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Research Article

Determination of Cell Permeabilization and Beta-Galactosidase Extraction from *Aspergillus oryzae* CCT 0977 Grown in Cheese Whey

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Aspergillus oryzae grown in cheese whey has the ability to produce beta-galactosidase. The objective of this work was to define the parameters for the determination of cell permeabilization and extraction of the enzyme from *Aspergillus oryzae* CCT 0977 biomass, with high enzymatic activity. The Box–Behnken design was used to determine cell permeabilization and extraction of beta-galactosidase conditions. The fermentation was carried out for a period of 5 days at 28°C, having as substrate the deproteinized cheese whey. To determine the effect of the variables on beta-galactosidase activity, enzymatic activity was determined by the lactose hydrolysis reaction. The most efficient condition for cell permeabilization was 25% ethanol at 30°C for 90 min, obtaining an enzymatic activity of 0.44 $U \cdot mL^{-1}$. For beta-galactosidase extraction from the biomass, the most efficient condition was 5.3% chloroform at 48°C, with an enzymatic activity of 0.17 $U \cdot mL^{-1}$. The use of ethanol was most efficient to promote cell permeability of *Aspergillus oryzae* CCT 0977.

1. Introduction

Biotechnological processes are widely used to obtain products with high added value [1]. The use of agroindustrial wastes in bioconversion by microorganisms has been the subject of extensive research, especially with reference to the production of metabolites of interest such as proteins, enzymes, organics acids, and secondary metabolites [2]. Cheese whey has been widely used as a fermentation medium, as it is a low-cost cheese residue and is nutritionally rich [3, 4]. Beta-galactosidase can be obtained by fermentation process by various microorganisms, such as *Aspergillus oryzae*, a thermotolerant fungus, without many environmental requirements for cultivation [5, 6].

The enzyme beta-galactosidase is one among other enzymes with industrial potential used in the hydrolysis of lactose in milk and cheese whey, generating food with low levels of lactose, which results in a better solubility and digestibility of milk and dairy products, making them ideal for consumers intolerant to this sugar [7].

When a metabolite is intracellular, the rupture of the cell wall is the first step performed in the downstream process. This allows separating the substance for later purification. Various methods may be used, but the process will depend on the location and stability of the metabolite. There are mechanical methods of cell disruption such as high-pressure homogenizers, ultrasonic waves, and glass beads. Chemical disruption methods use alkalis, detergents, and organic solvents. Enzymatic methods, however, consist of enzymatic lysis or inhibition of cell wall synthesis [1, 8, 9].

The use of organic solvents for cellular permeabilization and enzymatic extraction is the most common methodology, which was applied after the cell fermentation process. The permeabilization is a simple and fast method that allows

Table 1: The Box–Behnken design used to determine the effects of ethanol, temperature, and time on the enzymatic activity of permeabilized cells of *Aspergillus oryzae* CCT 0977.

Run	Variables			Beta-galactosidase activity ($U \cdot mL^{-1}$)	
	X_1 Ethanol (%)	X_2 Temperature (°C)	X_3 Time (min)	Experimental	Predicted
1	−1 (15)	−1 (20)	−1 (30)	0.22	0.17
2	−1 (15)	0 (30)	1 (90)	0.36	0.40
3	−1 (15)	1 (40)	0 (60)	0.29	0.29
4	0 (25)	−1 (20)	1 (90)	0.38	0.38
5	0 (25)	0 (30)	0 (60)	0.44	0.39
6	0 (25)	1 (40)	−1 (30)	0.17	0.22
7	1 (35)	−1 (20)	0 (60)	0.25	0.29
8	1 (35)	0 (30)	−1 (30)	0.23	0.23
9	1 (35)	1 (40)	1 (90)	0.40	0.35

measuring the enzymatic activity [10]. For the extraction of enzymes, it is necessary to promote the chemical breakdown of the cell wall. It is a simple and efficient method that does not leave cellular fragments and does not require high investments with the mechanical methods, but it demands more time, due to the chemical reactions. These solvents modify the cell wall structures causing disorganizations in their pores allowing the passage of small molecules, such as substrates or other molecules present in the fermentative medium [11].

Thus, the aim of this work was to cultivate the filamentous fungus *Aspergillus oryzae* CCT 0977 in cheese whey and determine the conditions of cell permeabilization and extraction of the enzyme beta-galactosidase in the biomass.

2. Materials and Methods

2.1. Microorganism, Inoculum, and Medium. The *Aspergillus oryzae* CCT 0977 strain was obtained from Tropical Culture Collection of Foundation Andre Tosello. The culture was maintained in tubes containing PDA (potato dextrose agar, Acumedia®) and stored at 4°C. For the inoculum, 0.85% saline solution with 1% Tween 80 was used, and the spore count was performed by using the Neubauer chamber at a count of 1×10^6 cells·mL^{-1}. An inoculum concentration of 1% v/v was used in relation to the culture medium. The cheese whey powder was obtained from a local dairy cooperative. The cheese whey powder was solubilized in distilled water at a concentration of 5% w/v. For deproteinization of the cheese whey, 85% lactic acid was added until pH 4.6 (isoelectric point of the milk caseins) and heated at 90°C for 30 min. After precipitation, the protein fraction was removed by filtration using Whatman no. 1 filter paper, and the medium was adjusted to pH 5.0. Pasteurization of the cheese whey was at 65°C for 30 min. The deproteinized and pasteurized cheese whey was used as the culture medium in the fermentation experiments.

2.2. Fermentation Conditions. For culture, 1% v/v of the inoculum was added and the fermentation was performed on an orbital shaker (Tecnal®, TE-420) at 28°C and 120 rpm for 5 days. The biomass produced after five days of fermentation was ground with the homogenizer (IKA®, T10 Basic), and 5 mL of the suspension was collected and transferred to falcon tubes, followed by centrifugation (Quimis®, Q222G) for 10 min at 1100 rpm, for separation of the biomass and supernatant. The Biomass was used for the two methods: cell permeabilization and extraction of beta-galactosidase.

2.3. Cell Permeabilization. After fermentation of *Aspergillus oryzae* CCT 0977, the cells were collected by centrifugation (1100 rpm for 10 min) and washed once with distilled water. Cell permeabilization was performed under static mode and incubated at 30°C for 90 minutes in a water bath in Falcon tubes (15 mL) containing 5 mL of the reaction suspension consisting of ethanol, according to the experimental design (Table 1), approximately 50 mg wet biomass, and 0.1 M potassium phosphate buffer (pH 6.8). The flasks were incubated at temperature and time according to the statistical design (Table 1). The biomass was collected by centrifugation at 1100 rpm for 10 min for further analysis, and the cells were washed once with the same buffer. The biomass was suspended in 1 mL of the phosphate buffer, and the enzymatic activity was determined by the enzymatic hydrolysis of lactose.

2.4. Enzyme Extraction. After fermentation of *Aspergillus oryzae* CCT 0977, the cells were collected by centrifugation (1100 rpm for 10 min) and washed once with distilled water. For the extraction of the beta-galactosidase, the biomass was transferred to Erlenmeyer flasks (50 mL) and resuspended in 0.1 M potassium phosphate buffer (pH 6.8) with the addition of chloroform at the concentrations according to the experimental design (Table 2), with a final volume of 10 mL. After addition of the reagents, the vials were incubated on the orbital shaker (Tecnal, TE-420) at 120 rpm, at the temperatures described in Table 2, overnight. The supernatant and the biomass were separated by centrifugation at 1100 rpm for 10 min. In the supernatant, the enzymatic activity was determined.

2.5. Determination of Beta-Galactosidase Activity. The enzymatic activity was determined by the initial rates of the lactose hydrolysis reaction through the glucose dosage

Table 2: Analysis of variance (ANOVA) of the regression parameters for the Box–Behnken design used to determine the effect of ethanol, temperature, and time on the enzymatic activity of permeabilized cells of *Aspergillus orzyae* CCT 0977.

	Seq. SS	df	MS	F	p value
Ethanol (%) (L + Q)	0.00324	2	0.001619	0.251141	0.799271
Temperature (°C) (L + Q)	0.00617	2	0.003084	0.478510	0.676356
Time (min) (L + Q)	0.0451	2	0.022552	3.499239	0.222260
Error	0.01289	2	0.006445	—	—
Total SS	0.06739	8	—	—	—

Seq. SS: sequential sums of squares; df: the degrees of freedom; MS: adjusted mean square; F: F calculated; p: p value at 0.05%.

produced by the enzymatic-colorimetric glucose oxidase kit (Bioliquid®) method. The unit of activity used in the work was the glucose unit produced per minute, per milliliter of enzymatic suspension ($U{\cdot}mL^{-1}$), defined as μmol of glucose produced per minute, per mL of enzymatic suspension at 47°C at a initial concentration of lactose solution equal to $10\,g{\cdot}L^{-1}$, prepared in 0.1 M citrate-phosphate buffer (pH 6.5). For the hydrolysis, an enzymatic suspension with a concentration of 30% v/v was used, and the incubation in a water bath remained overnight. After the incubation time, the enzyme was inactivated at 90°C for 5 min followed by an ice bath. Analyses were performed in triplicate.

3. Results and Discussion

3.1. Cell Permeabilization. Taking into account that permeabilized cells are biocatalysts, that is, they function as a source of enzymes that naturally remains immobilized. The Box–Behnken design (BBD) was used to evaluate the effects among the significant variables and to determine their optimal values. BBD was developed to reduce the number of experimental runs and increase the efficiency. BBD has been applied and considered a very efficient statistical experimental design tool in several areas including chemical engineering optimization [12].

The determination of the enzymatic activity for permeabilized cells of *Aspergillus oryzae* CCT 0977 was carried out to find the optimal values of independent variables (ethanol, X_1; temperature, X_2; time, X_3), which would give maximum beta-galactosidase activity. Based on the Box–Behnken design, the experimental levels of beta-galactosidase activity under each set of conditions were determined and compared with the corresponding predicted levels suggested (Table 1). The maximum experimental value for beta-galactosidase activity was $0.44\,U{\cdot}mL^{-1}$ while the value of predicted response was $0.39\,U{\cdot}mL^{-1}$. Some authors report the supplementation of the culture medium during fermentation. Panesar et al. [13] used some salts, lactose, peptone and yeast extract and obtained a maximum activity of the *Aspergillus oryzae* NCIM 1212 beta-galactosidase of $0.50\,U{\cdot}mL^{-1}$, at a temperature between 45 and 50°C, a value similar to that obtained in the present study. Senm et al. [14], under submerged fermentation at pH 4.5 with *Aspergillus alliaceus*, obtained an enzymatic activity of 0.0486 U/mL.

The coefficient of determination R^2 of the model was 0.81266, which indicated that the model adequately represented the real relationship between the variables under consideration. An R^2 value of 0.81266 means that 81.26% of the variability was explained by the model, which is acceptable for the biological system and only 18.74% was as a result of chance. Approximately 81.26% of validity was achieved, indicating that the model exerted an adequate prediction on the enzyme activity. The close correlation between the experimental and predicted data indicates the appropriateness of the experimental design. The maximum beta-galactosidase activity ($0.44\,U{\cdot}mL^{-1}$) was achieved under the following conditions: ethanol (25%), temperature (30°C), and time (60 min) (Table 1). In Table 2, the analysis of variance (ANOVA) of the regression parameters for the Box–Behnken experimental design was evaluated.

Based on the results for high beta-galactosidase activity from permeabilized cells of *A. oryzae* CCT 0977, all linear (L) and quadratic (Q) effects of ethanol, temperature, and time were not significant ($p < 0.05$), indicating that the variables in the smaller range limit, 15%, 20°C, and 30 min, respectively, was enough for enzymatic activity.

In order to determine the ranges of variables that influence beta-galactosidase activity from permeabilized cells of *A. oryzae* CCT 0977, response surface plots were generated for this analysis. The response surfaces for beta-galactosidase activity evaluating the variables ethanol, temperature, and time were plotted as shown in Figure 1. Figure 1(c) shows the effects of temperature and ethanol on beta-galactosidase activity. Temperature and ethanol ranging from low to high values of process showed the high enzyme activity. The beta-galactosidase activity was higher in the temperature range of 26–33°C and ethanol between 20 and 30%. Figure 1(b) depicts the response surface plot as a function of time versus ethanol. Change of ethanol does not significantly affect the curvature of the surface. From a graphical representation, there is a dependence of beta-galactosidase activity on the time ranging from 60 to 90 min. Figure 1(c) shows high beta-galactosidase activity effectiveness within the temperature range of 20–40°C and time ranging from 60 to 90 min, while below and above these ranges, a significant decrease of activity can be observed. According to Kumari et al. [15], a lower temperature with the increase of the permeabilization time intensifies the activity of the enzyme, since an elevation of the temperature can generate the partial inactivation of the beta-galactosidase. This confirms that the range of these variables were chosen properly and sufficient for the process. A maximum beta-galactosidase activity of $0.44\,U{\cdot}mL^{-1}$ for permeabilized cells was defined under the following conditions: ethanol 25%, at 30°C, and 60 min.

Based on the results obtained, the temperature and time are fundamental factors in the process of cell permeabilization and can be explained by the fact that the cell walls of the fungi are more rigid than others, requiring a lower concentration of ethanol with more reaction time and a higher temperature for cellular disorganization.

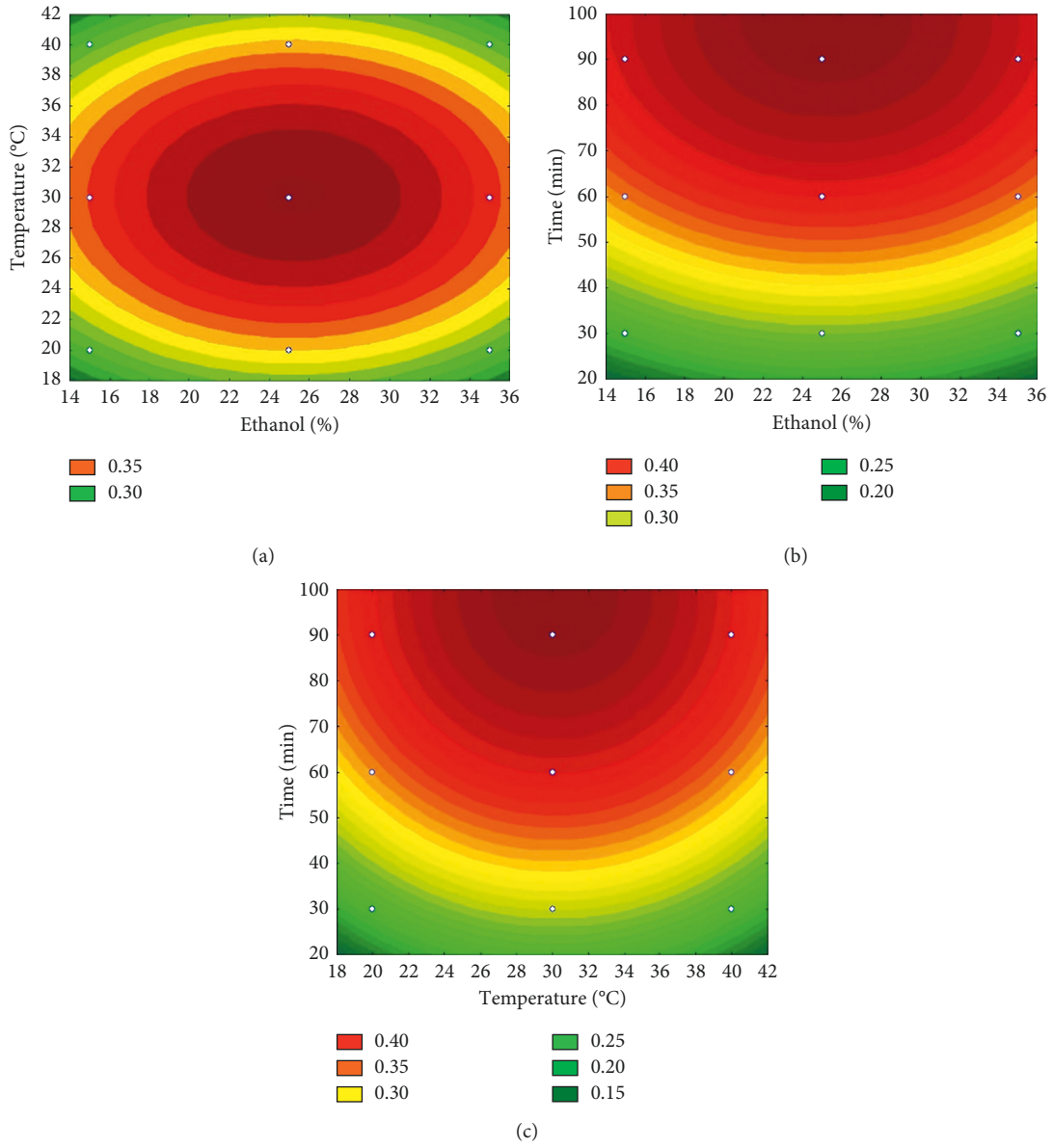

Figure 1: Response surface plot representing the effect of the variables on beta-galactosidase activity ($U \cdot mL^{-1}$) of permeabilized cells from *Aspergillus oryzae* CCT 0977.

3.2. Enzyme Extraction. The determination of the enzymatic activity for extracted beta-galactosidase of *Aspergillus oryzae* CCT 0977 was carried out to find the optimal values of independent variables (chloroform (X_1) and temperature (X_2)), which would give maximum beta-galactosidase activity. Based on the Box–Behnken design, the experimental levels of beta-galactosidase activity under each set of conditions were determined and compared with the corresponding predicted levels suggested (Table 2). The maximum experimental value for beta-galactosidase activity was 0.17 $U \cdot mL^{-1}$ while the value of predicted response was 0.15 $U \cdot mL^{-1}$. The coefficient of determination R^2 of the model was 0.87248, which indicated that the model adequately represented the real relationship between the variables under consideration. An R^2 value of 0.87248 means that 87.24% of the variability was explained by the model, which is acceptable for biological system, and only 12.76% was as a result of chance. Approximately 87.24% of validity was achieved, indicating that the model exerted an adequate prediction on the enzyme activity. The close correlation between the experimental and predicted data indicates the appropriateness of the experimental design. The maximum beta-galactosidase activity (0.17 $U \cdot mL^{-1}$) was achieved under the following conditions: chloroform (5.0%) and temperature (45°C) (Table 3). In Table 4, the analysis of variance (ANOVA) of the regression parameters for the Box–Behnken experimental design was evaluated.

Based on the results for high beta-galactosidase activity from extracted beta-galactosidase of Aspergillus oryzae CCT

Table 3: The Box–Behnken design used to determine the effect of chloroform and temperature on the enzymatic activity of extracted beta-galactosidase of *Aspergillus orzyae* CCT 0977.

Run	Variables		Beta-galactosidase activity ($U \cdot mL^{-1}$)	
	X_1 Chloroform (%)	X_2 Temperature (°C)	Experimental	Predicted
1	−1 (4.0)	−1 (40)	0.09	0.08
2	−1 (4.0)	0 (45)	0.12	0.13
3	−1 (4.0)	1 (50)	0.15	0.14
4	0 (5.0)	−1 (40)	0.10	0.11
5	0 (5.0)	0 (45)	0.17	0.15
6	0 (5.0)	1 (50)	0.16	0.16
7	1 (6.0)	−1 (40)	0.12	0.12
8	1 (6.0)	0 (45)	0.16	0.16
9	1 (6.0)	1 (50)	0.15	0.15

Table 4: Analysis of variance (ANOVA) of the regression parameters for the Box–Behnken design used to determine the effects of chloroform and temperature on the enzymatic activity of extracted beta-galactosidase of *Aspergillus orzyae* CCT 0977.

	SS	df	MS	F	p value
Chloroform (%) (L + Q)	0.001089	2	0.000544	2.63677	0.218346
Temperature (°C) (L + Q)	0.004689	2	0.002344	11.35426	0.039863
$X_1 * X_2$	0.0002225	1	0.000225	1.08969	0.373253
Error	0.000619	3	0.000206	—	—
Total SS	0.006622	8	—	—	—

Seq. SS: sequential sums of squares; df: the degrees of freedom; MS: adjusted mean square; F: F calculated; p: p value at 0.05%.

0977, the effect of temperature (L + Q) was significant ($p > 0.05$), indicating that higher temperature is more appropriate for high enzymatic activity. The linear (L) and quadratic (Q) effects of chloroform and the quadratic effect of temperature were not significant ($p > 0.05$), indicating that the variables remaining at the lowest levels, 4% and 40°C, respectively, were enough for enzymatic activity.

In order to determine the ranges of variables that influence enzymatic activity of beta-galactosidase extracted from *Aspergillus oryzae* CCT 0977, response surface plots were generated for this analysis. The response surfaces for beta-galactosidase activity evaluating the variables chloroform and temperature were plotted in Figure 2 that shows the effects of temperature and chloroform on beta-galactosidase activity. Chloroform ranging from lower to higher values of process showed the high enzyme activity. The beta-galactosidase activity was higher in the chloroform range of 5.0–6.0%. From a graphical representation, there is a dependence of beta-galactosidase activity on the temperature ranging from 45 to 50°C, while below these ranges, a significant decrease of activity can be noticed. A maximum beta-galactosidase activity of 0.17 $U \cdot mL^{-1}$ was defined under the following conditions: chloroform 5.0% and 47°C.

Several authors have studied the effect of solvents on the extraction of enzymes [8, 16, 17]. Nagy et al. [18] determined the activity of the intracellular beta-galactosidase of

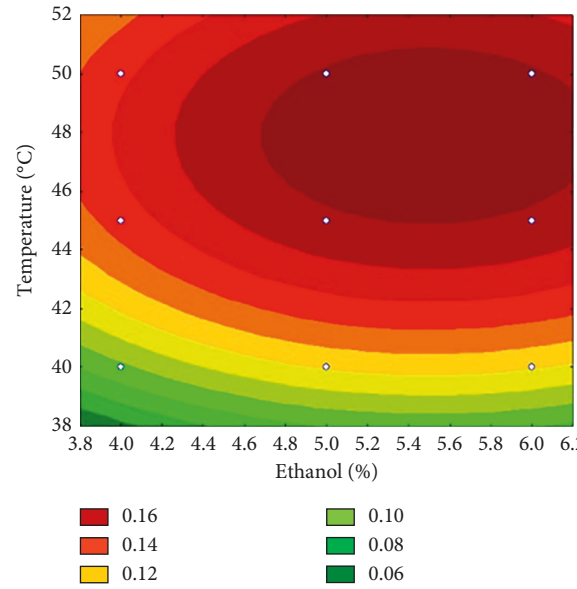

Figure 2: Response surface plot representing the effect of the variables on enzymatic activity ($U\ mL^{-1}$) of beta-galactosidase extracted from *Aspergillus oryzae* CCT 0977.

Penicillium chrysogenum NCAIM 00237 and verified an activity of approximately 0.16 $U \cdot mL^{-1}$. Mirdamadi et al. [19] tested different compositions of liquid media for their efficacy in the production of beta-galactosidase by *Aspergillus orzyae* PTCC 5163 and verified that in cheese whey-based media, the maximum enzymatic activity obtained was approximately 0.13 $U \cdot mL^{-1}$. Therefore, in our study, it was observed that, in the extraction process, there was an expressive reduction in the enzymatic activity. In the permeabilization, the activity was 0.44 $U \cdot mL^{-1}$; in the extraction, this value decreases to 0.17 $U \cdot mL^{-1}$, a reduction of 61.36% in beta-galactosidase activity. Probably, the process of extracting the enzyme from the inside of the cell by solvent causes damage to the enzyme that causes reduction in its activity.

4. Conclusion

Aspergillus oryzae CCT 0977 was successfully cultivated in deproteinized cheese whey for the production of the enzyme beta-galactosidase. Two chemical solvents were used: ethanol for cell permeabilization and chloroform for extraction of the enzyme. The ethanol as a permeabilizing agent obtained higher enzymatic activity when compared to the extraction by chloroform. The best condition in the permeabilization process, obtaining the highest activity of beta-galactosidase, was with ethanol 25%, temperature of 30°C, and for 60 minutes. Under these conditions, the maximum experimental value of beta-galactosidase activity was 0.44 $U \cdot mL^{-1}$. For the extraction process, the condition was 5.3% chloroform at 48°C, with an enzymatic activity of 0.17 $U \cdot mL^{-1}$.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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