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# Behavior of the conversion of glucose, galactose and lactose to lactic acid by kefir grains

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**INFO**

**Keyworks**
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**ABSTRACT**

Few studies have been reported on lactic acid production by kefir grains. Kefir has been widely associated with probiotic use due to its cell growth in food matrices such as milks, juices and sugary solutions. However, from industrial scale there are no reports of its use in lactic acid production. In this work, we carried out experiments to test and understand how glucose, galactose and lactose, during lactic-acid fermentation, were converted in lactic acid by kefir grains. Given the microbial complexity in coexistence in kefir grains, it is likely that kinetic studies are not the most appropriate for the positive or negative definition of the kefir use as a starter in the lactic acid fermentative production on an industrial scale. It was concluded that, although with a higher Lag phase, lactose was the substrate that best presented a product and cell conversion rate, although glucose and galactose can also be used as a substrate in the production of this carboxylic acid.

**Palavras-chaves**
bacteria do ácido láctico
cultura mista
metabolismo primário
fermentação

**RESUMO**

*Comportamento da conversão de glicose, galactose e lactose em ácido lático por grãos de kefir*
Poucos estudos foram relatados sobre a produção de ácido lático pelos grãos de kefir. O kefir tem sido amplamente associado ao uso de probióticos devido ao seu crescimento celular em matrizes alimentares como leites, sucos e soluções açucaradas. Entretanto, em escala industrial não há relatos de sua utilização na produção de ácido lático. Neste trabalho, realizamos experimentos para testar e entender como a glicose, galactose e lactose, durante a fermentação láctica, são convertidas em ácido lático pelos grãos de kefir. Dada a complexidade microbiana em coexistência nos grãos de kefir, é provável que os estudos cinéticos não sejam os mais adequados para a definição positiva ou negativa do uso do kefir como starter na produção fermentativa de ácido lático em escala industrial. Concluiu-se que, embora com maior fase Lag, a lactose foi o substrato que melhor apresentou produto e taxa de conversão celular, embora glicose e galactose também possam ser utilizadas como substrato na produção desse ácido carboxílico.

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## INTRODUCTION

Ninety percent of lactic acid produced worldwide is obtained through sucrose fermentation (Cock and Stouvenel, 2005) from sugarcane molasses (Ahmad et al., 2020; González-Leos et al., 2020) or sugarbeet juice (Olszewska-Widdrat et al., 2020; Malik et al., 2019; Kotzamanidis et al., 2002), but Abedi and Hashemi (2020) reported other substrates can be used such as lignocellulosic materials supplemented with nitrogen sources. It is also possible to use lactose from whey and glucose from hydrolyzed starch (Iskandar et al., 2019; Bulut et al., 2004; Lazarova and Peeva, 1994).

Bacteria have been employed in the lactic acid production. The main ones are Gram-positive cocci and bacilli, facultative anaerobes, non-sporulated and catalase negative, belonging to the genera *Lactobacillus* (Nagarajan et al., 2022; Sudhakar et al., 2022), *Carnobacterium* (Karolenko et al., 2022), *Leuconostoc* (Sarhir et al., 2023; Liu et al., 2022), *Pediococcus* (Song et al., 2021; Qiu et al., 2020; Yang et al., 2019), *Streptococcus* (Wu et al., 2021; Yu et al., 2019), *Tetragenococcus* (Kobayashi et al., 2004), *Lactococcus* (Taye et al., 2021; Aso et al., 2019), *Vagococcus* (Rawoof et al., 2021; Ringø et al., 2020), *Enterococcus* (Abdel-Rahman et al., 2021; Wang et al., 2020; Hassan et al., 2019), *Aerococcus* (Mostefa et al., 2021) and *Weissella* (Nagarajan et al., 2022; Montero-Zamora et al., 2022; Cock and Stouvenel, 2005).

Few studies have been reported on lactic acid production by kefir grains. Kefir has been widely associated with probiotic use due to its cell growth in food matrices such as milks, juices and sugary solutions. However, from industrial scale there are no reports of its use in lactic acid production. Thus, as an exploratory and innovative study, the complete kinetics of lactic acid biosynthesis was evaluated in a culture medium containing kefir grains (previously activated to be out of the Lag phase) in three different carbon sources: glucose, galactose and lactose. The study included the analysis of influence of the molecular structure of sugars on ability to absorb by kefir cells and, consequently, the ability to metabolize them into lactic acid.

In this work, we sought a better understanding of substrates uptake such as glucose, galactose and lactose, during lactic-acid fermentation, especially in the production of lactic acid by kefir grains.

## MATERIAL E METHODS

### Kefir grains preparation, maintenance and fermentation process

Kefir grains obtained from a local market in Londrina, Brazil were used as starter. The kefir grains were maintained in a broth with a 10% brown sugar solution in distilled water and at constant temperature of 26±2°C, without agitation. Weekly, the inoculum was transferred to a new broth, until obtaining an appropriate cell biomass for the experiments, equivalent to 80 g of cell biomass per liter of solution.

For the preparation of the fermentation broth, 8% (w/v) solutions of glucose, galactose and lactose were used, which were added with saline solution containing $FeSO_4$ (4.6 g $L^{-1}$), $KH_2PO_4$ (2 g $L^{-1}$) and $CaCl_2$ (0.3 g $L^{-1}$).

The fermentation processes with kefir grains were carried out in Erlenmeyer flasks (150 mL) with 50 mL of the respective broth, in triplicate. For the analyzes of concentrations of substrate consumption, product formation and cells growth, samples were taken at 24 h intervals and a control sample at time t = 0. In the control sample, the initial conditions of the fermentation process were determined.

### Determination of celular concentration of kefir grains during fermentation

The cell concentration, at the respective times, was obtained by analyzing the dry mass after total filtration of the broth, where the cells retained in Whatman nº. 1 paper filters (Maidstone, England), previously tared, were weighed to constant mass.

### Follow-up of the fermentation process by pH and titrable acidity analyses

The pH and titratable acidity analyzes were performed according to methodologies described by AOAC 10.163 and AOAC 22.060 (i.e., Association of Official Analytical Chemists Handbook of Analysis, 1990), respectively.

### Determination of glicose by enzymatic method

Glucose concentration was determined by the enzymatic-colorimetric method with aid of Sigma-Aldrich's kit. According to supplier, glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in presence of peroxidase. Oxidized o-dianisidine reacts with $H_2SO_4$ to form a stable pigment. The intensity of the pigment was measured at 540 nm. The glucose concentration


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were expressed in mg/dL against standardized glucose concentrations.

**Enzymatic determination of galactose**

Galactose is oxidized by galactose oxidase resulting in a colored intermediate which was colorimetric measured at 570 nm. The galactose assay kit (MAK012, Merck Co.) has a linear detection range of 2 to 10 nmol galactose for colorimetric assay. The concentrations of galactose were expressed by:

$$C_{galactose} = \frac{Sa}{Sv}$$

where: C = concentration of galactose in sample (nmol); Sa = amount of galactose in unknown sample (nmol) from standard curve; Sv = sample volume (µL) added into the curvette. To convert the results in g $L^{-1}$ consider the molecular weight of galactose equal to 180.16 g $mol^{-1}$.

**Determination lactose by enzymatic method**

Lactose was quantified by Boehringer-Mannheim method after hydrolysis to glucose and galactose in the presence of galactosidase and water. Galactosidase was oxidized by NAD to galactonic acid in the presence of galactose desidrogenase. The NADH formed is stoichiometric with the lactose contente and its is measured at 340 nm in a spectrophotometer (Kleyn, 1985). The initial galactose contentes was subs tracted by the final value.

**Spectrophotometric determination of lactic acid**

Lactic acid concentration was determined by spectrophotometer of the colored intermediate after reaction of lactate ions with $FeCl_3$. The samples (50 µL) containing lactic acid was mixed to 2 mL of 0.2% (w/v) $FeCl_3$. After stirred, the mixture was submitted to measurement at 390 nm (Borshchevskaya et al., 2016).

**Statistical analyzes**

Statistical differences in the values of total phenolics, total flavonoids, antioxidant activities and narigin and hesperidin contents by HPLC were determined by one-way analysis of variance (ANOVA) and Tukey's means test. The results are presented as the mean value±standard deviation (n = 9). Pearson correlation, hierarchical clustering analysis (HCA) and principal component analysis (PCA) using the statistical package MetaboAnalyst v. 5.0 (Xia et al., 2009). The data from UV-visible spectra of orange peels ethanolic extracts and directly analysis of color on the orange peel surface by CIELab system were used for PCA and hierarchical analysis (HCA) by software for metabolomics data analysis. The data were normalized by the sum with scaling performed by Pareto and did not undergo any data transformations.

## RESULTS AND DISCUSSION

Kefir or kefir grain consists of lactic bacteria and yeast in casein and gelatinous colonies (Figure 1). Once kefir are composed by *Lactobacillus*, *Fructobacillus*, *Lactococcus*, *Pediococcus*, *Acetobacter* and *Kluyveromyces* genera (Cui et al., 2022) and these microorganisms are closely associated with the production of lactic acid, so kefir would have a great performance in the lactic acid production. In fact, kefir has been associated not only in the lactic acid biosynthesis but also ethanol in concentrations from 1.4 to 17.4 g $L^{-1}$ (Magalhães et al., 2011a) and 7.8 to 8.3 g $L^{-1}$ (Magalhães et al., 2011b), respectively.

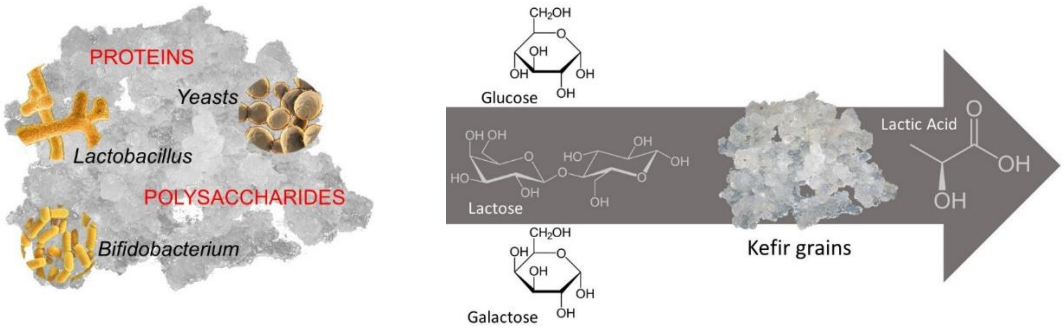

Figure 1 - Colony diversity of kefir grains and some substrates used to obtain lactic acid by kefir grains fermentation.

Indeed, these concentrations depend on the microorganism, the environmental conditions (pH, temperature, carbon and nitrogen sources), fermentation type and by-products formed (Hofvendahl and Hhan-Hägerdal, 2000). The ethanol contents potentially produced in fermentation assays were not the target of this study.

As described by Cui et al. (2022) and Magalhães et al. (2011a; 2011b) during fermentation tests with kefir grains purchased at the local market in Londrina, Brazil were able to synthesize lactic acid in glucose solution (analytical grade). The solutions were supplemented with P and N sources to promote cell growth, and after 24 h cell growth was exponential with a slight reduction in cell growth acceleration after 48 h. This is largely due to the reduction in glucose supply after 48 h of fermentation (Figure 2).

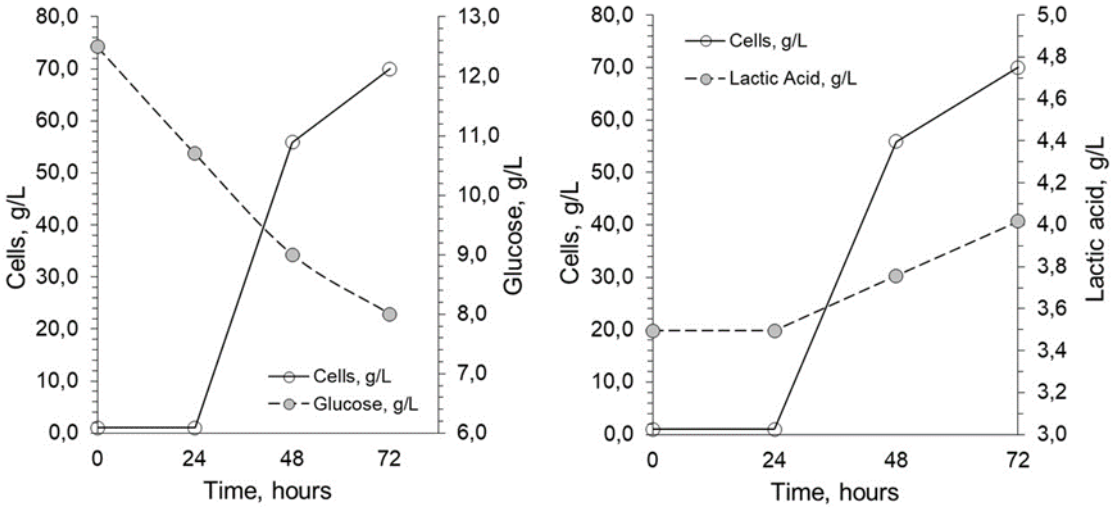


Figure 2 - Growth curve of kefir grains cells at glucose solution as carbon source and production of lactic acid during fermentation process.

Cell growth is directly associated with substrate consumption. The kinetic behavior was observed for the product formation, lactic acid, from glucose uptake. The kinetic profile demonstrated (Figure 2) denotes that the lactic acid formation by kefir grains in glucose is primary metabolism. Initially, there is a residual lactic acid content from the pre-incubation of kefir grains in brown sugar solution (maintenance solution). Although the kefir grains were washed in sterile distilled water before the fermentation tests, it is clear that a residual content (20 g $L^{-1}$) was retained in the polymeric structures of the colonies' biofilm. Thus, after 24 h the formation of lactic acid is exponential reaching linear rates of product formation, and the lactic acid concentration after 72 h reached approximately 4.8 g $L^{-1}$.

The same kinetic behavior was observed for galactose uptake by kefir grains (Figure 3). However, cell growth showed two distinct phases. There is a plateau in the cell growth curve between 24 and 48 h. Although cellular development stabilized in this time interval, galactose uptake was always continuous and linear. The galactose uptake rate by kefir cells was 1.72 g $L^{-1}$ $h^{-1}$. According to Pessione (2012), galactose to be catabolized into lactic acid initially needs to be converted, with ATP cost, to galactose-1-phosphate and then isomerized to glucose-1-phosphate, with the help of high energy via Leloir pathway (Figure 4).


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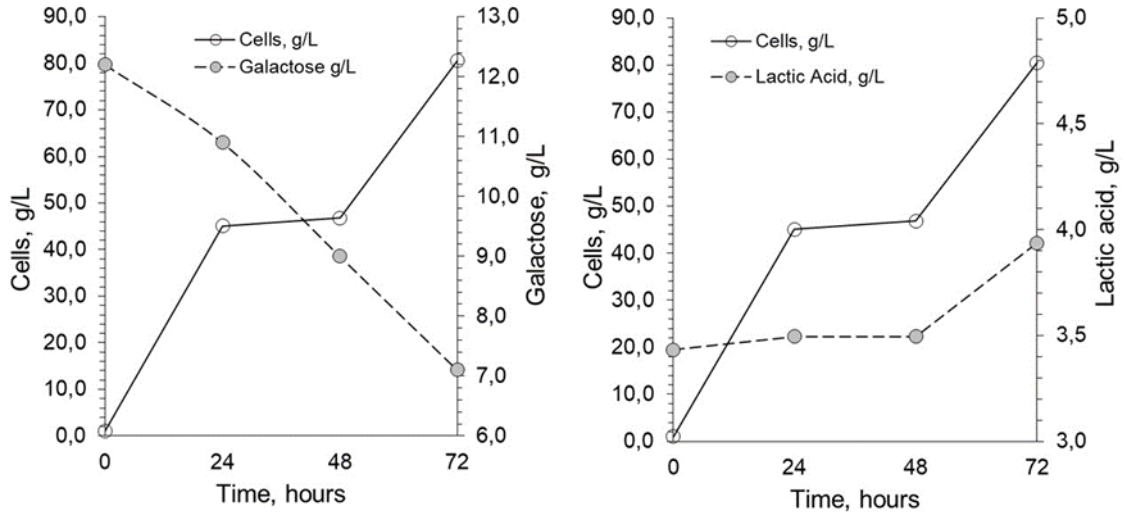


Figure 3 - Growth curve of kefir grains cells at galactose solution as carbon source and production of lactic acid during fermentation process.

The sugars' transport through the LAB cell membrane are classified according to the energy type used for transportation (Zaunmüller and Unden, 2008). Glucose has been reported as the most readily assimilable substrate by LAB, since it enters glycolysis directly, while other sugars, such as galactose or lactose, should be first be enzymatically transformed, which leads to greater energy cost for the microbial cells (Jeckelmann and Erni, 2020).

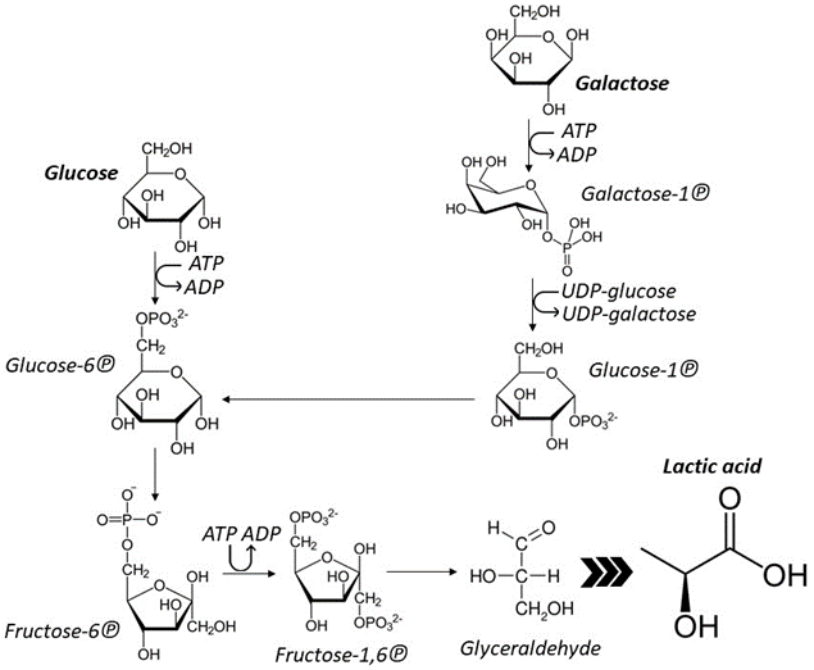


Figure 4 - Conversion of galactose to lactic acid via Leloir pathway. ATP: adenosine triphosphate; ADP: adenosine diphosphate; UDP: uridine diphosphate; P: phosphate.

Thus, the plateau observed in cell growth is associated with the interval between the uptake of only carbon source - galactose, and the isomerization to glucose, which is converted to lactic acid, common in lactic acid bacteria (LAB). Glucose and galactose are stereoisomers and differ


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in their orientation of hydroxyl group (OH) at carbon 4. This small stereochemical difference makes glucose greater absorbable by the LAB cell walls than galactose.

The formation of lactic acid in broth with galactose presented a plateau in the same interval of 24 to 48 h of fermentation, following the plateau of cell growth. This clearly demonstrates that lactic acid formation by kefir grains is also characterized as primary metabolism (Figure 3).

The gain in cell biomass in lactose-based solutions was soaring and linear (22.6 g $L^{-1}$ $h^{-1}$) up to 72 h of incubation (Figure 5). In the first hours there is a slow substrate uptake (0.9 g $L^{-1}$ $h^{-1}$) for a fast and intense uptake from 24 h (3.9 g $L^{-1}$ $h^{-1}$), significantly higher than that of glucose (1.52 g $L^{-1}$ $h^{-1}$) and galactose (1.72 g $L^{-1}$ $h^{-1}$). The estimate for the maximum specific speed of cell growth was ($\mu_{max}$) for glucose was 0.072 $h^{-1}$; for galactose was 0.063 $h^{-1}$; and for lactose was 0.051 $h^{-1}$ (Table 1). It is possible to notice that there is an inverse behavior when we analyze the maximum specific speed of cell growth compared to the general profile of cell growth in 72 h. Once the $\mu_{max}$ reveals the maximum relative growth peak as a function of substrate characteristics and reaction conditions adopted. On the other hand, after the lag phase (cell adaptation to substrate), cell growth is pronounced for the different substrates used in this study.

First, lactose is internalized into cells (LAB or some yeasts) by lactose permease by proton gradient, and then hydrolyzed into glucose and galactose by β-galactosidase (Corrieu and Béal, 2016). Onwards, we can consider that the catabolism of simple sugars follows the Leloir pathway (Figure 4).

This can be expressed in Figure 5, where the cell multiplication rate is linear and increasing while product formation is only perceived after 48 h of incubation. First moments the kefir grain cells consume the lactose substrate with consequent cell multiplication and only then start the biosynthesis of final products, as in this case, lactic acid. The rate of lactic acid formation up to 48 h of fermentation in lactose was 0.045 g $L^{-1}$ $h^{-1}$, whereas after 48 h it was 0.79 g $L^{-1}$ $h^{-1}$.

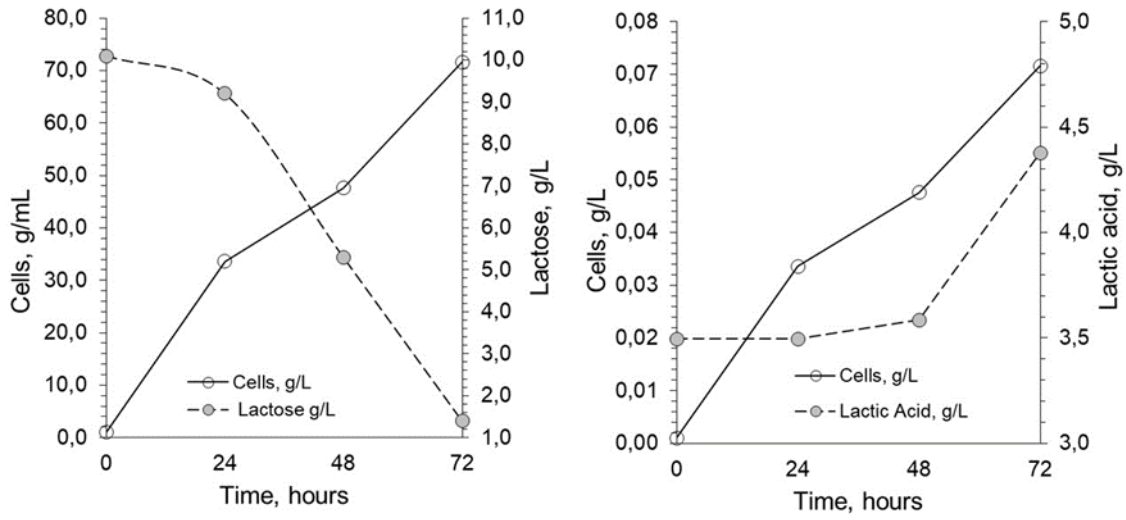


Figure 5 - Growth curve of kefir grains cells at lactose solution as carbon source and production of lactic acid during fermentation process.

Table 1 - Cinética da multiplicação celular, consumo de substrato e formação de produto.

| Time h | Cells g $L^{-1}$ | Glucose uptake g $L^{-1}$ | Lactic acid g $L^{-1}$ | Specific velocity $h^{-1}$ | $Y_{PS}$ g $g^{-1}$ | $Y_{XS}$ g $g^{-1}$ |
|---|---|---|---|---|---|---|
| 0 | 1.0 | 0.0 | 3.5 | 0.0 | 0.28 | 0.08 |
| 24 | 1.0 | 1.8 | 3.5 | 0.0 | 0.33 | 0.09 |
| 48 | 55.9 | 1.7 | 3.8 | 0.07 | 0.42 | 6.21 |
| 72 | 70.0 | 1.0 | 4.02 | 0.01 | 0.50 | 8.80 |

| Time h | Cells g $L^{-1}$ | Galactose uptake g $L^{-1}$ | Lactic acid g $L^{-1}$ | Specific velocity $h^{-1}$ | $Y_{PS}$ g $g^{-1}$ | $Y_{XS}$ g $g^{-1}$ |
|---|---|---|---|---|---|---|
| 0 | 1.0 | 0.0 | 3.43 | 0.0 | 0.28 | 0.1 |
| 24 | 45.1 | 1.3 | 3.50 | 0.063 | 0.32 | 4.1 |
| 48 | 46.8 | 1.9 | 3.50 | 0.002 | 0.39 | 5.2 |
| 72 | 80.5 | 1.9 | 3.94 | 0.023 | 0.55 | 11.3 |


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| Time h | Cells g $L^{-1}$ | Lactose uptake g $L^{-1}$ | Lactic acid g $L^{-1}$ | Specific velocity $h^{-1}$ | $Y_{PS}$ g $g^{-1}$ | $Y_{XS}$ g $g^{-1}$ |
|---|---|---|---|---|---|---|
| 0 | 1.0 | 0.0 | 3.50 | 0.0 | 0.28 | 0.10 |
| 24 | 33.6 | 4.8 | 3.50 | 0.05 | 0.31 | 3.65 |
| 48 | 47.6 | 7.8 | 3.59 | 0.01 | 0.61 | 8.98 |
| 72 | 71.6 | 5.3 | 4.38 | 0.02 | 3.06 | 51,14 |

Therefore, there are two scenarios to be analyzed. The first for lactic acid production focus of this study. Although we worked with a similar initial concentration of substrate equivalent to 80 g $L^{-1}$, the highest substrate uptake were observed for lactose with an upatake peak of 7.8 g $L^{-1}$ in 48 h of incubation. The substrate that showed the lowest upatake by kefir cells was glucose with a peak at 1.8 g $L^{-1}$, followed by galactose with 1.9 g $L^{-1}$ after 48 h. This does not necessarily represent high efficiency in the conversion rate to cell biomass (Yxs).

The rate of conversion to product was very similar between glucose and galactose, with Yps equal to 0.50 g $g^{-1}$ for glucose and 0.55 g $g^{-1}$ for galactose. In fact, the best substrate for lactic acid production proved to be lactose with Yps of 3.06 g $g^{-1}$. Although it is a more complex substrate, lactose showed the highest conversion rate into lactic acid, as well as the highest conversion rate into cell biomass (Yxs = 51.14 g $g^{-1}$). The cell multiplication of kefir grains in glucose was, at most, 8.80 g $g^{-1}$ and for galactose 11.3 g $g^{-1}$, in 72 h of submerged fermentation.

Thus, the microbial biomass production onto lactose is also the most recommended. Lactose was also the substrate with the longest Lag phase, i.e., 48 h. Although the kefir grains were pre-incubated, they were active but were grown in sucrose solution, a disaccharide of glucose and fructose. Thus, glucose readily available to be assimilated and metabolized into lactic acid, had the shortest Lag phase (= 24 h).

It is notable that the conversion rate of kefir grains is not readily high at the beginning of the fermentation process. In fact, it is noteworthy that given the complexity of the microbiota present in kefir grains, each genus or species of bacteria or yeast has its own physiological and biochemical characteristics.

Another aspect noted during the fermentation processes is the low stability of the kefir grains, especially in the polymeric network of the biofilms, which led us to consider the maximum fermentation time of 72 h, since at longer intervals the grains fell apart.

## CONCLUSIONS

Given the microbial complexity in coexistence in kefir grains, it is likely that kinetic studies are not the most appropriate for the positive or negative definition of the kefir use as a starter in the lactic acid fermentative production on an industrial scale. It was concluded that, although with a higher Lag phase, lactose was the substrate that best presented a product and cell conversion rate, although glucose and galactose can also be used as a substrate in the production of this carboxylic acid.

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