medium with 40 g/L of lactose, at 160 rpm.

Concerning the production of kefiran when 40 g/L of lactose were used, **Table 14** shows that the best result, 1.640 g/L, was attained at 160 rpm. Additionally, this value was also the highest between all assays with MRSL broth medium. As observed for the previous assay with a lactose concentration of 80 g/L, kefiran concentration was directly proportional to the stirring speed, and the difference between 80 and 160 rpm (91.8 % increase) was higher than between 0 and 80 rpm (31.5 % increase), which is in accordance with the fact that higher stirring speeds promote the release of kefiran into the medium (Zajšek et al., 2013). Once more, both kefiran productivity and yield on substrate rose with the increase of stirring speed.

The difference in the substrate consumption rate seen between 0 rpm (0.3821 g/L.h) and the higher stirring speeds (0.5126 g/L.h at 80 rpm and 0.4868 g/L.h at 160 rpm) is due to the longer time it took to reach depletion, as mentioned before. This agitation provides a higher contact between kefir grains and lactose since it promotes a better mass transfer which, in turn, eases and hastens its consumption.

With 40 g/L of lactose, kefir grains were only able to grow at 0 rpm. As the substrate was depleted before the end of the fermentation, the agitation seemed to damage the grains. As observed in **Table 14**, the depletion of lactose in conjunction to the stirring speed of 160 rpm, resulted in a loss of 33.06 % of the grains' initial biomass.

Table 14. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, during 100 h of batch fermentation of MRSL broth medium with 40 g/L of lactose, at different stirring speeds.

Parameters	**Stirring speed**		
	0 rpm	**80 rpm**	**160 rpm**
Kefiran concentration (g/L)	0.650 ± 0.040	0.855 ± 0.055	1.640 ± 0.050
$r_{kefiran}$ (g/L.day)	0.155 ± 0.0095	0.203 ± 0.0131	0.390 ± 0.0119
$Y_{kefiran/substrate}$ (g/g)	0.016 ± 0.0013	0.021 ± 0.0016	0.041 ± 0.0023
Lactic acid production (g/L)	43.13 ± 0.808	39.96 ± 0.497	41.79 ± 0.483
$r_{lactic\ acid}$ (g/L.h)	0.395 ± 0.0026	0.361 ± 0.0065	0.438 ± 0.0674
$Y_{lactic\ acid/substrate}$ (g/g)	1.076 ± 0.0020	0.963 ± 0.0013	1.043 ± 0.0137
Lactose consumption (g/L)	40.10 ± 0.677	41.48 ± 0.571	40.10 ± 0.991
$r_{substrate}$ (g/L.h)	0.382 ± 0.0041	0.513 ± 0.0031	0.487 ± 0.0090
Kefir grains biomass variation (%)	+ 11.38 ± 2.32	- 7.67 ± 0.18	- 33.06 ± 2.60

After analysing all the previous results obtained with MRSL broth medium, the effect of the different fermentation conditions tested is clear.

The optimal fermentation time for the production of kefiran, from an industrial point of view, was 100 h, due to the best productivity values obtained, which would result in the highest possible concentration of kefiran, produced in the shortest time possible. Therefore, based on this study, the fermentation time of 100 h was chosen for the following assays with cheese whey. All cheese whey samples (CWC, CWCD and CWS) were tested for a duration of 100 h, while, in parallel, CWC was also fermented for 220 h due to its higher concentration of lactose, whereas CWCD and CWS were tested for a duration of 50 h, due to their lower lactose concentration.

Relatively to the stirring speed, the best results in terms of kefiran production were attained at 160 rpm, in all cases, due to the superior mass transfer and kefiran release into the medium. However, this high stirring speed always led to a loss of kefir grains biomass, which was due to the visibly high grain fragmentation observed at the end of each assay at 160 rpm, as can be seen in **Figure 25**, which was in accordance with Zajšek et al. (2013). In the assays with stirring, there was enough mass transfer for the substrate to easily diffuse from where its concentration was higher to where it was lower. In static assays, this diffusion was much less efficient, leading to slower substrate consumption rates, since, in order to be consumed, lactose needs to reach the bottom of the glass jar, where the grains were deposited, contributing to the lower kefiran production values. Therefore, the stirring speed of 80 rpm was chosen for all the following assays with cheese whey, since it corresponded to the best compromise between the production of kefiran and a good condition of kefir grains for their reutilisation.

Figure 25. Visual aspect of kefir grains, after 100 h of batch fermentation of MRSL broth medium with 80 g/L of lactose, at 0, 80 and 160 rpm, respectively.

The optimal lactose concentration in the medium for starting the production of kefiran could not be so straightforwardly interpreted, because the results were not so clear. The lowest lactose concentration, 40 g/L, provided the best results in assays with stirring (0.855 g/L at 80 rpm and 1.640 g/L at 160 rpm) while, on the other hand, the highest lactose concentration, 120 g/L, resulted in the highest amount of kefiran produced in the static assays (0.775 g/L). With this in mind, MRSL broth containing 80 g/L of lactose provided the worst results at 0 and 160 rpm (0.545 g/L and 1.050 g/L, respectively), while only barely stepping ahead of the 120 g/L concentration in the assays at 80 rpm (0.670 and 0.640 g/L, respectively). Thus, it is not completely correct to assume that a high amount of lactose, such as 120 g/L, could have inhibited the kefir grains, as it provided better results overall than a lower concentration, 80 g/L, even though the best results were attained with the lowest lactose concentration of 40 g/L. Hereupon, the results of this part of the study were inconclusive, though, since cheese whey samples have their defined lactose concentration, there was no actual need to choose an optimal initial lactose concentration. Nevertheless, based on these results, it can be expected for the cheese whey samples containing a lower amount of lactose to provide a higher concentration of kefiran, however, cheese whey is an industrial by-product and it is not optimised for the growth of LAB species, responsible for the production of kefiran, and it certainly contains a trace of inhibitory compounds, even after the pre-treatment step, such as proteins or lipids, which are not present in the synthetic medium.

4.3 Kefiran production – Cheese whey

The study of kefiran production from cheese whey started after the selection of the optimal conditions with the synthetic medium. All assays using cheese whey were done at a stirring speed of 80 rpm and for a period of 100 h (additional assays with different durations were also performed depending on the initial lactose concentration of each cheese whey sample and on the objective on the assay).

4.3.1 Batch fermentation

Initially, all cheese whey samples (CWC, CWCD and CWS) were tested for the production of kefiran in batch fermentation.

Cheese whey from cow's milk, CWC, had an initial lactose concentration of approximately 80 g/L. According to **Figure 26**, during the 100 h of fermentation, about half of the lactose was consumed (42.79 g/L), producing 24.54 g/L of lactic acid, which represents a concentration much lower than when MRSL medium was used, under the same fermentation conditions. These lower values were expected, since cheese whey, even after pre-treatment, might still contains inhibitory compounds for the LAB, such as proteins and lipids (Bosco et al., 2018). The value of pH is this assay variated between 4.95 and 3.32. Glucose and galactose, which are the monomers that result from lactose hydrolysis, were almost immediately consumed, since they are the preferable carbon sources for the microbial community living in the kefir grains, thus, their concentration is kept extremely low for the entire assay duration. Ethanol and acetic acid are also produced during this fermentation (7.31 g/L and 4.25 g/L, respectively), mainly by yeasts and AAB, respectively, although in significantly lower concentrations than lactic acid.

When the fermentation of CWC was extended to 220 h (**Figure 27**), the depletion of lactose occurred before 170 h, which was very close to the time seen with the synthetic medium (174 h). Given the longer duration, the maximum lactic acid concentration, 31.65 g/L, was higher than in the previous assay (pH ranged from 4.97 to 3.31), while the concentration of both glucose and galactose was, again, continuously very low. On the other hand, the final ethanol concentration (30.27 g/L) was much higher than before (11.80 g/L), which is in accordance with the assays with a longer duration, discussed in **Chapter 4.2.1**.

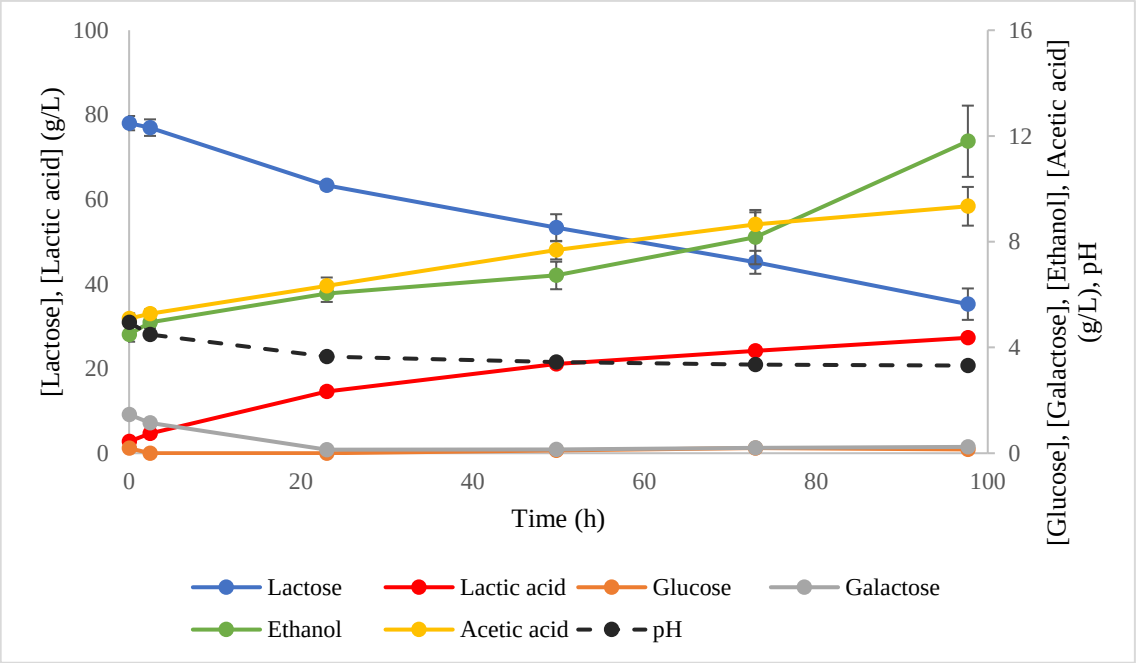

Figure 26. Lactose, lactic acid, glucose, galactose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of CWC medium, at 80 rpm.

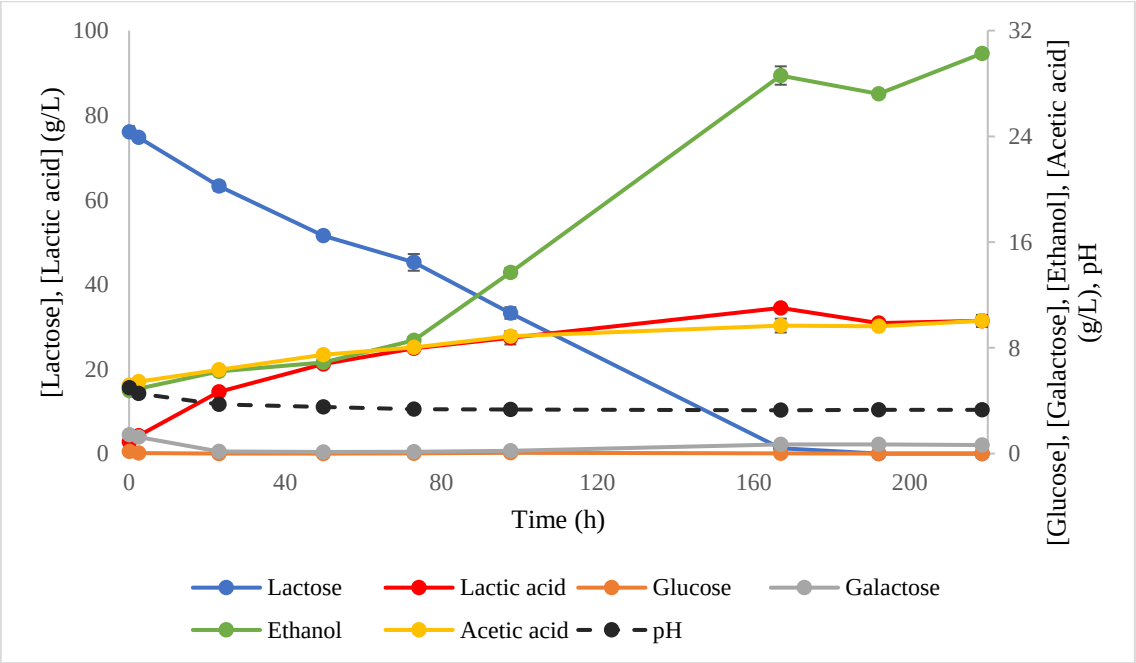

Figure 27. Lactose, lactic acid, glucose, galactose, ethanol and acetic acid concentrations and pH values, during 220 h of batch fermentation of CWC medium, at 80 rpm.

Table 15. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, during 100 and 220 h of batch fermentation of CWC medium, at 80 rpm.

Parameters	Fermentation time	
	100 h	**220 h**
Kefiran concentration (g/L)	0.585 ± 0.065	0.550 ± 0.010
$r_{kefiran}$ (g/L.day)	0.144 ± 0.016	0.060 ± 0.001
$Y_{kefiran/substrate}$ (g/g)	0.014 ± 0.001	0.007 ± 0.000
Lactic acid production (g/L)	24.54 ± 0.077	31.65 ± 0.331
$r_{lactic\ acid}$ (g/L.h)	0.250 ± 0.000	0.187 ± 0.000
$Y_{lactic\ acid/substrate}$ (g/g)	0.575 ± 0.025	0.416 ± 0.012
Lactose consumption (g/L)	42.79 ± 2.027	76.04 ± 1.326
$r_{substrate}$ (g/L.h)	0.435 ± 0.021	0.411 ± 0.005
Kefir grains biomass variation (%)	+ 23.72 ± 0.49	+ 5.90 ± 3.02

Kefiran production using CWC was slightly lower than when using the MRSL broth medium, under similar operational conditions. This was expected, since cheese whey samples can contain inhibitory compounds for LAB, such as some proteins or lipids (Bosco et al., 2018). However, these results were promising, as they were higher than in some reported studies, also without pH control, such as the kefiran concentration of 0.370 g/L, obtained by Blandón *et al*. (2018) using kefir grains, and the concentration of 0.568 g/L, achieved by Cheirsilp and Radchabut (2011) with a pure culture of *L. kefiranofaciens*. In this assay with CWC, pH had a very similar behaviour to what was observed in the assay using MRSL medium under the same conditions, as presented in **Chapter 4.2.1**: after 23 h of fermentation, pH was already at 3.72 (3.49 after 30 h with the synthetic medium), and by

the end of the assay, after 220 h, it was at 3.31 (3.35 after 220 h with the synthetic medium). In comparison, the study mentioned above, published by Cheirsilp and Radchabut (2011), reached a pH value of 3.51 at the end of fermentation, after 120 h.

In terms of kefiran concentration, the amounts produced after 100 and 220 h were similar, as shown in **Table 15**, but the productivity was considerably higher in the former, due to the lower assay duration. Kefiran yield on substrate was also higher in the shorter assay, because of the much lower amount of substrate consumed during fermentation.

Kefir grains were able to grow while fermenting CWC. As expected, this growth was more noticeable in the assay that lasted for 100 h, because lactose was not totally consumed. With the depletion of lactose and the longer exposure to a low pH value (> 4), which tends to occur in longer fermentations, as discussed in **Chapter 4.2.1**, kefir grains were severely affected, and their biomass started to decrease.

A different cheese whey sample, CWCD, which was a diluted version of CWC, with approximately 30 g/L of lactose, was tested for the production of kefiran for 100 and 50 h, due to the lower lactose concentration. Regarding the assay of 100 h (**Figure 28**), lactose was depleted after approximately 70 h. Lactic acid concentration, 20.64 g/L, was lower than with CWC, for the same assay duration, probably due to the lower amount of substrate consumed. This cheese whey sample had a lower initial pH value, 4.59, which decreased to 3.08 at the end of fermentation. Glucose and galactose, as always, were immediately consumed after lactose hydrolysis, while the productions of both ethanol (5.73 g/L) and acetic acid (1.59 g/L) were also lower to when CWC was tested for 100 h.

During the assay of 50 h (**Figure 29**), 22.04 g/L of lactose were consumed, and a lactic acid production of 18.54 g/L was reached, which was lower than in the previous assay, as expected, leading to a slightly higher final pH value (3.20). Concerning glucose, galactose and acetic acid, their concentrations were mostly identical to what was previously observed. However, ethanol production was significantly lower (2.82 g/L) because of the short assay duration, since ethanol tends to ramp up later in the fermentation.

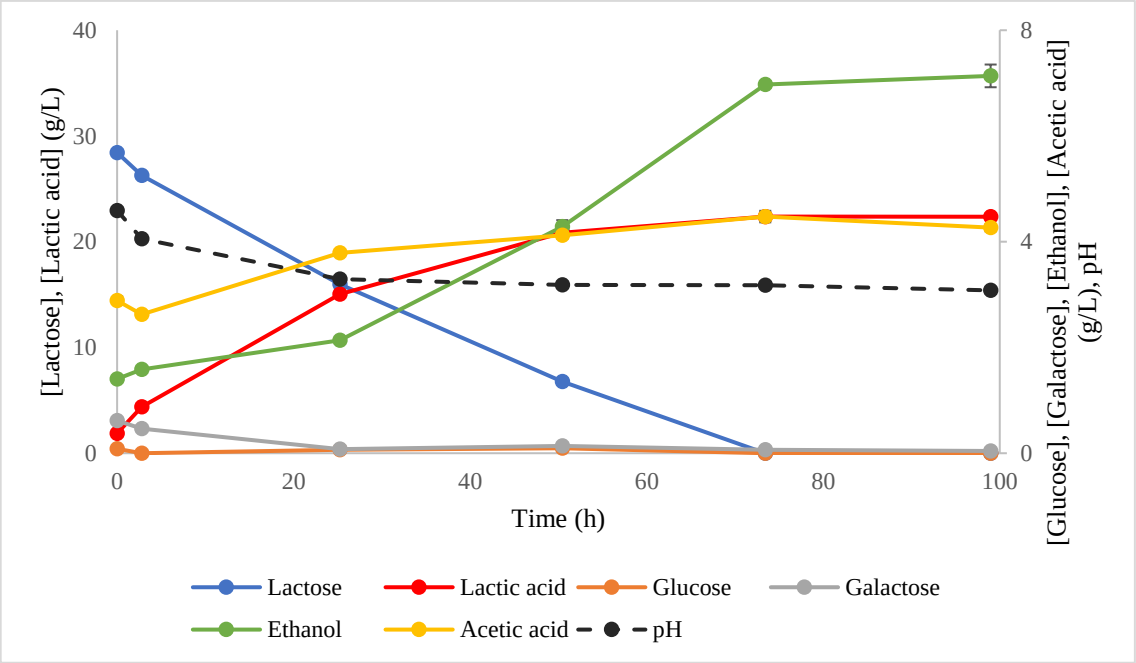

Figure 28. Lactose, lactic acid, glucose, galactose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of CWCD medium, at 80 rpm.

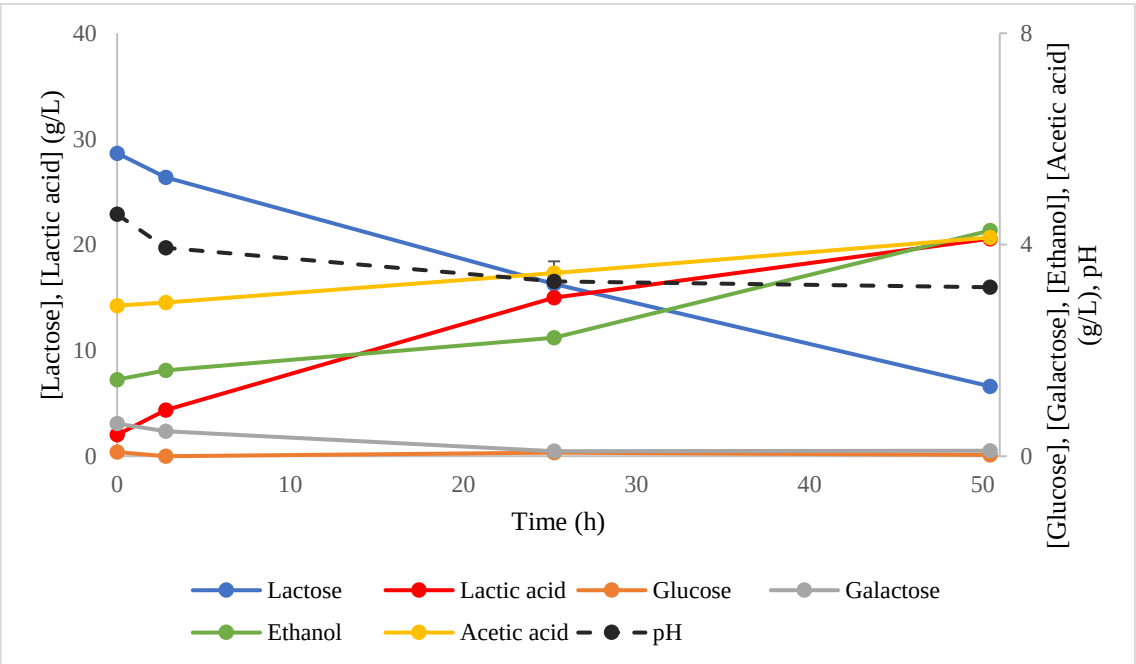

Figure 29. Lactose, lactic acid, glucose, galactose, ethanol and acetic acid concentrations and pH values, during 50 h of batch fermentation of CWCD medium, at 80 rpm.

Table 16. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, during 100 and 50 h of batch fermentation of CWCD medium, at 80 rpm.

Parameters	Fermentation time	
	100 h	**50 h**
Kefiran concentration (g/L)	0.690 ± 0.020	0.470 ± 0.040
$r_{kefiran}$ (g/L.day)	0.167 ± 0.005	0.224 ± 0.019
$Y_{kefiran/substrate}$ (g/g)	0.024 ± 0.001	0.021 ± 0.001
Lactic acid production (g/L)	20.64 ± 0.231	18.54 ± 0.118
$r_{lactic\ acid}$ (g/L.h)	0.248 ± 0.038	0.367 ± 0.002
$Y_{lactic\ acid/substrate}$ (g/g)	0.726 ± 0.009	0.842 ± 0.016
Lactose consumption (g/L)	28.43 ± 0.044	22.04 ± 0.552
$r_{substrate}$ (g/L.h)	0.387 ± 0.000	0.431 ± 0.011
Kefir grains biomass variation (%)	- 0.89 ± 2.18	+ 15.70 ± 0.56

The production of kefiran using CWCD as the substrate resulted in better results than using CWC, with a concentration of 0.690 g/L in the 100 h assay, which was the highest concentration so far in this study, using cheese whey (**Table 16**). Additionally, this kefiran concentration was very close to the one obtained by Ghasemlou *et al*. (2012) in an assay with pH controlled at 5.5, using cheese whey supplemented with yeast extract (0.700 g/L of kefiran), as presented in **Table 3**, therefore, the results obtained in this work, without pH control, seem promising at the industrial level. The pH values at the end of the assays were 3.08 and 3.20, after 100 and 50 h of fermentation, respectively, which are even lower than the ones presented earlier in this study.

The best productivity yet in this work, with cheese whey, 0.224 g/L.day, was also attained with CWCD, this time in the assay with the lower duration of 50 h. The yield of kefiran on substrate was very close for both fermentation times.

In the longer assay with CWCD, due to the depletion of lactose, kefir grains suffered a slight loss of biomass (0.89 %). On the other hand, in the 50 h fermentation, a significant growth was observed (15.70 %).

Finally, cheese whey from sheep's milk, CWS, with an initial lactose concentration of approximately 35 g/L was tested for kefiran production with 50 h and 100 h of fermentation. As expected, similarly to what happened with CWCD, in the assay of 100 h lactose was depleted before 70 h (**Figure 30**). Lactic acid production (20.46 g/L) was almost the same as in CWCD, and was, in turn, lower than with CWC, for the same fermentation time. This cheese whey sample had a considerably higher initial pH value, 5.26, which had decreased to 3.24 at the end of the fermentation. As usual, the concentrations of glucose and galactose were close to zero during the entire assay. Acetic acid was produced in a lower amount (1.98 g/L), while the production of ethanol (10.05 g/L) was higher than in the assays using CWCD.

In the assay with 50 h of duration (**Figure 31**), 31.02 g/L of lactose were consumed, and 19.61 g/L of lactic acid were produced, both higher values when compared with the results obtained with CWCD, while pH ranged from 5.23 to 3.31. The concentrations of glucose, galactose and acetic acid did not diverge significantly from previous assays, but it is worth noting that the production of ethanol (7.35 g/L) was more than two times higher than the one seen for CWCD, for the same duration.

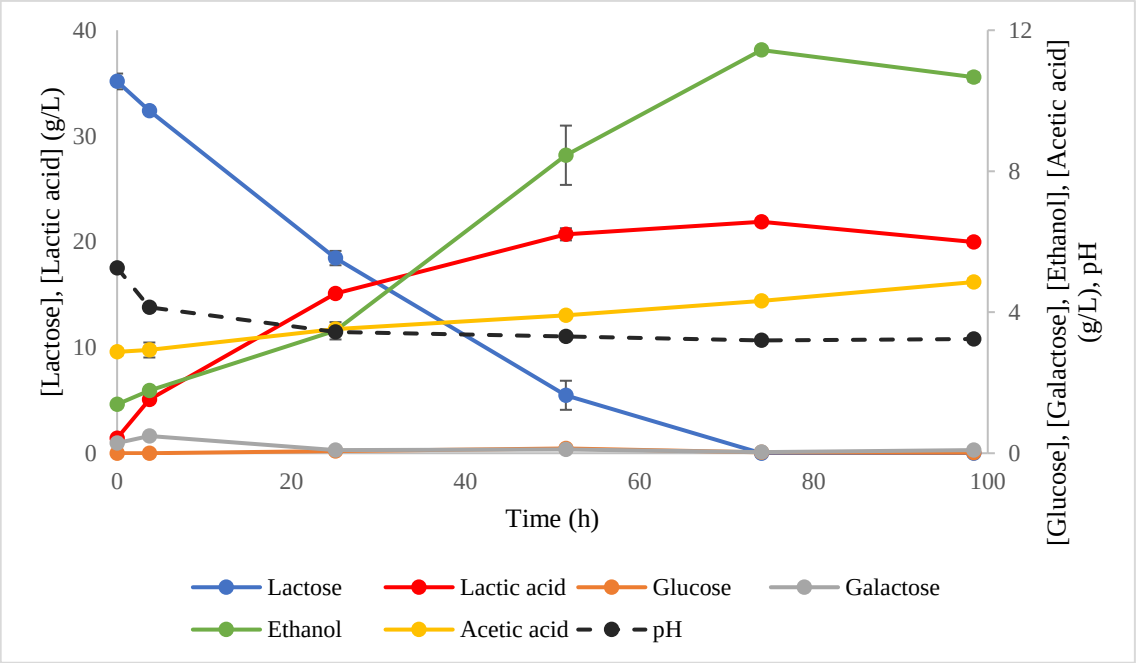

Figure 30. Lactose, lactic acid, glucose, galactose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of CWS medium, at 80 rpm.

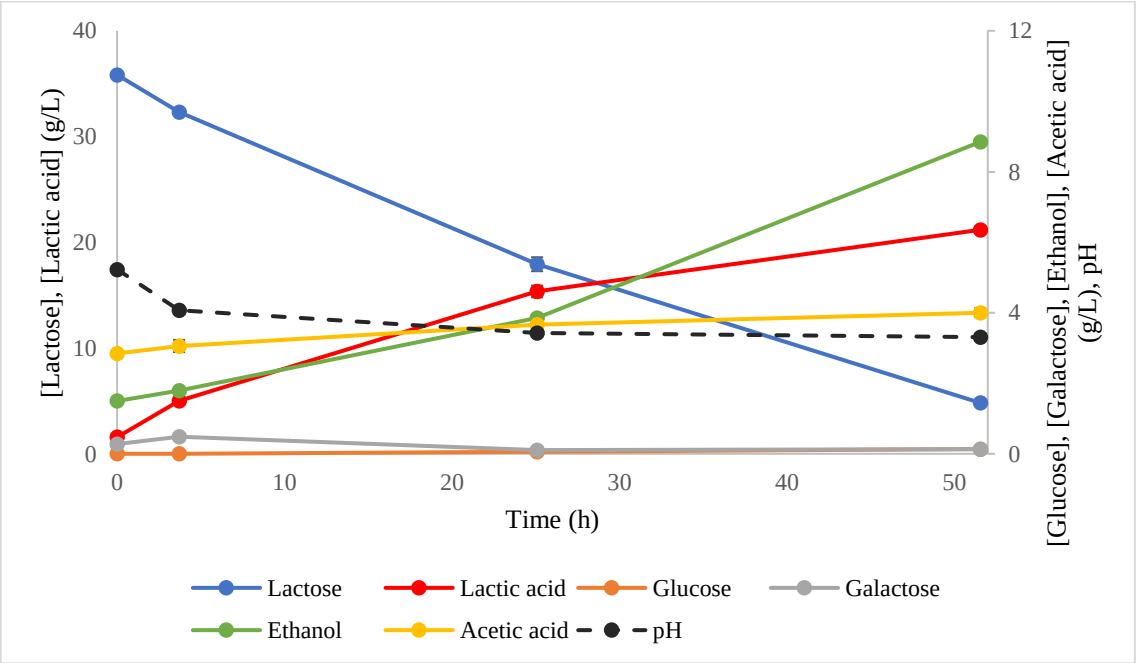

Figure 31. Lactose, lactic acid, glucose, galactose, ethanol and acetic acid concentrations and pH values, during 50 h of batch fermentation of CWS medium, at 80 rpm.

Table 17. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, during 100 and 50 h of batch fermentation of CWS medium, at 80 rpm.

Parameters	Fermentation time	
	100 h	**50 h**
Kefiran concentration (g/L)	0.315 ± 0.025	0.160 ± 0.020
$r_{kefiran}$ (g/L.day)	0.077 ± 0.006	0.074 ± 0.009
$Y_{kefiran/substrate}$ (g/g)	0.009 ± 0.001	0.005 ± 0.001
Lactic acid production (g/L)	20.46 ± 0.069	19.61 ± 0.204
$r_{lactic\ acid}$ (g/L.h)	0.275 ± 0.003	0.373 ± 0.003
$Y_{lactic\ acid/substrate}$ (g/g)	0.582 ± 0.010	0.632 ± 0.009
Lactose consumption (g/L)	35.16 ± 0.746	31.02 ± 0.131
$r_{substrate}$ (g/L.h)	0.488 ± 0.011	0.597 ± 0.002
Kefir grains biomass variation (%)	+ 7.49 ± 1.89	+ 26.48 ± 0.61

The CWS medium provided the worst results, in terms of kefiran production, of all cheese whey samples tested, for both fermentation times, as shown in **Table 17**. This could be explained by the higher conductivity value observed, 15.30 mS, which is related to a higher concentration of salts in this cheese whey sample (**Table 7**). The concentration of kefiran attained during the 100 h fermentation (0.315 g/L) was nearly double of what was produced in the assay that lasted for 50 h (0.160 g/L). As the assay duration is exactly half the fermentation time of the former, the productivities were almost identical in both assays. Nonetheless, the kefiran concentration obtained after 100 h of fermentation, 0.315 g/L, was still higher than the values obtained by Rimada and Abraham (2003), where 0.225 g/L of kefiran were obtained in a fermentation of UHT skim milk and 0.266 g/L of kefiran were

obtained in a fermentation of deproteinised cheese whey, both results attained with the use of kefir grains and without pH control.

On the other hand, CWS was the cheese whey sample in which kefir grains were able to grow the most. Interestingly, the 50 h fermentation, which provided the worst result for kefiran concentration, also offered the best result for the growth of kefir grains (+ 26.48%) of all cheese whey samples tested.

4.3.2 Batch fermentation – Kefiran concentration over time

In all previous assays presented in this study, kefiran concentration could only be assessed at the end of each fermentation, due to the complexity of its extraction and quantification without damaging kefir grains. Therefore, in order to get a better understanding of when the production of kefiran begins and how does its concentration vary during the fermentation of cheese whey, an extra assay in batch mode was performed, using CWS, at 80 rpm.

This assay consisted in carrying out several replicas of fermentation simultaneously, by collecting the kefir grains of two of the glass jars at the end of each day, for 5 days, and extract the kefiran produced. Consequently, it was possible to evaluate the variation in kefiran concentration during the fermentation. As shown in **Figure 32.A**, the variation of the several compounds during fermentation resembles, very closely, the evolution observed in the previous assay using the same cheese whey sample and operational conditions. The most noticeable difference resides in the time it took for the depletion of lactose to occur, around 50 h. This can be explained by the fact that, in this assay, glass jars were left undisturbed inside the incubator, until the end of each fermentation time. In contrast, in the previous assays, all jars were opened every day for sampling. This disturbance probably resulted in oxygen transfer into the medium, which can affect the kefir grains and slow the lactose consumption, since some of the existing microbial species in kefir grains were described as microaerophiles or facultative anaerobes (Cheirsilp and Radchabut, 2011; Fujisawa et al., 1988).

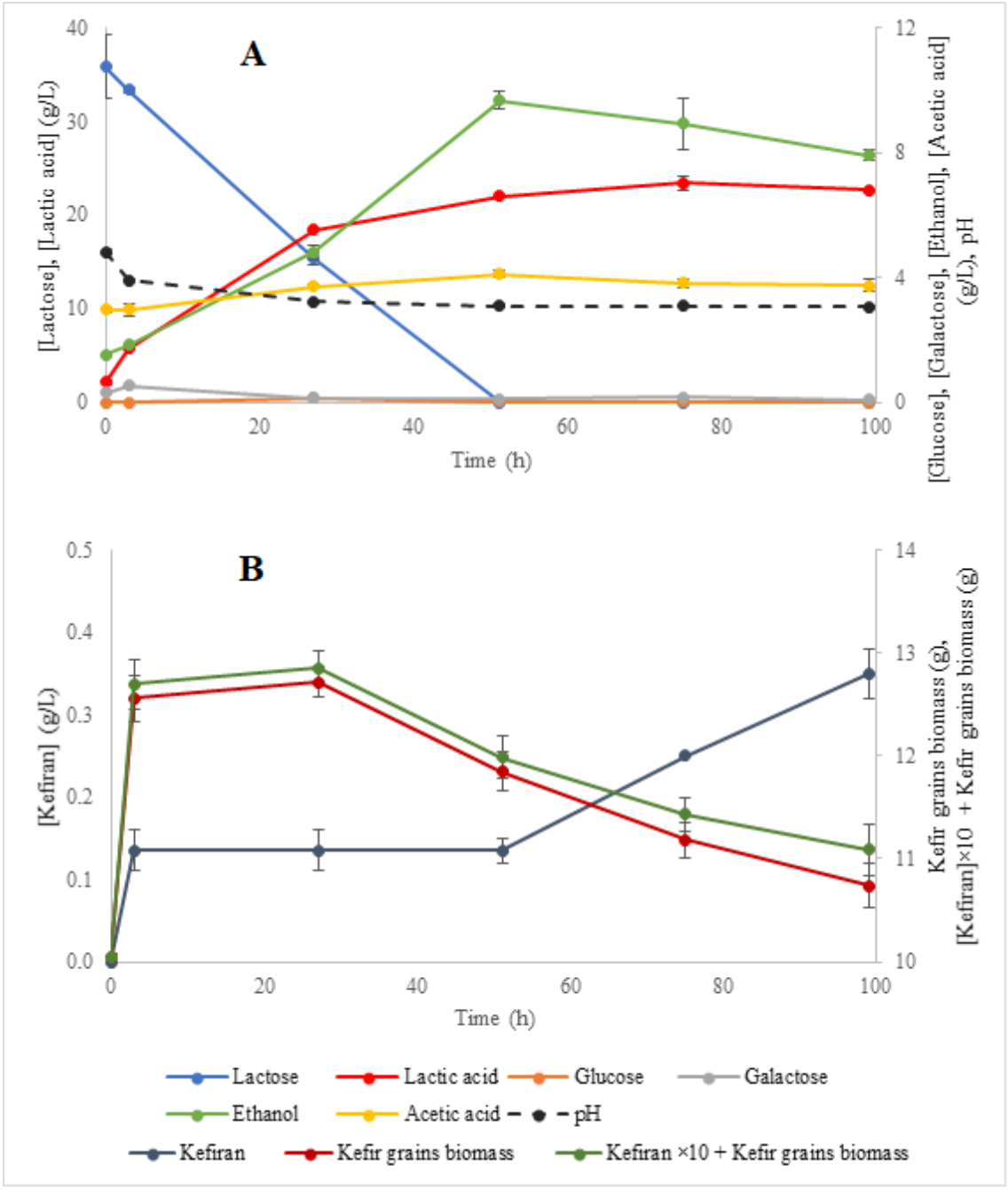

Figure 32. Lactose, lactic acid, glucose, galactose, ethanol and acetic acid concentrations and pH values, during 100 h of batch fermentation of CWS medium, at 80 rpm. (**A**). Kefiran concentration and kefir grains biomass over the course of fermentation (**B**).

Table 18. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, with different times of batch fermentation of CWS medium, each one representing the end of each consecutive day, at 80 rpm.

Parameters	Fermentation time				
	3.05 h (day 1)	**27.03 h (day 2)**	**51.17 h (day 3)**	**75.08 h (day 4)**	**99.15 h (day 5)**
Kefiran concentration (g/L)	0.135 ± 0.025	0.135 ± 0.025	0.135 ± 0.015	0.250 ± 0.000	0.350 ± 0.030
$r_{kefiran}$ (g/L.day)	1.062 ± 0.1967	0.120 ± 0.0222	0.063 ± 0.0070	0.080 ± 0.0000	0.085 ± 0.0073
$Y_{kefiran/substrate}$ (g/g)	0.056 ± 0.0162	0.007 ± 0.0015	0.004 ± 0.0004	0.007 ± 0.0000	0.010 ± 0.0008
Lactic acid production (g/L)	3.59 ± 0.082	16.26 ± 0.057	19.86 ± 0.124	21.29 ± 0.821	20.58 ± 0.025
$r_{lactic\ acid}$ (g/L.h)	1.177 ± 0.0269	0.602 ± 0.0021	0.388 ± 0.0024	0.284 ± 0.0109	0.208 ± 0.0003
$Y_{lactic\ acid/substrate}$ (g/g)	1.473 ± 0.1931	0.798 ± 0.0299	0.554 ± 0.0035	0.594 ± 0.0229	0.574 ± 0.0007
Lactose consumption (g/L)	2.47 ± 0.268	20.42 ± 0.838	35.85 ± 0.000	35.85 ± 0.000	35.85 ± 0.000
$r_{substrate}$ (g/L.h)	0.811 ± 0.0880	0.756 ± 0.0310	0.701 ± 0.0000	0.477 ± 0.0000	0.362 ± 0.0000
Kefir grains biomass variation (%)	+ 25.29 ± 2.26	+ 26.75 ± 1.21	+ 17.98 ± 1.82	+ 11.24 ± 1.69	+ 6.60 ± 2.24

The results shown in **Table 18** demonstrate that immediately after inoculation, and probably due to the stirring (80 rpm), a significant portion of the capsular kefiran must have been released from the kefir grains' surface, into the medium, which explains the 0.135 g/L broth kefiran concentration observed after only 3.05 h of fermentation. Additionally, as observed in **Figure 32.B**, kefiran concentration was kept constant for the first 50 h, and then started to increase, which may indicate that the kefir grains stopped the release of kefiran into the medium after the first impact. Yet, the grains also grew considerably after the initial 3.05 h (25.29%), which was surprising, since approximately half of their dry weight is considered relative to kefiran (Exarhopoulos et al., 2018a). Thus, due to the release of kefiran after inoculation, a decrease in their biomass was expected, and not the opposite. This might be explained by the role of kefiran in the kefir grains' growth and development, a phenomenon which may be emphasised at the early stages of fermentation. However, there seemed to be a turning point, when kefir grains slowed their growth and began to release kefiran into the medium, losing biomass in the process. In this assay, this point was somewhere between 50 and 75 h of fermentation, as seen in **Figure 32.B**. These results are in accordance with those obtained by Cheirsilp (2006). During kefiran production by a pure culture of *L. kefiranofaciens*, this author observed an increase in the concentration of capsular kefiran with cell growth and a decrease when the growth stopped, probably due to the excretion of kefiran into the medium.

Kefiran productivity and yield on substrate, as shown in **Table 18**, were extremely high in day 1, when compared to the subsequent days. Logically, these values were also considering the capsular kefiran released into the medium immediately after inoculation, as explained before, while the following days were only quantifying the broth kefiran, produced and excreted into the medium after the first initial impact.

4.3.3 Fed-batch fermentation

Generally, a fed-batch fermentation results in improved production values, when compared to batch operation mode, particularly when microorganisms are inhibited by high concentrations of substrate. Fed-batch mode allows the supplying of an increasing amount of substrate in time-spaced pulses. Several fed-batch fermentations were carried out, using the different cheese whey samples previously tested in batch (CWC, CWCD and CWS), in

order to evaluate their effect on the production of kefiran, with kefir grains. In these assays, two pulses of substrate were added, each one consisting of 50 mL of the corresponding cheese whey sample. HPLC analysis was performed for all fermentation samples, but both kefiran concentration and kefir grains biomass were only assessed at the end of each fed-batch fermentation.

The fed-batch fermentation of the CWC medium lasted for 510 h, due to the higher lactose concentration. It is relevant to note that the sudden increase in the concentration of lactose and the drops in the concentration of lactic acid, ethanol and acetic acid after the addition of a pulse of substrate, were a result of medium dilution. The first pulse of substrate was added after 167 h of fermentation, which was, approximately, the time it took to deplete the substrate in the corresponding assays in batch mode, as shown previously, in **Figure 27**. However, in this assay, this duration was not enough to completely consume the substrate, as can be observed in **Figure 33**. Before the first pulse, lactic acid was produced in very high amounts (32.28 g/L), very similar to what occurred in the batch fermentation with the same medium and conditions. It was also only during this period of time that lactic acid was actually produced, since its concentration began to slightly decrease after the first pulse of substrate. Similarly, the concentrations of acetic acid, glucose and galactose did not considerably vary over the course of the assay, while, on the other hand, ethanol concentration continued to increase until the end of the fermentation, since yeasts are known to have higher resistance to a low pH, when comparing to LAB. In order to add the second pulse of substrate, samples were injected daily into the HPLC, until a low concentration of lactose was reached, which happened after 341 h of fermentation, when one of the replicas (jar 2) shown a depletion of lactose in the medium. However, the other replica still had a considerable amount of lactose (23.93 g/L), which led to a very noticeable difference in its concentration in the period between both pulses, as seen in **Figure 33**. After the second pulse, the same situation occurred, with a much more pronounced lactose consumption in one replica (this time in jar 1) than in the other. The final concentration of ethanol was also very distinct between replicas (32.24 g/L in jar 1 and 22.10 g/L in jar 2), with higher production in the jar where the consumption of lactose was also superior. Due to medium dilution, caused by the addition of the substrate pulses, pH value at the end of this fed-batch fermentation (510 h) was at 3.56, which was considerably higher than the value observed after 220 h of batch fermentation of CWC (3.31), in the same operational conditions.

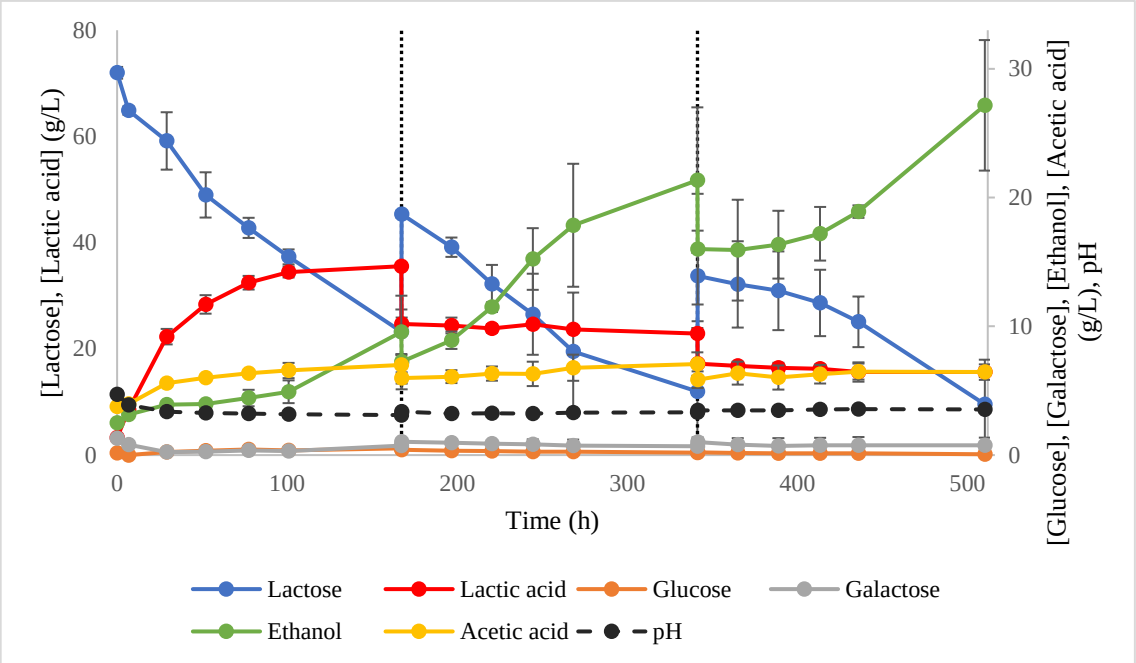

Figure 33. Lactose, lactic acid, glucose, galactose, ethanol and acetic acid concentrations and pH values, during 510 h of fed-batch fermentation of CWC medium, at 80 rpm. Each dotted vertical line corresponds to a pulse of substrate.

As shown in **Table 19**, the fed-batch fermentation of the CWC medium resulted, up to this point, in the highest kefiran concentration, 1.190 g/L, using a cheese whey sample. While this was a promising result, the extremely long assay duration (510 h) also needs to be considered, since the concentration of kefiran in the medium is deeply connected to the fermentation time, as previously discussed. However, this higher kefiran concentration may also be related to the addition of each pulse, diluting the lactic acid present in the medium which, in turn, leads to a considerable rising in pH, alleviating its inhibition potential upon the LAB, which did not occur in the batch fermentations, where the effect of pH inhibition must have had a greater impact. It is an established fact that the accumulation of lactic acid and the consequent exposure to a low pH inhibits the growth of LAB, effectively lowering kefiran production (Cheirsilp et al., 2018). Even with the physical protection conferred by the kefiran-protein matrix, LAB still get significantly inhibited when kefir grains are exposed to a low pH during a long period of time. Due to the long assay duration, kefiran productivity was lower compared to the assays in batch mode (**Table 15**). Additionally, during this entire fed-batch fermentation, 106.37 g/L of lactose were consumed, which explains the somewhat

low yield of kefiran on substrate value. Given the assay duration, even with available lactose in the medium, kefir grains lost some of their biomass (8.48 %).

Table 19. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, during 510 h of fed-batch fermentation of CWC medium, at 80 rpm.

Parameters	**After fed-batch fermentation (510 h)**		
Kefiran concentration (g/L)	1.190 ± 0.130		
$r_{kefiran}$ (g/L.day)	0.056 ± 0.0061		
$Y_{kefiran/substrate}$ (g/g)	0.011 ± 0.0001		
Kefir grains biomass variation (%)	- 8.48 ± 3.52		
Parameters	**Before pulse 1 (167 h)**	**After pulse 1 and before pulse 2 (174 h)**	**After pulse 2 (169 h)**
Lactic acid (g/L)	production of 32.28 ± 0.162	consumption of 1.84 ± 0.625	consumption of 2.04 ± 1.207
$r_{lactic\ acid}$ (g/L.h)	0.187 ± 0.0009	- 0.010 ± 0.0050	- 0.018 ± 0.0138
$Y_{lactic\ acid/substrate}$ (g/g)	0.670 ± 0.0694	- 0.069 ± 0.0423	- 0.234 ± 0.2140
Lactose consumption (g/L)	48.77 ± 5.299	33.41 ± 11.322	24.19 ± 16.945
$r_{substrate}$ (g/L.h)	0.282 ± 0.0330	0.199 ± 0.0738	0.144 ± 0.0996

The fed-batch fermentation of the CWCD medium only lasted for 167 h, since its initial lactose concentration (26.81 g/L) was much lower than the one found in the CWC medium (71.99 g/L). Again, the first pulse was decided according to the previous assays in batch mode (**Figure 28**), which was added after 52 h. As seen in **Figure 34**, the substrate was somewhat close to depletion at this point, while lactic acid concentration was already

considerably high (22.22 g/L). Contrary to the previous fed-batch assay with CWC, where it completely stopped after the first pulse, the production of lactic acid continued for the full course of fermentation, leading to the pH value of 3.15 at the end of the assay. The concentrations of glucose, galactose and acetic acid, on the other hand, were practically constant, from the beginning to the end, identical to what was observed in the previous assay. As expected, ethanol concentration kept rising, which is in accordance with all assays mentioned before, reaching a final concentration of 9.65 g/L. The second pulse of substrate was added after 101 h of fermentation and, by the next medium sample taken, 66 h after the pulse, lactose was depleted.

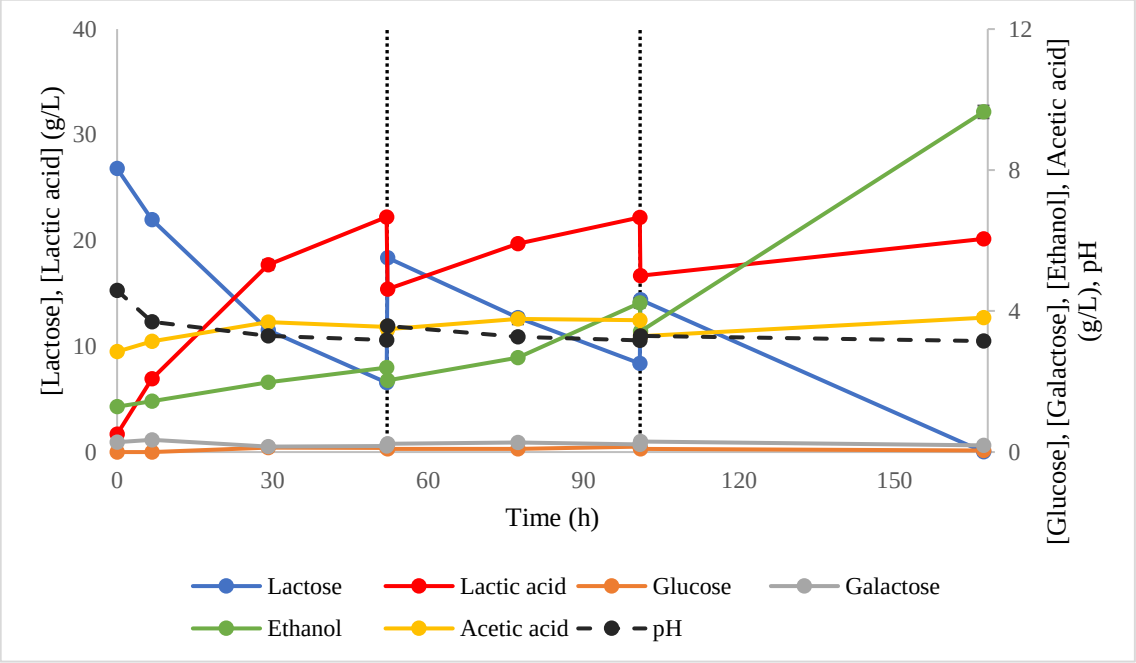

Figure 34. Lactose, lactic acid, glucose, galactose, ethanol and acetic acid concentrations and pH values, during 167 h of fed-batch fermentation of CWCD medium, at 80 rpm. Each dotted vertical line corresponds to a pulse of substrate.

As seen in **Table 20**, the kefiran concentration obtained after the fed-batch fermentation of CWCD (0.495 g/L) was much lower than with its undiluted version, CWC (1.190 g/L). It was also significantly inferior than in its 100 h batch counterpart (0.690 g/L), and only slightly higher when compared to the 50 h batch fermentation (0.470 g/L), in the

same conditions (**Table 16**). Moreover, because of the longer assay duration, kefiran productivity was much lower in the fed-batch fermentation, as well as the kefiran yield on substrate, due to the much higher total lactose consumption in this assay (44.56 g/L). Despite the long term fermentation, kefir grains were able to grow considerably (15.76 %), which may be explained by the renewing of lactose in the medium and the increase in pH, both resulting from the addition of the substrate pulses.

Table 20. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, during 167 h of fed-batch fermentation of CWCD medium, at 80 rpm.

Parameters	**After fed-batch fermentation (167 h)**		
Kefiran concentration (g/L)	0.495 ± 0.095		
$r_{kefiran}$ (g/L.day)	0.071 ± 0.0136		
$Y_{kefiran/substrate}$ (g/g)	0.011 ± 0.0021		
Kefir grains biomass variation (%)	+ 15.76 ± 0.20		
Parameters	**Before pulse 1 (52 h)**	**After pulse 1 and before pulse 2 (49 h)**	**After pulse 2 (66 h)**
Lactic acid production (g/L)	20.53 ± 0.027	6.78 ± 0.374	3.47 ± 0.086
$r_{lactic\ acid}$ (g/L.h)	0.390 ± 0.0016	0.140 ± 0.0076	0.052 ± 0.0013
$Y_{lactic\ acid/substrate}$ (g/g)	1.014 ± 0.0084	0.682 ± 0.0382	0.242 ± 0.0006
Lactose consumption (g/L)	20.24 ± 0.140	9.94 ± 0.009	14.38 ± 0.324
$r_{substrate}$ (g/L.h)	0.386 ± 0.0022	0.205 ± 0.0007	0.217 ± 0.0049

Lastly, the fed-batch fermentation of the CWS medium was carried out, lasting for 167 h. Due to the similar lactose concentrations between CWS and CWCD, as well as nearly identical lactose depletion times achieved in the batch fermentations, both pulses of substrate were added at the same fermentation times as before, 52 and 101 h, respectively. The concentration of the analysed compounds over time (**Figure 35**) was similar to what was seen in the previous assay, and the final pH value was of 3.10. Lactose concentration was higher than expected when the first pulse was added, which is explained by the lower substrate consumption rate in this period of time (0.384 g/L.h), when compared to the values seen in **Table 17**, relative to the assays in batch mode, using the same medium and in the same fermentation conditions. This concentration was lower when the second pulse was added and, by the end of the assay, after 167 h, lactose had been completely consumed. Glucose and galactose concentrations were, again, both very low for the entire fermentation, while acetic acid was produced more notoriously than in the previous assay (3.66 g/L and 1.76 g/L, respectively). The concentration of ethanol at the end of this fermentation (11.31 g/L) was also higher than in the assay with CWCD, but not by a very noticeable margin.

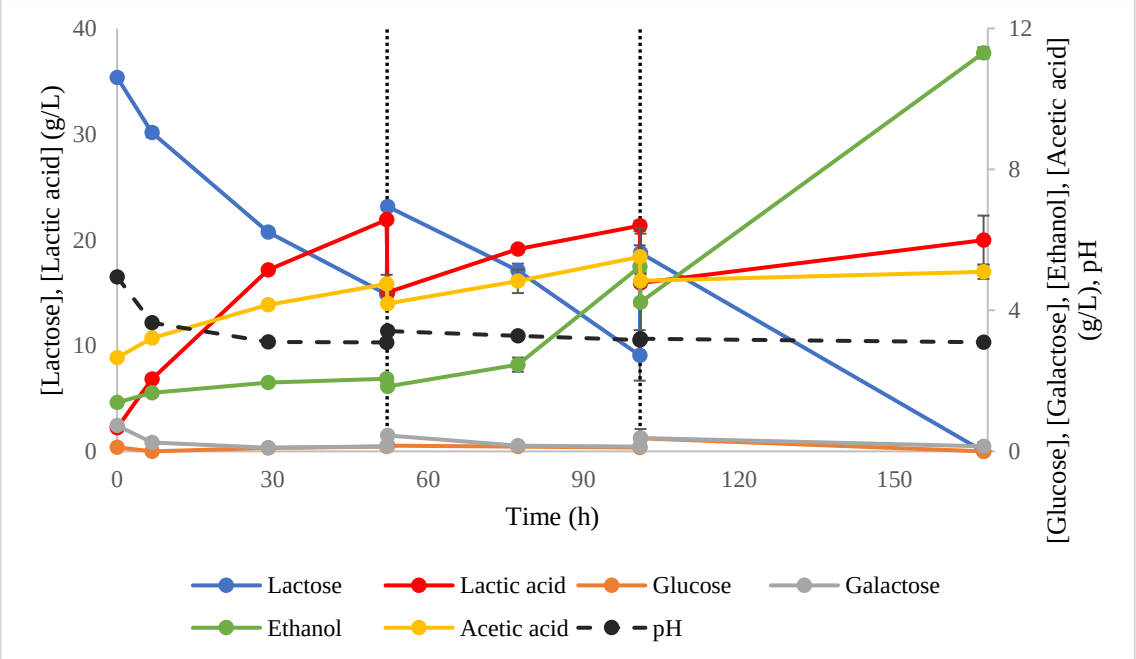

Figure 35. Lactose, lactic acid, glucose, galactose, ethanol and acetic acid concentrations and pH values, during 167 h of fed-batch fermentation of CWS medium, at 80 rpm. Each dotted vertical line corresponds to a pulse of substrate.

Table 21. Parameters for kefiran and lactic acid production, lactose consumption and kefir grains biomass variation, during 167 h of fed-batch fermentation of CWS medium, at 80 rpm.

Parameters	**After fed-batch fermentation (167 h)**
Kefiran concentration (g/L)	0.535 ± 0.075
$r_{kefiran}$ (g/L.day)	0.077 ± 0.0108
$Y_{kefiran/substrate}$ (g/g)	0.010 ± 0.0014
Kefir grains biomass variation (%)	+ 9.82 ± 0.69

Parameters	**Before pulse 1 (52 h)**	**After pulse 1 and before pulse 2 (49 h)**	**After pulse 2 (66 h)**
Lactic acid production (g/L)	19.68 ± 0.118	6.29 ± 0.341	4.07 ± 2.141
$r_{lactic\ acid}$ (g/L.h)	0.376 ± 0.0003	0.130 ± 0.0069	0.061 ± 0.0323
$Y_{lactic\ acid/substrate}$ (g/g)	0.959 ± 0.0125	0.460 ± 0.0910	0.208 ± 0.0939
Lactose consumption (g/L)	20.51 ± 0.145	14.07 ± 2.041	18.76 ± 1.827
$r_{substrate}$ (g/L.h)	0.384 ± 0.0038	0.289 ± 0.0416	0.284 ± 0.0276

It is shown in **Table 21** that kefiran concentration (0.535 g/L) was slightly higher than in the previous assay with CWCD (0.495 g/L), but considerably superior than the one obtained after 100 h of batch fermentations (0.315 g/L), using the same CWS medium and under identical conditions (**Table 17**). Kefiran productivity matched the one achieved in the 100 h batch assay, while kefiran yield on substrate was slightly higher in the fed-batch fermentation, even with the substantially superior total lactose consumption (53.34 g/L and 35.16 g/L, respectively). Kefir grains were, once more, able to grow in a long fermentation (9.82 %), even though not as much as in the previous assay with the CWCD medium, possibly due to the reasons mentioned before.

On a final note, in all fed-batch fermentations, the more noticeable variations in terms of lactic acid production, productivity and yield on substrate, as well as substrate consumption and consumption rate, were observed on the period of time before the first pulse, slowing down after that point, which is in accordance with the results obtained in the fermentations in batch mode, where, in general, both production and consumption rates tend to be inversely proportional to the fermentation time. This can be explained, once more, by the severe inhibition caused by a low pH resulting from the accumulation of lactic acid in the medium, which usually aggravates over the course of fermentation, slowing consumption and production rates.

4.4 Kefiran characterisation

The determination characteristics of kefiran is very important to assess its functionality and behaviour in order to assess its possible applications.. Additionally, these characteristics are known to have a role in the interactions between kefiran and other biopolymers, such as milk proteins (Exarhopoulos et al., 2018a), and in determining its ability to form kefiran-based hydrogels and scaffolds. With this in mind, lyophilised kefiran samples **(Figure 36**) were characterised by two distinct analytical techniques, FTIR and SEM, in order to better understand the characteristics of the obtained exopolysaccharide.

Figure 36. Lyophilised kefiran sample, produced through the fermentation of cheese whey by kefir grains.

4.4.1 Glucose to galactose ratio quantification

At the end of the downstream processing, samples were hydrolysed and analysed by HPLC, as detailed in **Chapter 3.8.1**. Then, the glucose to galactose ratio was assessed. No lactose residues were detected in any of the hydrolysed kefiran samples. The analysed kefiran sample produced from MRSL broth medium was obtained after 100 h of batch fermentation of MRSL broth with 120 g/L of lactose, at 80 rpm, while kefiran samples produced from cheese whey were obtained after 100 h of batch fermentation of the respective cheese whey medium, at 80 rpm. The ratio of glucose to galactose in the structure of kefiran is known to be approximately 1.00 : 1.00 (Exarhopoulos et al., 2018a; Piermaría et al., 2016), such as the ratios of 1.00 : 1.05 attained by Maeda *et al*. (2004) and of 1.00 : 0.90, obtained by Exarhopoulos *et al*. (2018a), as seen in **Table 4**, which were very close to what was observed in the samples produced from each different medium used in this study (**Table 22**).

Table 22. Glucose to galactose ratio of the kefiran samples produced with each different fermentation medium.

Fermentation medium	Glucose : Galactose
MRSL broth	1.00 : 0.89
CWC	1.00 : 0.95
CWCD	1.00 : 0.82
CWS	1.00 : 0.98

4.4.2 Fourier-transform infrared spectroscopy

Infrared spectroscopy analysis was performed as a method of polysaccharide identification, and as a technique of identification of the fundamental chemical groups in kefiran structure. These groups define the physical behaviour of polysaccharides and are one of the major factors, which dictate the range of applications of kefiran in the various fields (Radhouani et al., 2018a). FTIR was applied to kefiran samples produced with the different media, which provided very similar infrared spectrums, hence, only the spectrum for kefiran

produced after 100 h of batch fermentation of CWC, at 80 rpm (**Figure 37**) is discussed, while the other spectra can be seen in the **Appendix**. Furthermore, these results were also in accordance with what was reported by Radhouani *et al*. (2018a). As shown in **Figure 37**, different bands are associated with specific fundamental groups. The band at 3300 cm^{-1} is related to hydroxyl groups, since this band region is usually attributed to the stretching vibration of O–H in the sugar residues. The band observed at 2920 cm^{-1} (stretching vibration of C–H) can be associated with methyl and methylene groups. The band seen at 1640 cm^{-1} is also related to the stretching vibration of O–H, which is akin to hydroxyl groups. The band at 1450 cm^{-1} corresponds to CH_2 groups. Finally, the band observed at 1130 cm^{-1} is associated with the stretching vibration of C–O–C. It is important to note that the band seen at 2360 cm^{-1} corresponds to the atmospheric CO_2 in the room where this technique was performed, therefore, it is not related to kefiran.

Reactive functional groups in polysaccharides, such as kefiran, confer more flexibility to several modifications, which is very important for applications in the medical field. In addition, hydrogels can also be effortlessly obtained, making these polysaccharides promising candidates for applications in the tissue engineering and regenerative medicine fields (Radhouani et al., 2018a).

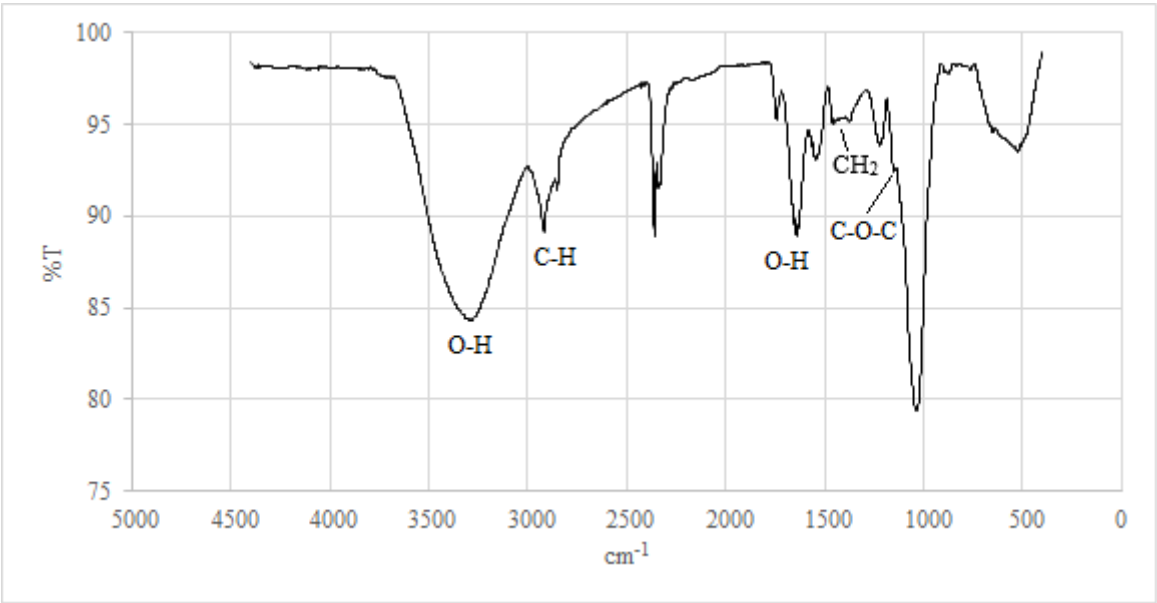

Figure 37. Infrared spectrum of kefiran between 500 and 4000 cm^{-1}, produced after 100 h of batch fermentation of CWC, at 80 rpm.

4.4.3 Scanning electron microscopy

Kefiran samples, obtained after 100 h of batch fermentation of MRSL broth medium with 80 g/L of lactose, at 80 rpm, were also analysed by SEM in order to observe the microstructure of this polysaccharide. As can be observed in **Figure 38**, its surface is neither smooth nor regular, and resembles a foam-like structure. Additionally, these kefiran samples, obtained after lyophilisation, can be considered scaffolds, presenting a high level of porosity. This is a very desirable feature in scaffolds, since it guarantees cellular penetration, proliferation and differentiation, while providing a porous network for the formation of new tissue, making kefiran-based scaffolds a great candidate in the development of drug delivery systems and tissue engineering applications (Radhouani et al., 2019).

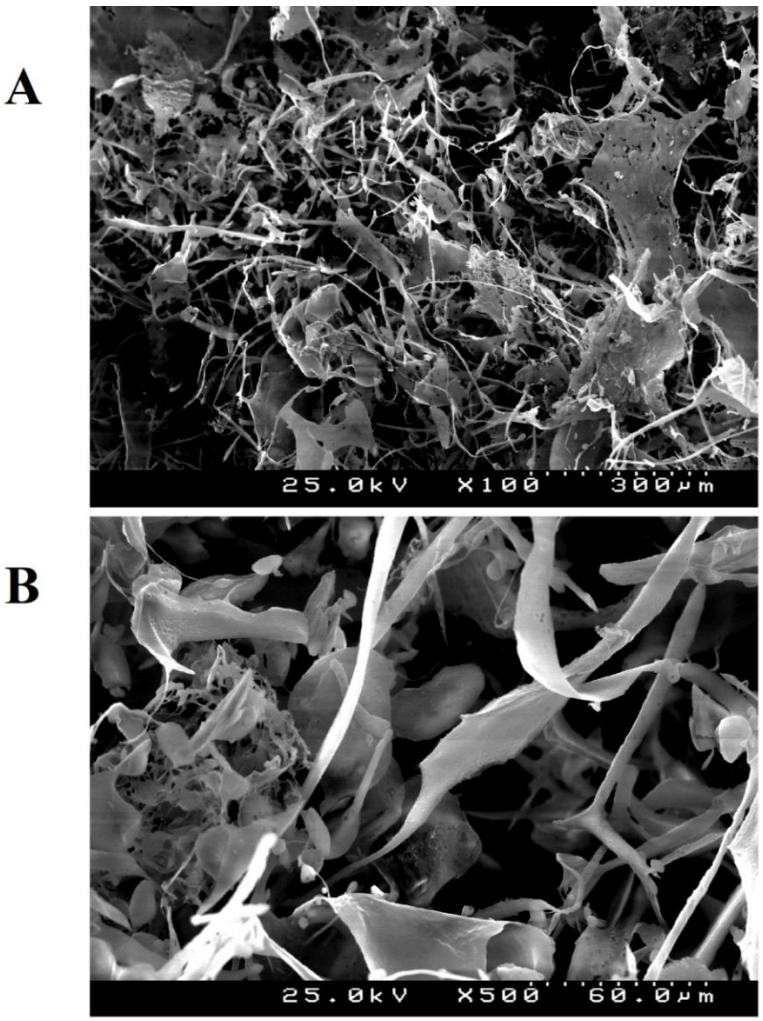

Figure 38. Scanning electron micrographs of kefiran, with a magnification of ×100 (**A**) and ×500 (**B**).

4.5 Kefiran-based films

An attempt to produce kefiran-based films was carried out at the end of this study. Unfortunately, as this polysaccharide was extracted in very low quantities, it was difficult to replicate the procedure described by Piermaria *et al*. (2009), thus, only kefiran produced with MRSL broth medium was used (**Figure 39.A**), since its availability was higher. Due to the low volume of the film-forming solution, illustrated in **Figure 39.B**, even with the addition of glycerol as a plasticiser, the resultant kefiran film was extremely thin and, as such, it was not possible to remove from the bottom of the beaker, after drying, without damaging it permanently, as seen in **Figure 40**. However, this preliminary effort has shown that by using a higher kefiran biomass and, consequently, a higher volume of filmogenic solution, it seems possible to produce kefiran-based films with the samples obtained in this study.

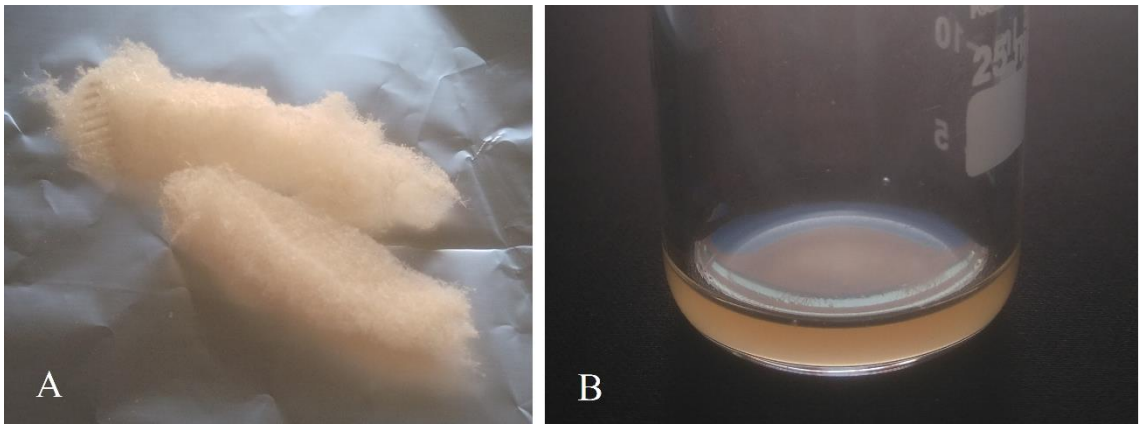

Figure 39. Kefiran produced after 100 h of batch fermentation of MRSL broth medium with 120 g/L of lactose, at 160 rpm (10 mg) (**A**) and the respective prepared filmogenic solution (1 g) (**B**).

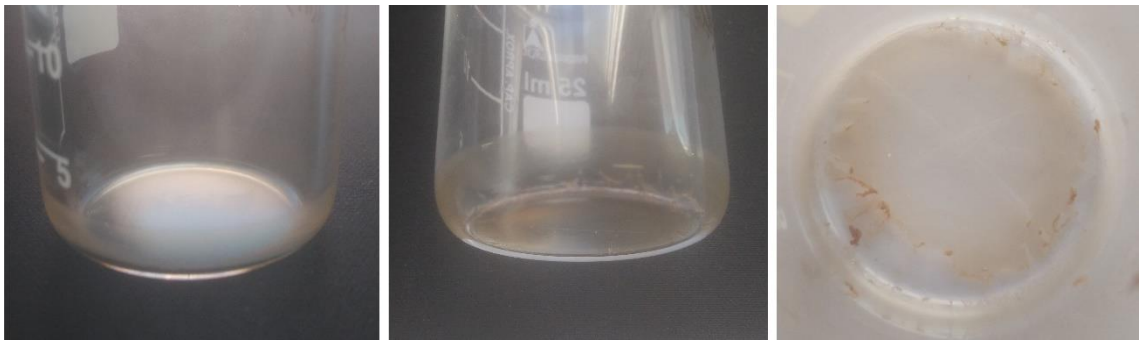

Figure 40. Kefiran-based film, showing an extremely low thickness.

5. Conclusion

This study showed that it is possible to produce kefiran using cheese whey, an industrial by-product, through fermentation by kefir grains. This is very relevant for the circular economy model, since cheese whey is massively produced worldwide, and dairy companies need to spend valuable monetary resources to treat it as an industrial effluent. Therefore, applications such as the one presented in this study, can significantly contribute to its valorisation.

Initially, this work aimed to optimise the process conditions, using MRSL broth as the synthetic medium. Concerning the fermentation time, the best results were achieved with the lower duration, 100 h, since it provides higher productivity values, which is the most important factor from an industrial point of view. On the other hand, it was also concluded that kefiran concentration was directly proportional to the fermentation time, while, in most cases, kefiran productivity was inversely proportional to it. Regarding stirring speed, the best results were always attained at 160 rpm. However, this stirring speed consecutively led to an excessive fragmentation of the kefir grains, which have a deeply negative impact on their biomass. Since the stirring speed of 80 rpm provided the second-best results, while maintaining the grains in a good condition, it was chosen as the optimal for all the following assays. Relatively to the optimal initial lactose concentration, this study was not entirely conclusive, since the best kefiran production results were dependant on the stirring speed. The best result in terms of kefiran concentration, 1.640 g/L, and the highest kefiran productivity, 0.390 g/L.day, were both achieved in the same assay, using a MRSL broth medium with an initial lactose concentration of 40 g/L, which fermented for 100 h, at 160 rpm.

Kefiran production values with cheese whey, while understandably lower than the ones obtained with the MRSL broth medium, were fairly promising. The best result in terms of kefiran concentration using a cheese whey sample, 1.190 g/L, was achieved in the fed-batch fermentation of the CWC medium, which lasted for 510 h, at 80 rpm. In contrast, the highest productivity value, 0.224 g/L.day, was attained in the batch fermentation of the CWCD medium, at 80 rpm, lasting for 50 h. Additionally, it seems that there is a point during fermentation when kefir grains slow their growth and begin to release kefiran into the medium, which matches the results achieved throughout the study. Finally, the data obtained

from the characterisation techniques used with this polysaccharide, is in accordance to the information published in the literature, indicating that kefiran was, in fact, successfully produced in this study.

6. Further work

There is still plenty of room for improvement regarding the production of kefiran from cheese whey. First and foremost, a fermentation with kefir grains in a bioreactor would be very interesting, since it provides significantly more control over the fermentation conditions, over time.

This study only focused on the use of kefir grains, a mixed culture, for kefiran production. Since the lactic acid bacterium *L. kefiranofaciens* is reported to be the main responsible for the production of kefiran in all of the kefir grains' microflora, it would be pertinent to do a fermentation using a pure culture of *L. kefiranofaciens*, in the same fermentation conditions used in this study, with the aim of increasing the production of kefiran. Afterwards, this fermentation using a pure culture could, similarly, be scaled-up to bioreactor scale.

The best results found in the literature were achieved using a co-culture of *L. kefiranofaciens* and *Saccharomyces cerevisiae*, in a bioreactor. This is due to the fact that this yeast is capable of consuming lactic acid, lowering its concentration and accumulation in the medium, effectively reducing its inhibition potential upon the kefiran-producing LAB, which significantly increases its production. Therefore, it would also be very relevant to carry out a fermentation with this specific co-culture, in a bioreactor, maximining the kefiran production values.

Kefiran samples should also be further characterised, by performing additional analytical techniques, such as GPC-SEC, DSC, TGA and NMR, to have a broader and more detailed understanding of its properties, which are crucial to evaluate target applications.

Lastly, another attempt to produce kefiran-based films should be carried out, using a higher volume of the film-forming solution, in order to attain thicker films. Additionally, similar to kefiran samples, kefiran-based films should also be characterised, to assess for their potential applications in the industry.

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8. Appendix

Appendix 1 - Lactose calibration curve

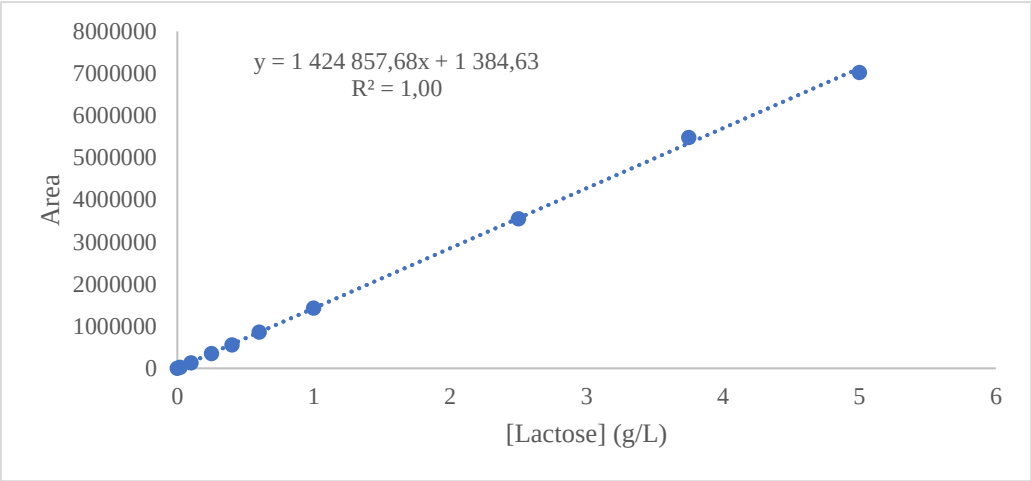

Figure 41. Lactose calibration curve, which relates the area of the peaks determined by HPLC with lactose concentration.

Appendix 2 - Lactic acid calibration curve

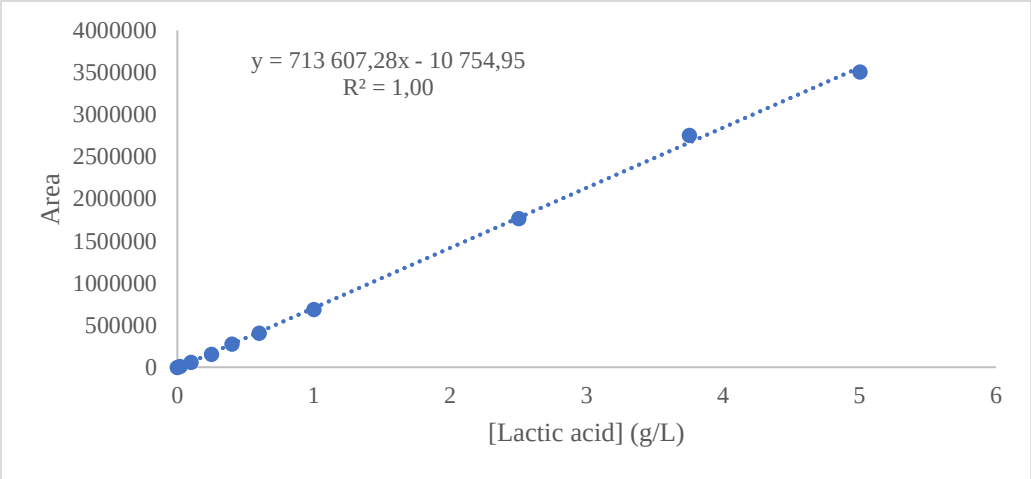

Figure 42. Lactic acid calibration curve, which relates the area of the peaks determined by HPLC with lactic acid concentration.

Appendix 3 - Glucose calibration curve

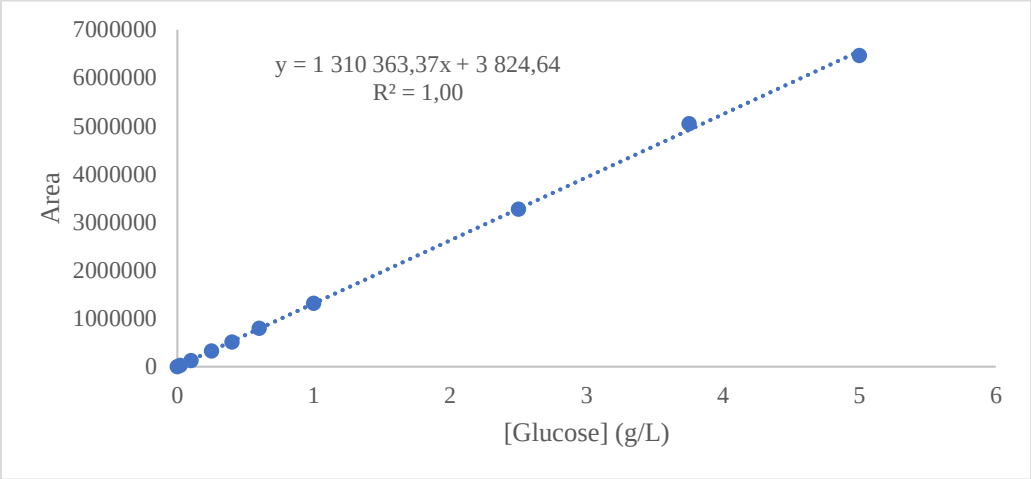

Figure 43. Glucose calibration curve, which relates the area of the peaks determined by HPLC with glucose concentration.

Appendix 4 - Galactose calibration curve

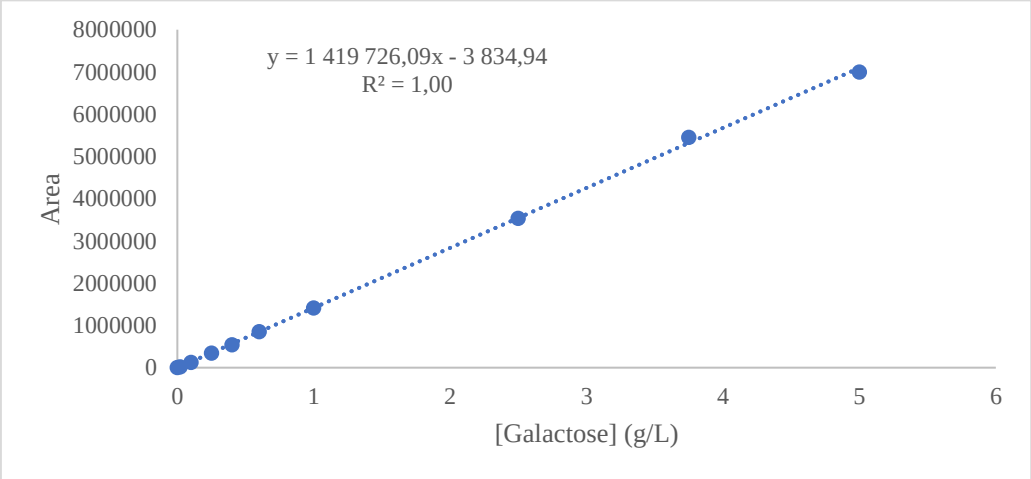

Figure 44. Galactose calibration curve, which relates the area of the peaks determined by HPLC with galactose concentration.

Appendix 5 - Ethanol calibration curve

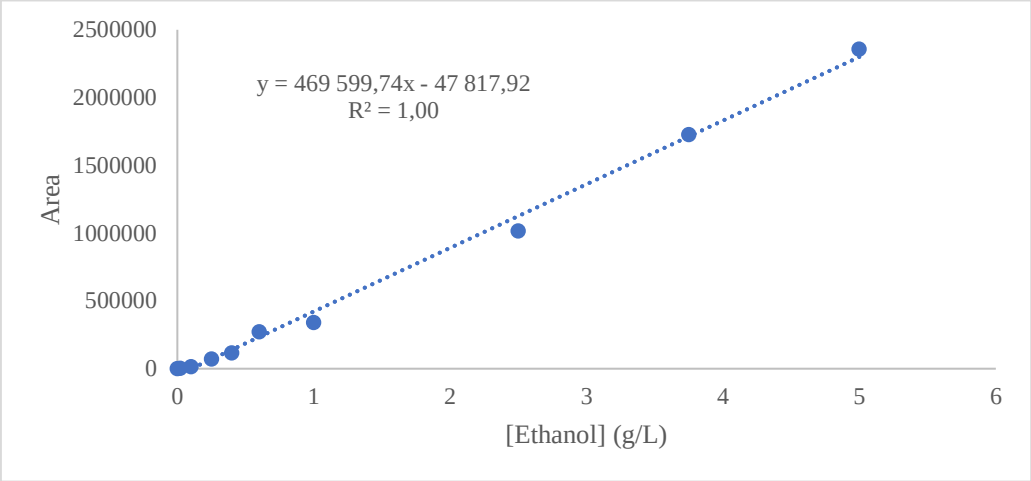

Figure 45. Ethanol calibration curve, which relates the area of the peaks determined by HPLC with ethanol concentration.

Appendix 6 - Acetic acid calibration curve

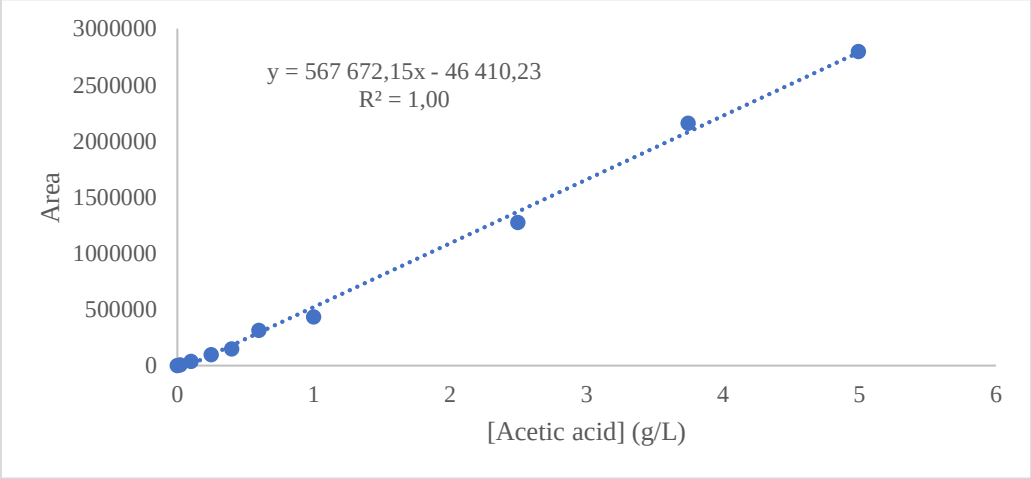

Figure 46. Acetic acid calibration curve, which relates the area of the peaks determined by HPLC with acetic acid concentration.

Appendix 7 - Infrared spectrum of kefiran (MRSL broth)

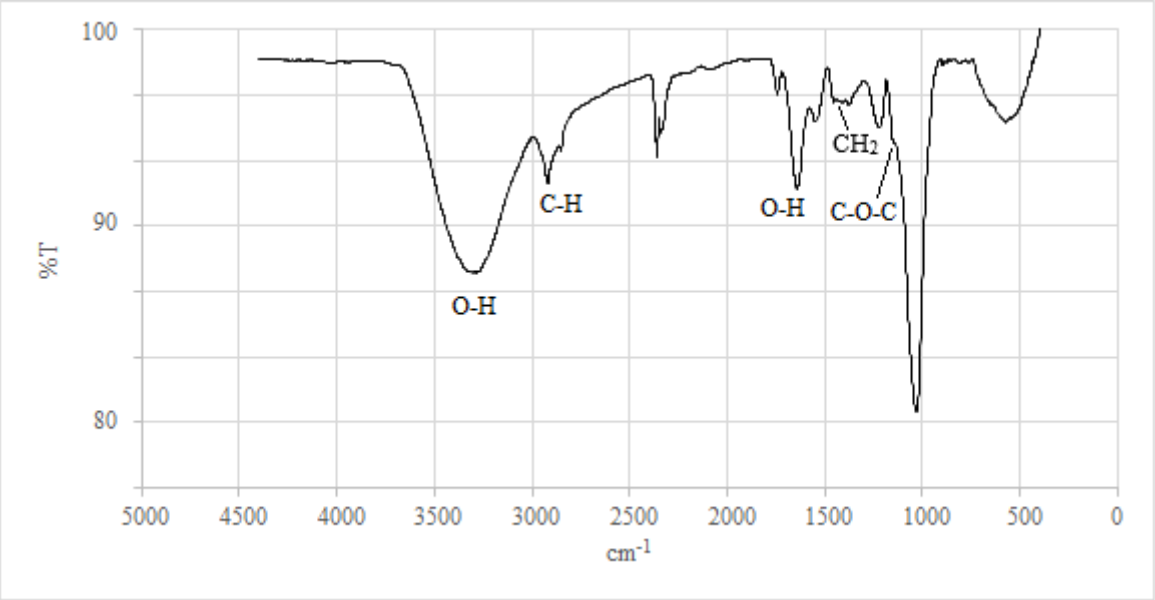

Figure 47. Infrared spectrum of kefiran between 500 and 4000 cm^{-1}, produced after 100 h of batch fermentation of MRSL broth medium with 80 g/L of lactose, at 80 rpm.

Appendix 8 - Infrared spectrum of kefiran (CWCD)

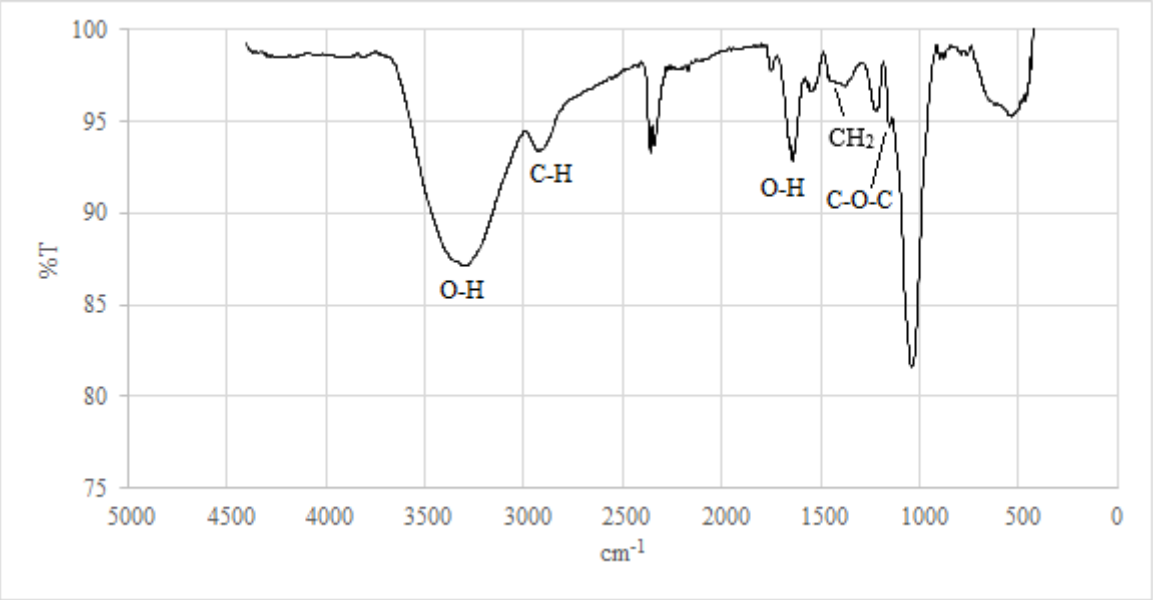

Figure 48. Infrared spectrum of kefiran between 500 and 4000 cm^{-1}, produced after 100 h of batch fermentation of CWCD, at 80 rpm.

Appendix 9 - Infrared spectrum of kefiran (CWS)

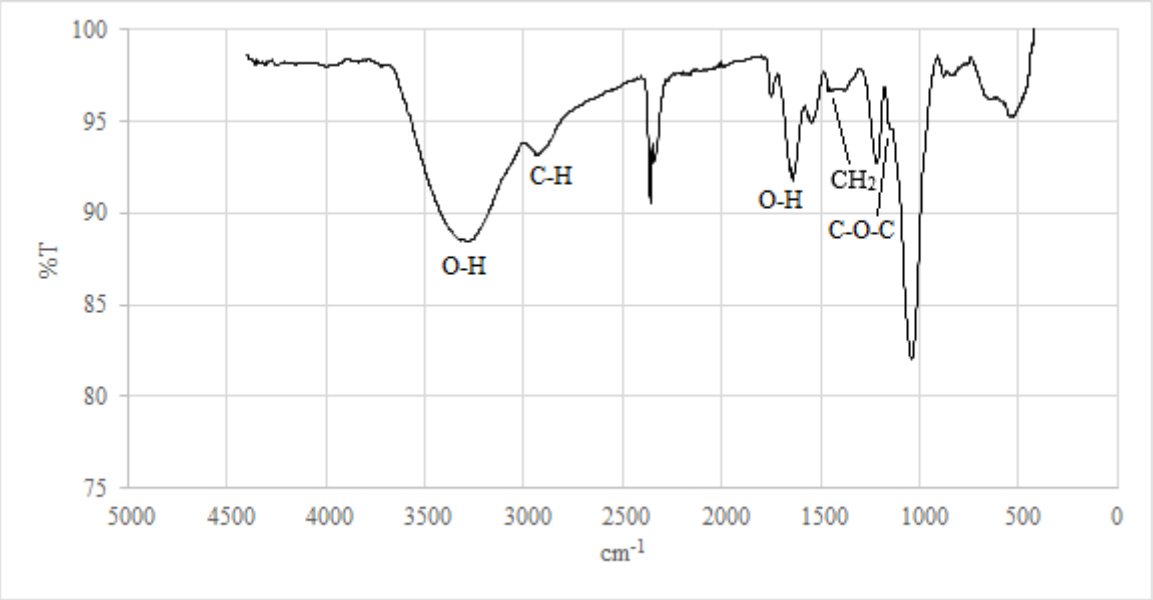

Figure 49. Infrared spectrum of kefiran between 500 and 4000 cm^{-1}, produced after 100 h of batch fermentation of CWS, at 80 rpm.