# Optimization by response surface methodology for production of β-galactosidase from *Enterococcus faecium* using recycled medium

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# Optimización por metodología de superficie de respuesta para la producción de β-galactosidasa a partir de *Enterococcus faecium* utilizando medio reciclado

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**Abstract**

In this study, recycled medium from three photosynthetic microorganisms (*Chlorella vulgaris, Dunaliella tertiolecta* and *Arthrospira platensis*) was evaluated for use in producing

β-galactosidase, an enzyme traditionally used to degrade lactose in dairy products. Recycled medium from *Chlorella vulgaris* was selected to optimize culture medium to be used to produce β-galactosidase by *Enterococcus faecium* in submerged fermentation. Response Surface Methodology (RSM) was used to optimize the levels of the variables: temperature (30-40°C), lactose concentration (0-5%), fermentation time (12-24h), pH (6-8) and their interaction. All variables studied had a statistically significant effect on the production of β-galactosidase. The optimal conditions for producing the enzyme were achieved: temperature of 31ºC, lactose concentration of 5.34%, fermentation time of 12h and pH of 8.0. Under these conditions, the β-galactosidase activity was 29.85 U/mL which was quite close to the predicted value (30.83 U/mL). Finally, it can be concluded that recycled medium from optimized *C. vulgaris* supernatant may well be important for the biotechnology industry as it is an abundant low-cost source for producing β-galactosidase by *Enterococcus faecium.*

**Keywords:** Recycled medium; Response surface methodology; Enzyme.

**Resumo**

Neste estudo, o meio reciclado de três microrganismos fotossintetizantes (*Chlorella vulgaris, Dunaliella tertiolecta e Arthrospira platensis*) foi avaliado para uso na produção de β-galactosidase, enzima tradicionalmente usada para degradar a lactose em produtos lácteos. O meio reciclado de *Chlorella vulgaris* foi selecionado para otimizar o meio de cultura para produção da β-galactosidase por *Enterococcus faecium* em fermentação submersa. A Metodologia de Superfície de Resposta (MSR) foi aplicada para otimizar os níveis das variáveis de produção como: temperatura (30-40 °C), concentração de lactose (0-5%), tempo de fermentação (12-24h) e pH (6-8). Todas as variáveis estudadas apresentaram efeitos estatisticamente significativos na produção da β-galactosidase nas seguintes condições: temperatura de 31 °C, concentração de lactose de 5,34%, tempo de fermentação de 12h e pH de 8,0. Com a otimização, a atividade da β-galactosidase foi de 29,85 U/mL, próximo ao valor previsto (30,83 U/mL). Com isso, pode-se concluir que o meio de cultivo otimizado com resíduo de cultivo da microalga *C.vulgaris* pode ser importante para a indústria de biotecnologia, por ser uma fonte abundante e de baixo custo pra produção de bioativos de microrganismos como o probiótico *Enterococcus faecium*.

**Palavras-chave:** Meio de cultura reciclado; Metodologia de Superfície de Resposta; Enzima.

**Resumen**

En este estudio, se evaluó el medio reciclado de tres microorganismos fotosintéticos (*Chlorella vulgaris, Dunaliella tertiolecta* y *Arthrospira platensis*) para su uso en la producción de β-galactosidasa, una enzima utilizada tradicionalmente para degradar la lactosa en productos lácteos. El medio de *Chlorella vulgaris* reciclado se seleccionó para optimizar el medio de cultivo para la producción de β-galactosidase por *Enterococcus faecium* en fermentación sumergida. Se aplicó la Metodología de Superficie de Respuesta (MSR) para optimizar los niveles de variables como: temperatura (30-40 °C), concentración de lactosa (0-5%), tiempo de fermentación (12-24h) y pH (6-8). Todas las variables estudiadas mostraron efectos estadísticamente significativos sobre la producción de β-galactosidasa en las siguientes condiciones: temperatura de 31 °C, concentración de lactosa de 5,34%, tiempo de fermentación de 12 hy pH de 8,0. Con la optimización, la actividad de la β-galactosidasa fue de 29,85 U / mL, cerca del valor predicho (30,83 U / mL). Con esto, se puede concluir que el medio de cultivo optimizado con cultivo residual de la microalga *C. vulgaris* puede ser importante para la industria biotecnológica, ya que es una fuente abundante y de bajo costo para la producción de microorganismos bioactivos como el probiótico *Enterococcus faecium*.

**Palabras clave:** Medio reciclado; Metodología de superficie de respuesta; Enzima.

## 1. Introduction

The use of microalgae is considered a future way to source the needs of various products and processes such as biofuel, feed/food, chemicals, pharmaceuticals and cosmetic formulations (Gangl et al., 2015). They constitute a broad biodiversity and have been used for many years as an important source of metabolites (pigments, antioxidants and polysaccharides) (Hadj-Romdhane et al., 2012). A wide range of microalgae can excrete abundant, extracellular, bioactive compounds (proteins, polysaccharides and various small molecules) into culture medium during growth or they can be suddenly released when cell lysis occurs (Berry, 2008; De Jesus Raposo, De Morais, & De Morais, 2013; Fogg, 1983; Hadj-romdhane et al., 2013). After microalgae are cultivated, only the biomass is processed for current products, while huge volumes of algae-free media are unexploited and after harvesting biomass from batch cultures (Liu et al., 2016).

Spent medium recovered from harvesting microalgal biomass can contain not only varying amounts of nutrients, but also microalgae cells, debris, bacteria and excreted organic compounds (Rodolfi et al., 2003). Furthermore, the volume of water needed to cultivate

microalgae results in a large water footprint. Producing microalgal biomass has been estimated to consume around one metric ton of water per kg of microalgal biomass produced (Farooq et al., 2015; Fret et al., 2017). Recycled culture medium of *Chlorella vulgaris* with nutrient limited to the physiological requirements of microalgae would save about 75% of water (Hadj-Romdhane et al., 2012). Some studies have measured microalgae growth in reused medium (*Scenedesmus obliquus, Arthrospira platensis* and *Chlorella vulgaris*) (Depraetere et al., 2015; Hadj-Romdhane et al., 2012, 2013; Rodolfi et al., 2003). It had already been reported that molecules excreted by microalgae were metabolized by lactic acid bacteria (Parada et al., 1998). However, to our knowledge, no information is available about bacteria cultivation in extracellular products from microalgae to produce bioactive, for example.

β-galactosidase or lactase is an important enzyme in the food industry that catalyzes the conversion of lactose to monosaccharides (galactose and glucose). This process facilitates the absorption of lactose by people who are lactose intolerant who constitute 70% of the world's population (Chanalia et al., 2018; Panesar,.2006). In addition, lactose has unpleasant effects when present in dairy products, such as low sweetness, low solubility and high crystal formation, resulting in a sandy texture. β-galactosidase is also prominent in the pharmaceutical industry due to the production of prebiotics (nondigestible ingredients present in foods that affect the host beneficially by stimulating the selective growth of the native microbiota) via another metabolic process known as transgalactosylation (Ansari & Satar, 2012; Chanalia et al., 2018).

The industrial need to large-scale of β-galactosidase production requires good cost-effective production methods to guarantee the economic viability on a commercial scale leading to an increased interest. The search for a suitable and inexpensive medium has shown that there are components that are readily available such as residues which appear to be attractive alternatives to replace or complement commercial media. In this context, an alternative to using a recycled medium from photosynthetic microorganisms would be to produce β-galactosidase. The aim of this study was to evaluate the influence of recycling a medium of photosynthetic microorganisms on β-galactosidase produced by *Enterococcus faecium.*

## 2. Materials and Methods

### 2.1 Microorganism and maintenance

*Enterococcus faecium* was isolated from coalho cheese produced in the semi-arid region of Pernambuco, Brazil. All procedures for genotyping this isolate were carried out by Stab Vida (Oeiras, Portugal).

The culture was maintained at - 80°C in sterile vials in Man Rogosa Sharpe (MRS) broth medium (Kasvi, Paraná - Brazil) containing 20% (v/v) of glycerol as a cryoprotective agent. The culture was activated and propagated by two successive transfers every 24h into MRS broth under static conditions at 37°C.

### 2.2 Screening of the recycled medium for β-galactosidase production

A quantitative test was carried out in order to obtain recycled medium indicated for the β-galactosidase production. The recycled medium (RM), cultivated from photosynthetic microorganisms *(C.vulgaris, D.tertiolecta* and *A.platensis)*, was provided by the Laboratory of Bioactive Products of the Federal Rural University of Pernambuco and stored at 4°C. The first step of the study consisted of supplementing the MRS medium with different concentrations of RM from each microalga (Table 1) and sterilizing the mixture at 121°C for 15 min. Then, 2% of inoculum previously standardized to $10^8$ was added to the medium, which was incubated at 37 °C, without stirring, for 24 h. The initial and final pH of cultivation were evaluated.

**Table 1.** Composition of culture medium.

| Experiment | Medium composition |
|:---:|:---:|
| M1 | MRS + 25% of RS from microalgae |
| M2 | MRS + 50% of RS from microalgae |
| M3 | MRS + 75% of RS from microalgae |
| M4 | 100% of RS from microalgae |

Concentration of recycled medium of each photosynthetic microorganism. Source: Elaborated by the authors.

### 2.3 Extraction of β-galactosidase

After fermentation time, the cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C, then washed once with 0.1M phosphate buffer (pH 7.0) and re-suspended in the same buffer. The suspension of cells was sonicated by an ultrasonic sonicator (Bandelin sonopuls, HD 2070, Germany) during 6 min in an ice bath. Then the suspension was centrifuged under the same conditions described above and the supernatant was used for measuring β-galactosidase activity.

### 2.4 β-galactosidase assay

The activity of β-galactosidase was measured as described by Nagy et al. (2001) with slight modification. Using 96 well plate, the reaction consisted of 50 μl of enzyme incubated with 50 μl of 3 mM o-nitrophenyl-b-D-galactopyranoside (ONPG - Sigma), a chromogenic substrate prepared in 0.1M phosphate buffer (pH 7.0). The reaction was stopped by adding 200 μl of 0.1M sodium carbonate. The enzyme activity was monitored by using a Microplate Absorbance Reader (Bio-Rad, iMark) at 405 nm. One unit of β-galactosidase was defined as the amount of enzyme that produced one μmol of o-nitrophenol per min. The test was performed in triplicate.

### 2.5 Optimization of β-galactosidase production

### 2.5.1 Complete factorial design on the β-galactosidase production

The enzyme production was optimized in accordance with the composition of the medium which had the highest β-galactosidase activity (2.2 Screening of recycled supernatant for β-galactosidase production). The full factorial design ($2^4$) proposed by Barros Neto et al. (1996) was used to screen the important factors affecting β-galactosidase production. The four selected factors were lactose concentration, pH, temperature and fermentation time. These factors were selected due to their known effects on β-galactosidase production by Lactic Acid Bacteria as described in the literature. Each factor was analyzed at three different levels: low (-1) medium (0) and high (+1), as can be seen in Table 2. To obtain a good estimate of an experimental error, the center points were repeated five times.

**Table 2.** Full factorial design for β-galactosidase production by *E.faecium.*

| | Independent variables (real and coded values) | | | | Dependent variable |
|---|---|---|---|---|---|
| Run | Lactose (%) | Temperature (°C) | pH | Fermentation time (h) | β-galactosidase (U/mL) |
| 1 | 0(-1) | 30(-1) | 6(-1) | 12(-1) | 11.8 |
| 2 | 5(+1) | 30(-1) | 6(-1) | 12(-1) | 14.2 |
| 3 | 0(-1) | 40(+1) | 6(-1) | 12(-1) | 10.2 |
| 4 | 5(+1) | 40(+1) | 6(-1) | 12(-1) | 11.0 |
| 5 | 0(-1) | 30(-1) | 8(+1) | 12(-1) | 12.5 |
| 6 | 5(+1) | 30(-1) | 8(+1) | 12(-1) | 30.2 |
| 7 | 0(-1) | 40(+1) | 8(+1) | 12(-1) | 9.85 |
| 8 | 5(+1) | 40(+1) | 8(+1) | 12(-1) | 10.7 |
| 9 | 0(-1) | 30(-1) | 6(-1) | 24(+1) | 10.5 |
| 10 | 5(+1) | 30(-1) | 6(-1) | 24(+1) | 16.6 |
| 11 | 0(-1) | 40(+1) | 6(-1) | 24(+1) | 10.5 |
| 12 | 5(+1) | 40(+1) | 6(-1) | 24(+1) | 12.0 |
| 13 | 0(-1) | 30(-1) | 8(+1) | 24(+1) | 14.0 |
| 14 | 5(+1) | 30(-1) | 8(+1) | 24(+1) | 10.9 |
| 15 | 0(-1) | 40(+1) | 8(+1) | 24(+1) | 11.5 |
| 16 | 5(+1) | 40(+1) | 8(+1) | 24(+1) | 11.2 |
| 17 | 2,5(0) | 35(0) | 7(0) | 18(0) | 11.7 |
| 18 | 2,5(0) | 35(0) | 7(0) | 18(0) | 13.7 |
| 19 | 2,5(0) | 35(0) | 7(0) | 18(0) | 13.3 |
| 20 | 2,5(0) | 35(0) | 7(0) | 18(0) | 13.1 |
| 21 | 2,5(0) | 35(0) | 7(0) | 18(0) | 13.3 |

Source: Elaborated by the authors.

### 2.5.2 Central composite design of the β-galactosidase production

A central composite design ($2^3$) was used to obtain the optimal experimental conditions and central points to estimate the pure process variability with β-galactosidase production as response (Y). Temperatures of 25 and 35°C, initial lactose concentrations of 4 and 6 %, and pH of 7.5 and 8.5 were the lowest and highest levels, respectively. The combination was tested, resulting in a set of 8 experiments with 5 central points (Table 3, Part

A). The regression coefficients for linear, quadratic and interactions for each variable were determined and adjusted to a polynomial second order equation:

$$Y= \beta_0 + \beta_i X_1 + \beta_i X_2 + \beta_i X_3 + \beta_{ii} X_1 + \beta_{ii} X_2 + \beta_{ii} X_3 + \beta_i X_2 X_3$$

(1)

Where Y is the response, $\beta_0$ is the coefficient of fitted response at the center point of design, $X_1$ $X_2$ and $X_3$ are the variables temperature, lactose concentration and pH, respectively, $\beta_i$ the coefficients of linear terms, βii- the coefficients of square terms and βij- the coefficients of interactive terms.

**Table 3.** Experimental conditions and β-galactosidase activity values obtained for *E. faecium* using $2^3$ complete factorial design.

| Independent variables (real and coded values) | | | Dependent variable |
|---|---|---|---|
| Run | Temperature (°C) | Lactose (%) | pH | β-Galactosidase (U/mL) |
| Part A: Initial experimental design | | | | |
| 1 | 25(-1) | 4(-1) | 7.5(-1) | 5.20 |
| 2 | 35(1) | 4(-1) | 7.5(-1) | 13.5 |
| 3 | 25(-1) | 6(1) | 7.5(-1) | 7.00 |
| 4 | 35(1) | 6(1) | 7.5(-1) | 15.1 |
| 5 | 25(-1) | 4(-1) | 8.5(1) | 10.0 |
| 6 | 35(1) | 4(-1) | 8.5(1) | 16.5 |
| 7 | 25(-1) | 6(1) | 8.5(1) | 12.4 |
| 8 | 35(1) | 6(1) | 8.5(1) | 19.2 |
| 9 | 30(0) | 5(0) | 8(0) | 28.9 |
| 10 | 30(0) | 5(0) | 8(0) | 29.3 |
| 11 | 30(0) | 5(0) | 8(0) | 30.6 |
| 12 | 30(0) | 5(0) | 8(0) | 29.0 |
| 13 | 30(0) | 5(0) | 8(0) | 29.0 |
| Part B: Additional tests for optimization | | | | |
| 14 | 35(+1) | 8(3) | 9(2) | 3.40 |
| 15 | 35(+1) | 5(0) | 9(2) | 1.20 |

| 16 | 35(+1) | 8(3) | 8(0) | 4.00 |

Part C: Additional tests for optimization confirmation

| 17 | 31(1) | 5.34(0) | 8(0) | 29.76 |
| 18 | 31(1) | 5.34(0) | 8(0) | 29.12 |
| 19 | 31(1) | 5.34(0) | 8(0) | 30.68 |

Source: Elaborated by the authors.

The validations of the proposed mathematical models were based on the F-test and determination coefficient ($R^2$). Each factor and interaction was studied by considering their significant influences on the respective responses at a 95% confidence level ($p < 0.05$). The response surface methodology (RSM) was used to describe the individual and cumulative effects of the test variables and their subsequent effects on the respective responses. The central composite design was conducted using Statistica 7.0® software by considering all experiments. The variance and regression analyses were used with the aim of determining the statistically significant parameters of the model and regression coefficients. For the statistical calculation, the variables were coded according to equation (Eq. 2).

$$X_I = x_I - x_0 / \Delta x_i$$

(2)

where $X_i$ is the codified value of the independent variable, $x_i$ is the actual value of the independent variable, $X_0$ is the actual value of the independent variable at the central point and $\Delta X_i$ is the step change value.

## 3. Results and Discussion

Table. 4 shows the β-galactosidase production in MRS medium supplemented with different concentrations of RM from each microalgae (*C.vulgaris, D.tertiolecta* and *A.platensis*) and pH values of each medium before and after fermentation of *E.faecium* in comparison with the MRS control. All media tested showed β-galactosidase activity. However, the enzyme production rate decreased when the RM concentration was increased.

**Table 4.** β-galactosidase production by *E. faecium* and pH values during 24h of fermentation.

| Medium | β-galactosidase activity (U/mL) | Initial pH | Final pH |
|---|---|---|---|
| Control | 35.64 ± 0,26 | 6.3 | 4.2 |
| *C.vulgaris* | | | |
| M1 | 22.68 ± 0,64 | 7,6 | 4,7 |
| M2 | 21.78 ± 0,02 | 7,8 | 4,4 |
| M3 | 15.17 ± 0,42 | 7,8 | 4,2 |
| M4 | 3.80 ± 0,19 | 7,6 | 5,6 |
| *D.tertiolecta* | | | |
| M1 | 13.44 ± 0,21 | 6,8 | 4,1 |
| M2 | 6.27 ± 0,43 | 6,6 | 4,1 |
| M3 | 2.18 ± 0,05 | 6,5 | 4,3 |
| M4 | 0.39 ± 0,04 | 6,1 | 4,6 |
| *A.platensis* | | | |
| M1 | 21.78 ± 0,19 | 8,6 | 4,8 |
| M2 | 7.98 ± 0,01 | 9,2 | 8,8 |
| M3 | 3.56 ± 0,04 | 9,9 | 9,3 |
| M4 | 3.33 ± 0,02 | 10,7 | 9,7 |

The relation between acidification and enzyme activity during the bacteria fermentation in each recycled medium. Source: Elaborated by the authors.

The highest β-galactosidase activity was obtained in medium M1 and M2 of *C. vulgaris* and M1 of *A.platensis,* which represents more than 60% of enzyme activity when compared with control (Table.4). Although the enzyme activity rate in M1 of *A.platensis* was the same as that observed in M2 of *C.vulgaris,* it showed a reduction of more than 50% on enzyme activity in M2. This may be due to the pH level in medium as can be seen in Table 4, which shows the pH values of each medium before and after fermentation of *E.faecium*. RM of *A.platensis* presented a high pH level in all media tested, except M1. Due to the low concentration of RM in this sample, an increase in acidification could be observed. This confirms that there was fermentation of *E.faecium* in this medium and it is also a possible explanation for the high enzyme activity obtained in M1 of RM from *A.platensis* (Table 4), which suggests that the high concentrations of RM from *A.platensis* inhibited the

fermentation of *E. faecium* and consequently reduced the enzyme production. Hsu et al. (2005) observed that higher initial pH values resulted in a sharp reduction in the production of β-galactosidase by *Bifidobacteria*. In other study, the maximum enzyme production was observed in initial pH of 6.5-7.5 with β-galactosidase from *Streptococcus thermophilus* (Ramana Rao & Dutta, 1977).

The longest and shortest time to acidification during fermentation of *E. faecium* was found in the RM of *D. tertiolecta* (Table.4). This was also found by Freitas et al. (1999) when they observed a slight acidification on fermentation of *E.faecium* cultivated in milk. However, the presence of salt in RM from *D.tertiolecta* may cause a decline in enzyme activity. This microalga is known as a halotolerant that uses inorganic nutrients present in artificial sea water (Chen et al., 2011). According to our results, increasing the concentration of RM from *D.tertiolecta* could have affected the enzyme synthesis, while the bacteria growth was not affected. There are two possible reasons to explain the fermentation from *E.faecium* in saline medium: (1) The *Enterococcus* genus is associated to strong survival traits that can overcome high NaCl concentrations (6.5%) and (2) our strain was isolated from coalho cheese, a highly sought-after product because of its sensory properties, including a salty flavor (Silva et al., 2012). In other words, the fermentation of *E. faecium* in RM from *D.tertiolecta* was possible due to this strain having adapted to the saline environment.

The result obtained of the enzymatic activity of β-galactosidase in the present study corroborate with other studies found in the literature involving different culture medium. Prasad et al. (2013) reached a maximum enzyme activity of 7.77 U/mL for β-galactosidase from *L. delbrueckii* ssp. *bulgaricus* cultivated in cheese whey. The highest activity of β-galactosidase from *S. thermophillus* grown in permeate-based medium was observed by Murad et al. (2011) with 7.85 U/mL. Martarello et al. (2019) obtained β-galactosidase activity of 24.64 U/mL from *Aspergillus niger* grown in a medium containing a soybean residue. Braga et al. (2012) reported an activity of 10.4 U/mL expressed by *K. marxianus* in an effluent from rice cultivation.

It is already known that it is worthwhile to recover, for further use, extracellular metabolites from photosynthetic microorganisms which are rich in bioactive substances. For example, exopolysaccharides (EPS) have been a topic of current interest as evidenced in some studies (El-naggar et al., 2020; Calderón et al., 2018; Zhang et al., 2019). The presence of EPS and other compounds in RM from *C.vulgaris* may be responsible for the growth of *E.faecium* and consequently, this may have stimulated the production of β-galactosidase. Some studies have already reported extensive research on the effect of EPS on lactic acid

bacteria growth (Das et al., 2015; Hongpattarakere et al., 2012; Liu et al., 2020). On the other hand, conditions such as the salinity and pH in RM from *D.tertiolecta* and *A.platensis,* apparently caused an inhibitory effect on the synthesis of the enzyme.

The addition of RM of *C.vulgaris* enhanced the fermentation rate of *E.faecium* and did not drastically affect the production of β-galactosidase, which has been observed in other media (Table.4). This medium had sufficient conditions to support the growth of the culture and β-galactosidase production. Thus, the composition of the M2 medium, formulated with the RM of *C.vulgaris*, was selected for further optimization tests of β-galactosidase production due to its being close to the control and being able to half the use of commercial medium.

## 3.1 Complete factorial design of the production of β-galactosidase

A $2^4$ complete factorial design was used to evaluate the interaction of the independent variables (fermentation time, temperature, pH and lactose concentration) on the production of β-galactosidase. The highest β-galactosidase activity was 30.2 U/mL in conditions of 12h of fermentation, at 30°C, pH 8.0 and 5% (w/v) of lactose (run 6, Table 2).

Figure 1 shows the Pareto chart used to estimate effects of the independent variables and their interactions on the response. The purpose of the Pareto chart is to highlight the significance level among a set of factors. The individual values and their interactions are represented in descending order by bars, and the cumulative total is represented by the line. The results observed at the right of the red line are statistically significant 95% confidence level.

**Figure 1.** Pareto chart for the standardized effects of the variables fermentation time, temperature, pH and lactose on the production of β-galactosidase.
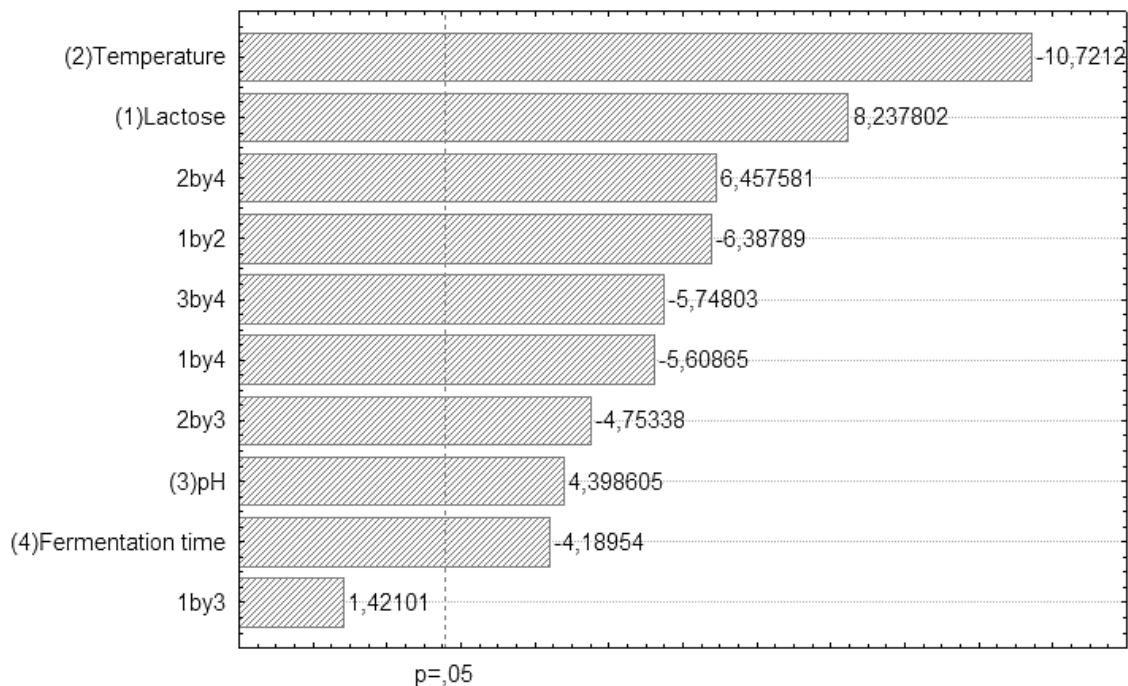


Standardized effect estimated (absolute values). Source: Elaborated by the authors.

All variables studied had a statistically significant effect on the production of β-galactosidase, except the interaction between lactose concentration and pH (Figure1). In order of magnitude, temperature was the most important variable. It can be seen from the Pareto chart that a decrease in temperature can increase β-galactosidase production. This result is in agreement with those of Murad et al. (2011) and Hsu et al. (2005) who demonstrated that the highest β-galactosidase production by *L.reuteri, S.thermophillus* and *B.longum* was obtained at temperatures below 40°C.

The lactose concentration was the second most significant variable (Figure 1). Many studies show that having lactose as a carbon source in the medium is an important factor in the production of β-galactosidase in bacteria. The presence of lactose in culture medium probably triggers β-galactosidase activity by the lac operon mechanism (Reznikoff et al., 1978). This interpretation is in agreement with our results and the findings of Hsu et al. (2005) and Murad et al. (2011) who showed that lactose in the media led to enhancing the production of enzyme by *Bifidobacteria, L.reuteri* and *S.thermophillus*.

The pH and fermentation time had a low influence compared with the other variables (Figure 1). pH affects at least two aspects of microbial cells i.e., the functioning of the enzyme and the transport of nutrients into the cell, also affect the RNA and protein synthesis

(Khovrytchev et al., 1977). When the bacteria grow on either side of their optimum pH range, the lag phase may increase. The authors observed that maximum β-galactosidase production is directly related to biomass production, total lactose consumption and lower pH (Duan et al., 2017; Hsu et al., 2005). Bosso et al. (2019) found that the peak of β-galactosidase activity occurred during the stationary phase when lactose was fully consumed. This explains the high influence of the interaction between the fermentation time and lactose when compared with the influence of only the fermentation time.

Taking all these considerations into account, the influence of temperature, lactose concentration and pH was further optimized using a $2^3$ central composite experimental design. The fermentation time was set as constant for all the further production, due to the production of enzymes being high in a short fermentation time, as indicated by the Pareto chart. Thus, the fermentation time was fixed at 12h of cultivation.

## 3.2 Response Surface Methodology

The analysis of multiple regression (Table 3, part A) showed that this parameter was a linearly increasing function of both lactose concentration and pH. Therefore, to obtain a maximum value of this parameter, the experimental design was expanded, and additional experiments (runs 14–16) were carried out to optimize β-galactosidase production (Table 3, part B). It was then possible to obtain a quadratic polynomial equation, derived from equation (1), to express the relationship between β-galactosidase and the selected independent variables. The optimal conditions to maximize the enzyme production, the variables (temperature, lactose concentration and pH) were estimated by deriving Equation (3).

The regression equation of the second-order model provides the levels of β-galactosidase activity based on temperature, lactose concentration and pH:

$$Y = 29.36 + 3.24\,X_1 - 9.90\,X_1{}^2 + 0.90\,X_2 - 2.06\,X_2{}^2 + 1.93\,X_3 - 5.49\,X_3{}^2 + 2.0\,X_2 X_3$$

(3)

Where Y is the predicted value of β-galactosidase and $X_1$, $X_2$ and $X_3$ are the coded values for temperature, lactose concentration and pH, respectively.

**Table 5.** Analysis of variance (ANOVA) for the second-order model for level optimization of β-galactosidase production by *Enterococcus faecium*.

| Source | Sum of squares | df | Mean of square | *F* value | *p* value |
|---|---|---|---|---|---|
| A-Temperature (L) | 86.954 | 1 | 86.95 | 171.55 | 0.0001 |
| A-Temperature (Q) | 146.678 | 1 | 146.67 | 289.38 | 0.0000 |
| B-Lactose concetration (L) | 89.802 | 1 | 89.80 | 177.16 | 0.0226 |
| B-Lactose concentration (Q) | 117.831 | 1 | 117.87 | 232.46 | 0.0001 |
| C-pH (L) | 26.015 | 1 | 26.01 | 51.32 | 0.0015 |
| C-pH (Q) | 185.902 | 1 | 185.90 | 366.76 | 0.0000 |
| B (L) by C (L) | 62.827 | 1 | 62.82 | 123.95 | 0.0003 |
| Pure error | 2,027 | 1 | 0,5069 | | |
| Lack of fit | 57.776 | 4 | 14,4441 | | |
| Total | 1.681.277 | 15 | | | |

$R^2$ =0.964 and Adj $R^2$ = 0.933. Significant for p values <0.05. Source: Elaborated by the authors.

The analysis of variance (ANOVA) was used to evaluate the significance of the quadratic polynomial model as shown in Table 5. The results indicated that the linear and quadratic effects of temperature, pH and lactose concentration were significant factors as their p values were <0.05. The interactive effect of lactose and pH was also found to be significant. The determination coefficient ($R^2$) and adjusted determination coefficient ($R^2$ adj) were 96.4% and 93.3%, respectively. According to Chauhan & Gupta (2004) a model that has an $R^2$ value higher than 75% can be accepted. In the present study, the design was an efficient tool to determine the effects of the condition of the culture on β-galactosidase by *E. faecium,* indicating that the model as fitted explained 96.4% of the variability in enzyme production.

The plots of response surface methodology can be used to study the effect of the interaction of variables on β-galactosidase production and also to predict the optimal values of those variables. The 3D surface plots were generated by plotting the response (β-galactosidase activity) against any two variables, while other variables were fixed at their middle levels. The model for β-galactosidase production was used to construct the response surfaces, which

is shown in Figure 2. The oval shape curve suggests optimum conditions and combinational effects on enzyme production considering all the possible combinations. The interactive roles of temperature, pH and lactose concentration on β-galactosidase production by *E. faecium* are illustrated in the three-dimensional curves of the calculated response surface

The combination of variables pH and temperature on β-galactosidase production is shown in Figure 2A. The high β-galactosidase production was observed in the temperature at the central point, while alkaline pH induced the enzyme production. In previous reports, several studies achieved high enzyme activity between 28-30°C and optimum pH from acid to neutral (5.0 - 7). The highest β-galactosidase activity was found from *Aspergillus niger* at 28°C and pH 7 (Martarello et al., 2019), *Kluyveromyces marxianus* at 30°C and pH 5 (Panesar, 2008) and *Kluyveromyces lactis* at 30°C and pH 7.5 (Dagbagli & Goksungur, 2008).

Figure 2B shows the interaction between temperature and the lactose concentration. The same behavior of temperature found in Figure 2A, while the variable lactose combined with other variables induced the increase in enzyme production with a concentration between 4-5.5% as shown in Figure 2B-C. Domingues et al. (2005) observed that recombinant *Saccharomyces cerevisiae* showed high β-galactosidase production when the fermentation medium was supplemented with 5.0% lactose as the sole carbon source. On the other hand, Kamran et al. (2016) observed that when lactose concentration was increased beyond 1% up to 3%, the production of enzyme by *Bacillus* strains declined and only 15% enzyme production was noticed with reference to optimum level produced at 1% lactose. Akolkar et al. (2005) reported that the maximum β-galactosidase activity from *Lactobacillus acidophilus* was 810 U/ mL and was at 1.5 % of lactose.

The interaction between pH and lactose on β-galactosidase production is shown in Figure 2C. Unlike other combinations, pH and lactose in low levels were found to be significant for β-galactosidase production. Substrate concentration has an important role in enzyme production. In this paper, a low concentration of lactose leads to a high production of β-galactosidase. However, the concentration of lactose may vary from strain to strain. A high substrate concentration can decrease enzyme production due to the increase in the viscosity of the growth medium that can create a hurdle for the appropriate availability.

**Figure 2.** Response surface plots showing interaction between different variables on b-galactosidase production by *E.faecium*. (A) effect of interaction between temperature and pH. (B) effect of interaction between temperature and lactose (C) effect of interaction between pH and lactose concentration.
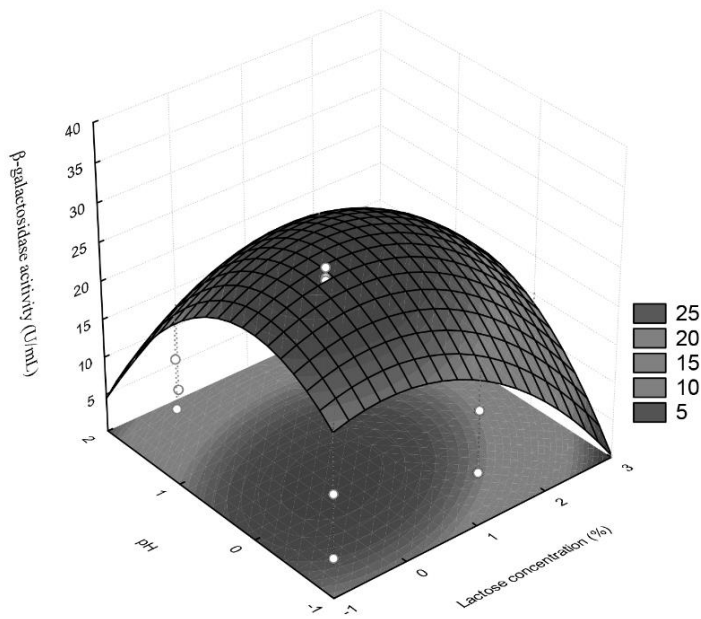
(a)
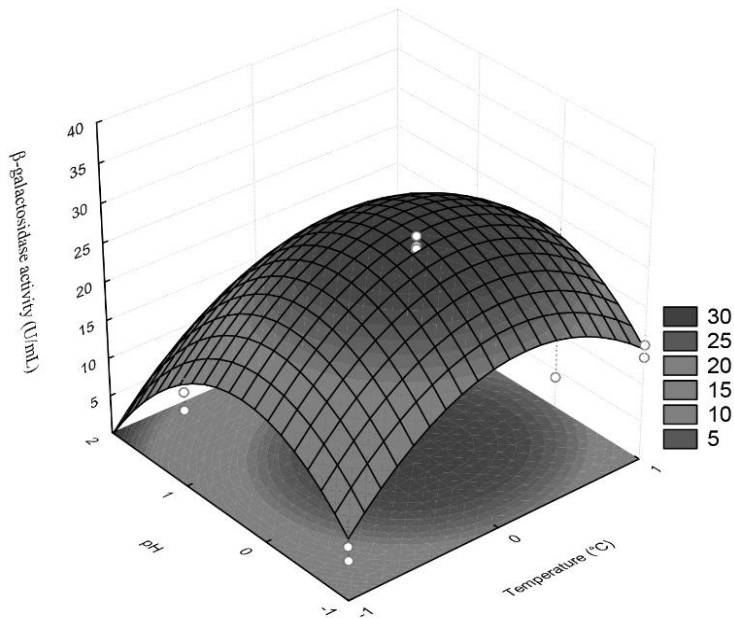


(b)
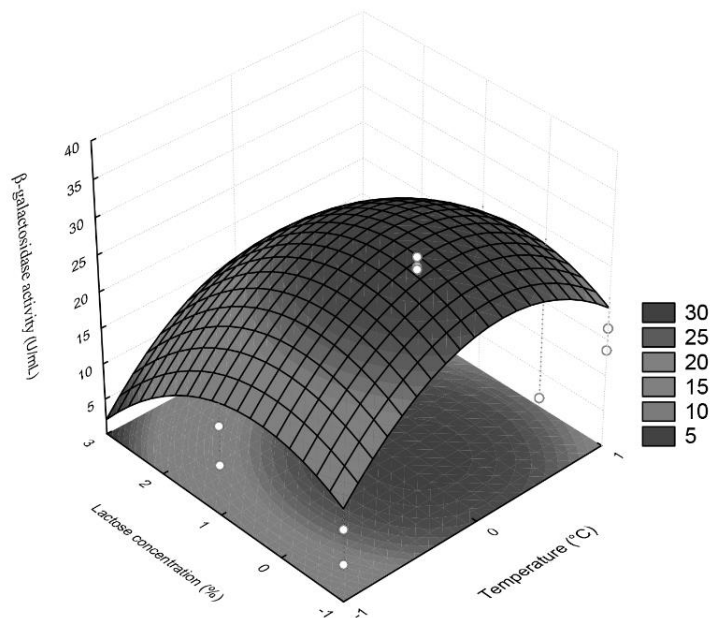
**(c)**



Source: Elaborated by the authors.

Most studies on the optimization of β-galactosidase production found in the literature involve yeasts. The major industrial enzymes are obtained from *Aspergillus sp.* and *Kluyveromyces sp*. Thus, there are still few studies that investigate the involvement of bacteria in β-galactosidase production. The result obtained in this study regarding the enzymatic activity of β-galactosidase shows some differences in variables when these are compared with other reports due to the difference in the strains used in each study. *E.faecium*, for example is a new source of β-galactosidase, as cited at the beginning of this paper.

**3.4 Validation of RSM model**

Based on the results of the statistical analysis, tests were performed in triplicate to confirm the predictions of the mathematical models. The optimal conditions used for β-galactosidase production was estimated to be a temperature of 31°C, pH 8.0 and a lactose concentration of 5.34% (Table 3, part C). Under these conditions, the galactosidase production was 29.85 U/mL which was slightly different from the predicted value of 30.83 U/mL. Thus, the model developed was accurate and reliable for predicting the optimized conditions, and for maximizing the production of β-galactosidase.

**4. Conclusions**

The use of RM from *C.vulgaris* as a complement of MRS medium for β-galactosidase production is a promising alternative since the enzyme activity obtained was considerably higher after optimization. To the best of our knowledge, this is the first time that a statistical approach has been used and it showed a significant improvement in results for optimizing a new alternative medium as the production of β-galactosidase from *E. faecium* under submerged fermentation is maximized. In future perspectives, study in a bioreactor for larger scale production using the results shown here will be perfomed, and the economic viability study of the enzyme production.

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