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α-L-Fucosidase from *Thermotoga maritima*: hydrolytic and transfucosylation activities

α-L-Fucosidasa de *Thermotoga maritima*: actividades hidrolíticas y de transfucosilación

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Abstract

Fucosylated oligosaccharides play several biologically relevant roles. They are naturally present in human milk and offers to infants, short- and long-term health benefits. These compounds can be obtained also by enzymatic synthesis. In this work, the effects of pH and temperature on hydrolytic and transfucosylation activities of α-L-fucosidase from *Thermotoga maritima* were evaluated. The optimal pH for the enzyme-catalyzed hydrolysis was found in a range from 6 to 8 while the highest conversions for transfucosylation reactions were observed within the range of 7-10. The best temperature for both enzymatic activities was 95 °C. Fucosylated oligosaccharides were synthesized with the highest productivity of 3.54 mM/h at pH 8 and 95 °C. Overall, optimization of the conditions of transfucosylation reaction catalyzed by the α-L-fucosidase from *Thermotoga maritima*, allowed for higher yields of fucosylated oligosaccharides as well as shorter reaction time and a lower concentration of the employed enzyme.

Keywords: Fucosylated oligosaccharides, fucosidase, *Thermotoga maritima*.

Resumen

Los oligosacáridos fucosilados desempeñan varias funciones biológicamente relevantes. Están presentes de forma natural en la leche materna, lo que ofrece a los bebés beneficios a la salud a corto y largo plazo. Estos compuestos pueden obtenerse también mediante síntesis enzimática. En este trabajo, se evaluaron los efectos de pH y temperatura en las actividades hidrolíticas y de transfucosilación de la α-L-fucosidasa de *Thermotoga maritima*. El pH óptimo para la hidrólisis catalizada por la enzima se encontró en un rango de 6 a 8, mientras que la mayor conversión en las reacciones de transfucosilación se observó en el rango 7-10. La mejor temperatura para ambas actividades enzimáticas fue 95 °C. Los oligosacáridos fucosilados fueron sintetizados con la productividad más alta de 3.54 mM/h a pH 8 y 95 °C. En general, la optimización de las condiciones de la reacción de transfucosilación catalizada por la α-L-fucosidasa de *Thermotoga maritima* permitió mayores rendimientos de oligosacáridos fucosilados así como un tiempo de reacción más corto y una menor concentración de enzima.

Palabras clave: Oligosacáridos fucosilados, fucosidasa, *Thermotoga maritima*.

1 Introduction

L-Fucose is present in many biological entities such as blood group antigen, glycoproteins, glycolipids and in human milk oligosaccharides (HMO) such as fucosylated oligosaccharides (Becker and Lowe, 2003; Lezyk *et al.*, 2016). The latter represent between 50-80% of the total HMO, in fact, 2'-fucosyllactose is one of the most abundant components in human milk (2.43 g/L) (Chaturvedi *et al.*, 2001). Fucosylated oligosaccharides show many important beneficial effects on infant health, for example, they act as prebiotics, modulators of gut motility, and pathogen adhesion blocking agents, that help to avoid enteric infections (Rudloff and Kunz 2012; Zehra *et al.*, 2018).

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Fucosylated oligosaccharides are synthesized in vivo in the Golgi apparatus of the alveolar cells in mammary glands through the activity of specific transferases (Gabrielli *et al.*, 2011). In vitro, fucosylated oligosaccharides can be obtained by biotransformations with α-L-fucosidases (E.C. 3.2.1.51) that belong to GH29 family. These glycosidases are capable of catalyzing the synthesis of fucosylated oligosaccharides, provided that the reaction conditions are manipulated, so that they favor the transfucosylation activity over hydrolysis. The mechanism of enzymatic catalysis leading to the formation of fucosylated oligosaccharides involves, in the first step, hydrolysis of a fucosyl residue at the non-reducing end of fucosylated oligosaccharides, and fucoglycoconjugates resulting in a glycosyl-enzyme intermediate. Then, this intermediate undergoes nucleophilic attack either by a water molecule, leading to an undesirable hydrolysis reaction, or by another glycosidic acceptor resulting in the formation of fucosylated oligosaccharides (Escamilla-Lozano *et al.*, 2015; Zeuner *et al.*, 2014; Guzmán-Rodríguez *et al.*, 2018).

In order to improve the yield of the fucosidase-catalyzed synthesis of carbohydrates, it is a decisive factor to work at the optimum reaction conditions of pH and temperature. In this way, the kinetic equilibrium of the reaction is forced towards transfucosylation (Zeuner *et al.*, 2016; Abdul-Manas *et al.*, 2018, Guo *et al.*, 2018). Furthermore, thermodynamic parameters such as energy activation must be considered while analyzing the efficiency of an enzyme (Haq *et al.*, 2020; Munir *et al.*, 2020). The aim of this work was to study the effects of pH and temperature on the hydrolytic and transfucosylation activities in the synthesis of fucosylated oligosaccharides using the α-L-fucosidase from *Thermotoga maritima*.

2 Materials and methods

2.1 Material

4-Nitrophenyl-α-L-fucopyranoside (pNP-Fuc), 4-nitrophenol (pNP), D-lactose, L-fucose and 2'-fucosyllactose were purchased from Sigma-Aldrich (St. Louis MO, USA). Sodium monophosphate, sodium diphosphate and sodium hydroxide were purchased from J.T. Baker (Mexico State, Mexico). Recombinant α-L-fucosidase from *Thermotoga maritima* (E.C. 3.2.1.51) was purchased from Megazyme (Leinster, Ireland) at 5 U/mL (2.6 U/mg) in 3.2 M ammonium sulphate solution. The working solution was obtained by 1:1000 dilution in 0.1 M phosphate buffer at the corresponding pH. Milli-Q® water (Merck, Germany) was used throughout all experiments.

2.2 Effect of pH on the α-L-fucosidase hydrolytic and transfucosylation activities

The hydrolytic activity of α-L-fucosidase was determined in a pH range from 5 to 10 at 60 °C during 5 min, following the protocol previously reported by Guzmán-Rodríguez *et al.* (2018) with minor modifications. Briefly, 50 μL of α-L-fucosidase (0.0065 U/mL) from *T. maritima* were added to 400 μL of pNP-Fuc (3.5 mM) in phosphate buffer (0.1 M). The reaction progress was analyzed at 0.5, 1, 1.5, 2, 3, and 5 min. The reaction was stopped by adding 50 μL of NaOH (1 M). Released pNP was quantified spectrophotometrically (Shimadzu UV-1800, Tokyo, Japan) at 410 nm by plotting the obtained value on a standard curve. Hydrolytic activity was represented as initial velocity (V_0).

The transfucosylation activity was evaluated in a similar way, except that D-lactose (438 mM) was added to the mixture. The reaction was incubated at 60 °C for 60 min and analyzed at 10, 20, 40, and 60 min. Transfucosylation activity was monitored through the synthetized fucosylated oligosaccharides which were quantified as described in section 2.5. The activity was represented as V_0.

2.3 Effect of temperature on the α-L-fucosidase hydrolytic and transfucosylation activities

Hydrolytic activity of the α-L-fucosidase was determined at pH 8 in a temperature range from 60 to 95 °C. The reaction progress was analyzed every minute during 15 min. Briefly, 50 μL of α-L-fucosidase (0.0065 U/mL) were added to 400 μL of pNP-Fuc (3.5 mM) in phosphate buffer (0.1 M). The reaction was stopped by placing the vial in an ice bath for 1 min and subsequent addition of 50 μL of NaOH (1 M). Released pNP was quantified spectrophotometrically as described previously in section 2.2. Hydrolytic activity was represented as V_0.

The transfucosylation activity was evaluated in a similar way, except that D-lactose was added to the reaction mixture (438 mM). The reaction was incubated for 60 min and analyzed at 10, 20, 40 and 60 min. The synthesized fucosylated oligosaccharides were quantified as described in section 2.5. Transfucosylation activity was represented as V_0. To determine the corresponding activation energy (Ea) of the studied reactions, the Arrhenius plot was obtained by plotting the natural logarithm of the initial velocity (ordinate) versus inverse temperature (1/T) on the abscissa (Fig. 3). Finally, Ea was calculated by applying the Arrhenius equation expressed as Equation 1:

$$Ea = -mR. \quad (1)$$

where m is the slope of the interpolation curves described above and R is the universal gas constant (1.987 kcal/mol K).

2.4 α-L-Fucosidase thermal stability

The α-L-fucosidase thermal stability was evaluated at 90 and 95 °C in 0.1 M phosphate buffer pH 8 for different time intervals with duration up to 30 min. Briefly, 100 μL of α-L-fucosidase (0.0065 U/mL) were suspended in phosphate buffer (0.1 M) and left to incubate. For each time interval, reactions were stopped by placing the vials in an ice bath for 1 min. Afterwards, an aliquot of 50 μL was withdrawn and added to 450 μL of pNP-Fuc solution (3.5 mM) to determine the residual hydrolytic activity. The mixture was incubated at 60 °C for 10 min in a Temperature-Controlled Cell-Holder. The released pNP was quantified spectrophotometrically as already described.

2.5 Carbohydrate quantification

The quantification of the synthesized fucosylated oligosaccharides and the released fucose was performed on a HPLC apparatus (LabAlliance, State College, PA, USA) with an ion-exclusion column Rezex RNO-Oligosaccharides Na+ (4%) (60 x 10 mm; particle size 12 μm) (Phenomenex; Amstelveen, Netherlands) and oven temperature of 75 °C. The HPLC was equipped with a SOFTA 300S light scattering detector (Chrom Tech, Minnesota, USA) with a nitrogen flow of 62.5 psi, spray chamber temperature of 10 °C, and a drift tube temperature of 45 °C.

Prior to injection on HPLC, samples were filtered through 0.22 μm Millipore Durapore membranes and eluted with Milli-Q water at a flow rate of 0.3 mL/min. A calibration curve of 2'-fucosyllactose was used to estimate the amount of product formed. Additionally, standard solutions of 2'-fucosyllactose, lactose and fucose were used to determine their corresponding retention times in order to identify the reaction products.

2.6 Statistical analysis

All experiments were performed in triplicate and results are reported as a mean value ± standard deviation. For statistical analysis, IBM SPSS Statistic version 25.0 for Windows (IBM, New York, USA) software was used to carry out a one-way analysis of variance (ANOVA) followed by the Tuckey's test for comparing all pairs of groups. A P value of 0.05 was considered statistically significant.

3 Results and discussion

3.1 Effect of pH on the α-L-fucosidase hydrolytic and transfucosylation activities

The highest hydrolytic activity of α-L-fucosidase from *T. maritima* was found in in the pH range between 6 and 8, with no significant difference between these values ($P<0.05$) (Fig. 1). The obtained results are in accordance with those reported by Lezyk *et al.* (2016) who described higher activity at pH range of 4 - 7 for the α-L-fucosidase from *T. maritima* at 30 °C and 1 mM of pNP-Fuc as substrate. In general, most of α-L-fucosidases exhibit hydrolytic activity at a wide pH range. For example, Berteau *et al.* (2004) found pH 4 as optimal for the performance of α-L-fucosidase from Pecten maximus, whereas, Cobucci-Ponzano *et al.* (2005) reported that the α-L-fucosidase from *Sulfolobus solfataricus* displayed its maximum activity at pH 5. In a study carried out by Benešová *et al.* (2013) the recombinant α-L-fucosidase from Paenibacillus thiaminolyticus, which was cloned and expressed in *E. coli*, worked most efficiently at pH of 8.2.

In addition, Fig. 1 also shows the transfucosylation activity of α-L-fucosidase from *T. maritima*.

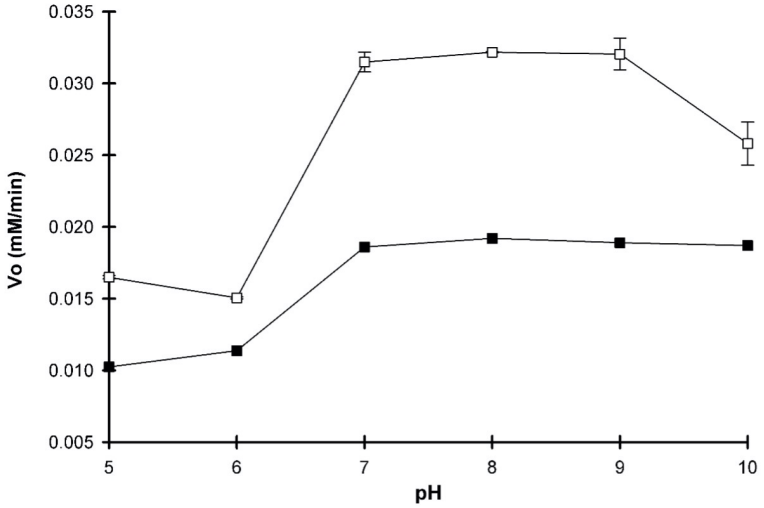

Fig. 1. Effect of pH on the hydrolytic (□) and transfucosylation (■) activities of the α-L-fucosidase from *T. maritima*. Hydrolytic activity was performed at 60 °C using 3.5 mM pNP-Fuc and 0.0065 U/mL α-L-fucosidase. Transfucosylation activity was performed under the same conditions, adding 438 mM D-lactose. Error bars represent the standard deviation of the mean of triplicates.

The highest activity was found in the pH range from 7 to 10 ($p<0.05$). The enhanced transfucosylation activity was observed when pH increased from 5 to 8 that led to a 6.7-fold higher yield (from 6.07 to 40.67%). The obtained results are in accordance with other studies on optimum reaction conditions of glycosidases from thermophilic sources. Wu *et al.* (2013) reported an enhancement of galactooligosaccharides (GOS) synthesis when pH was modified from 4 to 6 for β-glycosidase from *Sulfolobus solfataricus*, whereas for the two recombinant β-glycosidases expressed in *E. coli* (F441Y and F359Q) GOS increased when varying pH from 4 to 6.5. Additionally, Ji *et al.* (2005) observed an improvement in GOS yields for biotransformation catalyzed by a recombinant β-galactosidase from *Thermotoga maritima* expressed in *E. coli* when pH was changed from 5 to 6. In another study, the improved GOS production was linked to the pH rise from 5 to 5.5 using β-mannosidase from Pyrococcus furiosus as a catalyst (Hansson *et al.* 2001).

It is observed that hydrolysis and transglycosylation activities exhibit similar profiles at pH 5 - 9, conversely, at pH 10, the hydrolytic activity decreased meanwhile for transfucosylation activity remained unchanged. Sulzenbacher *et al.* (2004) and Tarling *et al.* (2003) suggested that changes in pH of the reaction medium affected the pKa of the key amino acids involved in the catalysis, while Abdul-Manas *et al.* (2018) concluded that the ionization of this key amino acid was a determinant factor that favored interactions either with water, resulting in hydrolysis reaction, or a sugar acceptor improving transfucosylation reaction yields as observed in this work.

3.2 Effect of temperature on the α-L-fucosidase hydrolytic and transfucosylation activities

As can be seen in Fig. 2, the highest hydrolytic activity was observed at 95 °C, conversely, no significance difference was observed in the temperature range 60-70 °C ($P<0.05$). Similar results were reported for other heat resistant glycosidases. For example, Turner *et al.* (2007) reported an optimum temperature of 90 °C for β-glycosidase B from Thermotoga neapolitana while Gumerov *et al.* (2015) found that 85 °C was an optimum temperature for a β-galactosidase from Acidilobus saccharovorans. In other studies, *Pyrococcus furiosus* α-glycosidase was shown to have the highest activity at temperatures ranging from 105 to 115 °C (Costantino *et al.*, 1990).

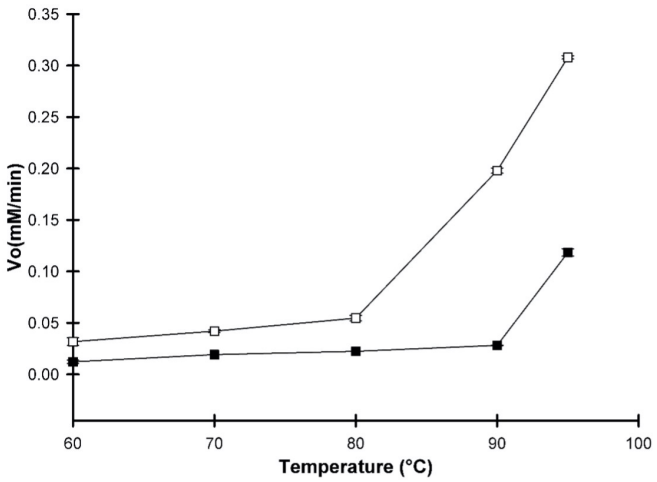

Fig. 2. Effect of temperature on the hydrolytic (□) and transfucosylation (■) activities of the α-L-fucosidase from *T. maritima*. Hydrolytic activity was performed using 3.5 mM pNP-Fuc and 0.0065 U/mL α-L-fucosidase. Transfucosylation activity was performed under the same conditions, adding 438 mM D-lactose. Error bars represent the standard deviation of the mean of triplicates.

Among numerous benefits of working with thermophilic enzymes at elevated temperatures, the most important are improved reaction kinetics and increased substrate solubility (Liu *et al.* 2015). Likewise, in our studies the rise of temperature enhanced the transfucosylation activity of α-L-fucosidase (Fig. 2). The reaction yield at 95 °C was three times higher than that obtained at 60 °C (32.01 and 10.58% respectively). Similar results were recorded by Vera *et al.* (2011) who reported an increase in the production yields of both GOS and lactulose with temperature. Authors suggested that the observed results might be related to the positive effect of temperature on the hydrolytic activity. Hence, it can be assumed that the enhanced yield of fucosylated oligosaccharides obtained in this work is associated with the effect of temperature on the improvement of enzymatic hydrolysis. In another work, the conversion yield of GOS in the presence of β-galactosidase from Bacillus circulans was three times greater at 60 than at 25 °C (5 and 15 g/L, respectively) (Warmerdam *et al.*, 2013). Moreover, the research group of Fourage *et al.* (2000) reported a 2.6 times higher reaction yield in the pNP-Fuc-Fuc synthesis (from 16 to 42%) catalyzed by β-glycosidase from Thermus thermophilus, in response to the temperature rise from 37 to 75 °C. Recently, Zeuner *et al.* (2016) reported a six-fold increase of the reaction yield (from 0.9 to 5.4%) in the N-acetyllactosamine synthesis catalyzed by β-galactosidase from Pyrococcus furiosus when temperature was risen from 40 to 60 °C.

Additionally, the Ea values for the α-L-fucosidase catalyzed hydrolysis and transfucosylation reactions were determined at 13.83 and 14.27 kcal/mol, respectively. According to Segel (1976) and Keleti (1983), log Vmax versus 1/T is the ideal approach to determine Ea values. As reported in our previous work (Guzmán-Rodríguez *et al.*, 2021) Vmax was reached at pNP-Fuc concentration of 0.136 mM, as the concentration of donor substrate employed in this work was 3.5 mM, we can assume to be working at Vmax, hence, data obtained from the Arrhenius plot (Fig. 3) of this work were deemed valid. The obtained values indicate that transfucosylation is a thermodynamically disfavored reaction, and the enzyme would have to overcome a higher energy barrier to perform the synthesis of fucosylated oligosaccharides in comparation to the energy required for hydrolysis. Simila r results were obtained by Yang *et al.* (2018) who determined a higher Ea value for the synthesis of galactotrisaccharides (7.42 kcal/mol) than that for the lactose hydrolysis (6.4 kcal/mol) in a reaction catalyzed by recombinant β-glucosidase from *Thermotoga naptophila* RKU-10.

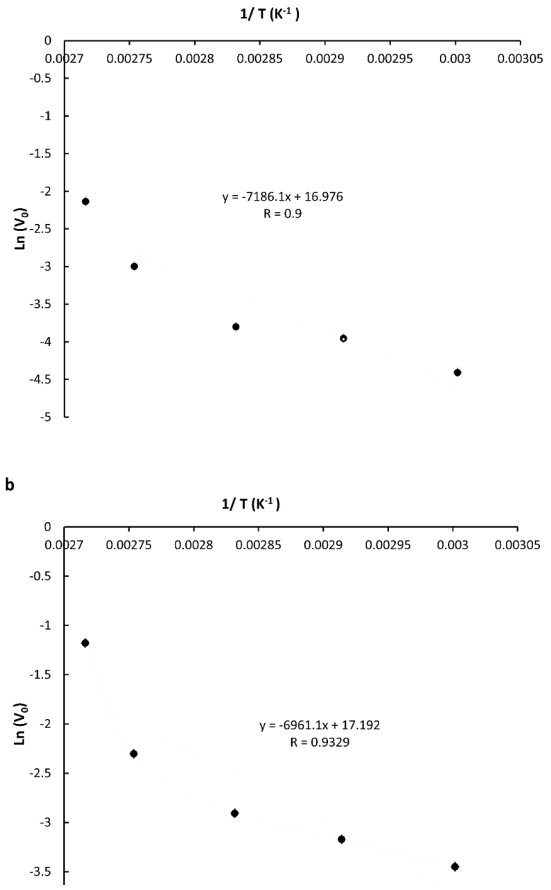

Fig. 3 Arrhenius plots of Ln (V_0) as a function of $1/T$. a) Hydrolytic activity, b) Transfucosylation activity. Error bars represent the standard deviation of the mean of triplicates.

Furthermore, the Ea for the hydrolytic activity obtained in this work is lower than that reported by Pouwels *et al.* (2000) for the hydrolysis of para-nitrophenyl-β-D-galactopyranoside catalyzed by a β-glycosidase from P. furiosus (15.52 kcal/mol) and for a β-glycosidase from S. solfataricus (16.96 kcal/mol). When an enzyme binds its substrate, residues in the active site may form interactions with substrate molecules which lowers the reaction's Ea (Marana *et al.*, 2002). Difference between hydrolytic and transfucosylation activities can be because of the different effect of the acceptor substrates on the enzyme active site.

It is remarkable that Arrhenius plots are distinctly nonlinear (Fig. 3). Vieille & Zeikus (2001) reported that hyperthermophilic enzymes are an important exception to the typical Arrhenius behavior. Furthermore, as temperature increase, this kind of enzymes suffers catalytically structural changes, which are frequently correlated with functionally conformational alterations (Londesborough, J., 1980; Hensel *et al.*, 1987).

It is noteworthy that by using an activated donor substrate (such as pNP-Fuc) for α-L-fucosidase, as described in this study, the energy liberated by the cleavage of glycosyl bond may contribute to lowering the activation energy of transglycosylation (Escamilla-Lozano *et al.*, 2019). Then, it is possible that higher Ea values could be obtained for non-activated substrates.

3.3 Thermal stability of the α-L-fucosidase

As shown in the previous section, the α-L-fucosidase showed the highest activity at pH 8 and temperatures of 90 and 95 °C. Therefore, its thermal stability was determined by incubating the enzyme at these temperatures. The half-lives of enzyme at 90 and 95 °C were 30.13 and 17.28 min, respectively. Zeuner *et al.* (2018) studied the thermal stability of the α-L-fucosidase from *T. maritima* at pH 5. These authors stablished that this enzyme presented half-lives of 15 and 4.4 min when incubated at 90 and 95 °C, respectively. The results reported by these authors differ from those obtained in this study, which can be attributed to the different concentrations of enzyme and substrate used, as well as a different pH. The α-L-fucosidase from *T. maritima* showed higher activity in alkaline media as discussed in section 3.1. In another study, a recombinant β-glucosidase expressed in *E. coli* showed half-life times of 71 and 9.2 h at 90 and 100 °C, respectively (Mehmood *et al.*, 2014).

3.4 Synthesis of fucosylated oligosaccharides

As the α-L-fucosidase from *T. maritima* exhibited the highest activity at pH 8 and good stability between 90 and 95 °C, these conditions were applied to synthesize fucosylated oligosaccharides. The highest conversion rates of fucosylated oligosaccharides at these temperatures were 0.76 and 3.54 mM/h, respectively (Fig. 4). However, it was observed that after 20 min at 95 °C the concentration of fucosylated oligosaccharides started to decrease and show no significance difference compared to 90 °C in the interval from 40-60 min ($P<0.05$). This decline could be explained in two ways; either product accumulation shifted the reaction equilibrium towards hydrolysis or long chain oligosaccharides were formed. The former explanation was discarded due to the lack of evidence in HPLC chromatograms.

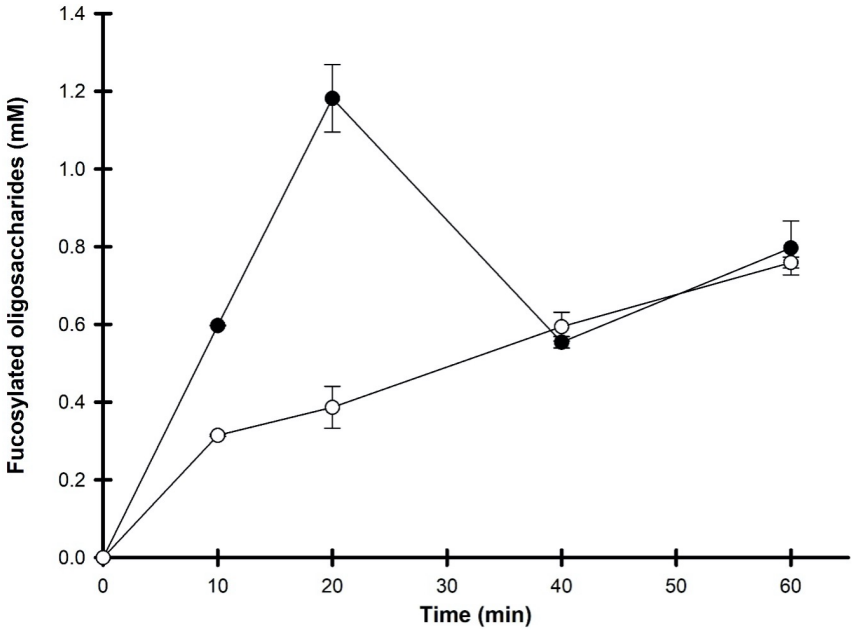

Fig. 4. Kinetics of transfucosylation reaction catalyzed by the α-L-fucosidase from *T. maritima*. Reaction was performed at 90 (○) and 95 °C (●) and pH 8, using 3.5 mM pNP-Fuc as donor substrate, 438 mM D-lactose as acceptor substrate and 0.0065 U/mL α-L-fucosidase. Error bars represent the standard deviation of the mean of triplicates.

However, the latter assumption may be a viable explanation since the HPLC column employed during analyses separated compounds according to molecular weight, and traces of long chain oligosaccharides produced in the reaction could be underestimated. This hypothesis needs to be further explored, for example, by performing tandem mass spectroscopy (MALDI-TOF) in order to identify all the possible compounds obtained during the transfucosylation reaction.

Lezyk *et al.* (2016) reported a conversion rate of 0.23 mM/h in the synthesis of 2'-fucosyllactose using 0.51 μg/mL α-L-fucosidase from *T. maritima*, 20 mM pNP-Fuc and 25 mM D-lactose at pH 5.0 and 30 °C. In another study, the α-L-fucosidase from *T. maritima* was employed at concentration of 0.13 μg/mL to catalyze the synthesis of fucosyllactose, using 3.5 mM pNP-Fuc and 584 mM D-lactose at pH 5.9 and 60 °C. The highest conversion rate achieved in these conditions was 0.29 mM/h (Guzmán-Rodríguez *et al.*, 2018). The conversion rate obtained in this study was higher than those reported by the aforementioned authors, noteworthy using lower concentration of α-L-fucosidase (0.013 μg/mL). However, in the present study different pH and temperature were employed.

Conclusions

α-L-fucosidase from *T. maritima* is already known for its capacity to synthesize fucosylated oligosaccharides through transfucosylation. Nevertheless, this enzyme has not been previously tested at the conditions presented in this research. At the best reaction conditions determined in this work (pH 8 and 95 °C), the fucosyl-oligosaccharides were yielded at 3.54 mM/h. It is possible that the fucosylated oligosaccharides synthesized in this study have biological functions like human milk oligosaccharides and can be used either for clinical applications or as an additive to infant formula.

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