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L-Fucose Synthesis Using a Halo- and Thermophilic L-Fucose Isomerase from Polyextremophilic Halothermothrix orenii

In Jung Kim and Kyoung Heon Kim *

Department of Biotechnology, Graduate School and Department of Food Bioscience and Technology, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Korea; ij0308@korea.ac.kr
* Correspondence: khekim@korea.ac.kr

Abstract: L-Fucose isomerase (L-Fucl)-mediated isomerization is a promising biotechnological approach for synthesizing various rare sugars of industrial significance, including L-fucose. Extremozymes that can retain their functional conformation under extreme conditions, such as high temperature and salinity, offer favorable applications in bioprocesses that accompany harsh conditions. To date, only one thermophilic L-Fucl has been characterized for L-fucose synthesis. Here, we report L-Fucl from Halothermothrix orenii (HoFucl) which exhibits both halophilic and thermophilic properties. When evaluated under various biochemical conditions, HoFucl exhibited optimal activities at 50–60 °C and pH 7 with 0.5–1 M NaCl in the presence of 1 mM Mn²⁺ as a cofactor. The results obtained here show a unique feature of HoFucl as a polyextremozyme, which facilitates the biotechnological production of L-fucose using this enzyme.

Keywords: L-fucose isomerase; L-fucose; L-fuculose; extremophile; halothermophilic bacteria; Halothermothrix orenii

1. Introduction

L-Fucose isomerase (L-Fucl) is a ketol isomerase that mediates the reversible reaction between L-fucose and L-fuculose (Figure 1) [1–3]. Biologically, L-Fucl is involved in the initial process of L-fucose catabolism in various bacteria, in which L-fucose obtained from the environment is converted into L-fuculose, eventually leading to the synthesis of L-lactaldehyde, a metabolically important intermediate, through successive reactions [4]. In addition to L-fucose, L-Fucl can metabolize D-arabinose due to its structural similarities [1,2,5,6].

In vitro assays have demonstrated that L-Fucl-mediated catalysis is not limited to the interconversion between L-fucose–L-fuculose and D-arabinose–D-ribulose; the enzyme is also involved in various isomerization reactions, such as reactions between D-altrose–D-psicose and L-galactose–L-tagatose [6]. Owing to its promiscuous activities, L-Fucl is considered to be a biotechnologically useful enzyme for producing rare sugars of industrial significance. Among them, L-fucose is of particular interest due to its diverse bioactive properties, such as anti-inflammatory, antitumor, immune-enhancement, skin-whitening, and skin-moisturization properties, that are beneficial to humans [7–9]. Although widely occurring in nature in the form of bacterial exopolysaccharides, brown seaweed polysaccharides (i.e., fucoidan), and human milk oligosaccharides, L-Fucl does not meet the industrial demands to directly acquire L-fucose from natural sources [10–12]. Conventional chemical synthesis is also industrially incompatible as it is a time-consuming and labor-intensive multistep process [13,14]. More importantly, the chemical method utilizes toxic reagents, which contradicts the consumer’s call for the use of “natural” products. Alternatively, biocatalysts based on either enzymes or whole cells can provide a more efficient and eco-friendly method for L-fucose synthesis. The in vitro L-Fucl-based biosynthetic routes for L-fucose known to date are directed towards L-fuculose production as the main
Material precursor, which is subsequently converted into L-fucose by L-FucI [15,16]. Although it is more expensive than L-fucose, strategies to obtain L-fuculose from cheap and common sugars using chemical or enzymatic methods have already been established [15,16]. Thus, L-FucI-mediated L-fucose production utilizing L-fuculose as the substrate is a key target for investigation. Previously, we identified and characterized two L-FucIs from Raoultella sp. (RdFucI) [2] and Thermaanaeromonas toyohensis (TtFucI) [3], demonstrating the applicability of L-FucI for L-fucose synthesis using L-fuculose.

Extremozymes are considered favorable in bioprocesses in the sugar, food, and pharmaceutical fields, which usually accompany high temperatures and high salt concentrations because they are stable and more active under harsh conditions. Such robust enzymes are generally sourced from organisms that have adapted to extreme environments, such as extremely hot and/or saline habitats. To date, three extremo-(thermophilic) L-FucIs (TtFucI, CsFucI from Caldicellulosiruptor saccharolyticus, and DtFucI from Dictyoglomus turgidum) have been characterized [3,5,6,17]. However, only TtFucI has been evaluated for L-fucose synthesis [3]. Therefore, it is of great importance to identify novel extremo-L-FucIs that could offer great advantages in industrial applications.

Halothermothrix orenii is a true anaerobic polyextremophile isolated from the Tunisian salt lake in the Sahara Desert [18]. Owing to its ability to thrive under dual extreme conditions, that is, high temperature and high salinity, with optimal growth at 60 °C and 1.7 M NaCl, it is classified as a halothermophile [18,19]. Polyextremophilic organisms are very rare and unique in nature, as they have evolved to develop clever strategies to adapt to more than two severe conditions. Accordingly, H. orenii could serve as a useful source of enzymes with beneficial features for industrial applications. Indeed, the genome of H. orenii revealed the presence of genes encoding enzymes of biotechnological significance, such as those associated with carbohydrate saccharification and metabolism of sugars, including L-fucose [18,19]. In this study, we identified L-FucI from H. orenii (HoFucI) and performed its functional characterization for L-fucose synthesis under various biochemical conditions, such as NaCl, temperature, pH, and metal ions, to explore its unique molecular features. The results obtained from this study will not only offer insights into the fundamental knowledge of extremozymes, but also allow the biotechnological production of L-fucose and other valuable rare sugars using HoFucI.

![Figure 1. A scheme for interconversion between L-fucose and L-fuculose catalyzed by HoFucI.](image)

2. Materials and Methods

2.1. Expression and Purification of HoFucI

The gene encoding HoFucI was synthesized (Bioneer, Daejeon, South Korea) and cloned into pET28a(+) as an expression vector. The vector containing the synthetic HoFucI gene was transformed into competent Escherichia coli BL21 (DE3) cells, which were grown at 37 °C on lysogeny broth (LB)-agar plates containing 50 μg/mL of kanamycin for the selection of transformants. E. coli transformants containing the HoFucI gene were cultivated in the LB medium containing 50 μg/mL of kanamycin at 37 °C. When the optical density at 600 nm (OD600) reached 0.6–0.8, the expression of recombinant HoFucI was induced by adding 0.5 mM isopropyl-β-d-1-thiogalactopyranoside to the medium, and the cells were further cultivated at 18 °C and 180 rpm overnight. After centrifugation at 4500×g at 4 °C for 20 min, the collected cells were resuspended in buffer A (50 mM Tris-HCl,
200 mM NaCl, and 10 mM imidazole; pH 8.0) and disrupted using an ultrasonicator to extract the intracellular proteins. Total cell lysates were centrifuged at 25,188×g at 4 °C for 30 min, and the supernatant containing the crude protein extract was loaded onto a His-Trap column pre-equilibrated with buffer A (GE Healthcare, Chicago, IL, USA). The HoFucI protein was purified using immobilized metal ion affinity chromatography. After thoroughly washing the column to remove the non-specifically bound proteins with buffer A, the bound protein was eluted with buffer B composed of 50 mM Tris-HCl, 200 mM NaCl, and 300 mM imidazole (pH 8.0). For further purification and determination of the oligomeric state of the native enzyme, the eluted protein fractions were concentrated and subjected to size-exclusion chromatography using a HiPrepTM 16/60 Sephacryl S-200 HR column (GE Healthcare), with a buffer composed of 20 mM sodium phosphate and 200 mM NaCl (pH 7.0) as the mobile phase.

2.2. Enzyme Assay

Generally, enzymatic reactions were performed in a 20 mM sodium phosphate (pH 7.0) buffer containing 1 mM Mn²⁺ as the cofactor and 0.5 M NaCl. Purified HoFucI (7 μg) was incubated with 10 mM L-fuculose at 60 °C for 5 min. The amount of L-fucose obtained from the enzymatic reactions was measured using the K-FUCOSE assay kit (Megazyme, Bray, Co. Wicklow, Ireland), according to the manufacturer’s instructions. The experimental data represent the means ± standard deviations. Enzyme activity was represented by specific activity (U/mg) where one unit (U) was defined as the amount of purified HoFucI enzyme required to yield 1 μmol of L-fucose per min from 10 mM L-fuculose under specified conditions.

2.3. Effects of Various Biochemical Conditions on HoFucI Activity

To investigate the effect of NaCl on the enzymatic activity of HoFucI, 7 μg of HoFucI and 10 mM L-fuculose were incubated at 60 °C in 20 mM sodium phosphate (pH 7.0) buffer in the presence of 1 mM Mn²⁺ for 5 min at different concentrations of NaCl (0, 0.5, 1, 2.5, and 5 M). Temperature and pH profiles were determined under the standard conditions described above. The optimum temperature was examined at various temperatures ranging from 30 to 100 °C with an interval of 10 °C, and the optimum pH was investigated with three buffer systems using 100 mM sodium acetate (pH 4–6), 100 mM sodium phosphate (pH 7–8), and 100 mM glycine-NaOH (pH 9–11). The effect of metal ions on the enzymatic activity of HoFucI was investigated using different concentrations of ethylenediaminetetraacetic acid (EDTA), Mn²⁺, Mg²⁺, or Co²⁺ (0, 1, and 10 mM) under standard conditions.

2.4. Phylogenetic Tree

A homolog search was conducted based on the HMMER algorithm with an E-value cutoff of 0.0001 using RdFucI as the query on the ConSurf server (https://consurf.tau.ac.il/ (accessed on 30 March 2022)). Extremely long and obvious fault sequences were discarded. After excluding redundant sequences at the N-terminus, the sequences for L-FucI homologs that were searched and previously characterized were aligned using Clustal Omega [20]. The aligned sequences were then applied to phylogenetic tree building using the ConSurf server. The tree was visualized using FigTree (https://tree.bio.ed.ac.uk/software/figtree/ (accessed on 30 March 2022)).

2.5. Sequence Alignment and Homology Modeling

Multiple sequence alignment of L-FucIcs was performed based on Clustal Omega [20] and graphically presented by ESPript [21]. The homology model of the HoFucI structure was constructed using SWISS-MODEL (http://swissmodel.expasy.org/(accessed on 30 March 2022)) with RdFucI (PDB code: 6K1F) as the template, which showed the 65% sequence identity with HoFucI. The root mean square deviation (rmsd) between HoFucI and
RdFuCl structures was 0.2427 Å. The structures were visualized using the PyMOL software [22].

3. Results
3.1. Oligomeric State of HoFucI

Commonly, native l-Fucls exist as oligomers [2,3,23]. According to crystal structure analysis, l-Fucls cause homohexameric structures to be functional for substrate interactions [2,23]. However, the oligomeric states of l-Fucls in a solution can vary, existing as trimers or tetramers, depending on the biochemical conditions [3,6,17]. In this study, size-exclusion chromatography, which is commonly used to examine the enzyme’s molecular mass at the native state (i.e. under the non-denaturing condition), was performed to determine the oligomeric state of HoFucI (Figure 2). The chromatogram showed a single peak corresponding to HoFucI (Figure 2a). By comparing its elution volume with that of standard proteins, the molecular mass of the enzyme was predicted to be approximately 204 kDa, corresponding to a trimer (Figure 2b).

![Figure 2](image_url)

**Figure 2.** (a) Size-exclusion chromatography profile of l-fucose isomerase (l-Fucl) from *Halothermothrix orenii* (HoFucI). (b) Calibration curve obtained using various protein standards (cytidine [Cyd], cytochrome C [Cyt C], β-lactoglobulin [β-LG], bovine serum albumin [BSA], and immunoglobulin G [IgG]) to determine the native molecular mass of HoFucI.

3.2. Effect of Salinity on HoFucI Activity

Since the origin of HoFucI, *H. orenii*, is a halophilic bacterium that grows optimally at 1.7 M NaCl [18,19]. The effect of salinity on the enzymatic activity of HoFucI was evaluated using various concentrations of NaCl from 0 to 5 M (Figure 3). Without NaCl supplementation (control), HoFucI showed substantial activity, with a specific activity of 106.2 U/mg. When the NaCl concentration was increased up to 1 M, the isomerization activity of HoFucI toward L-fuculose also increased. The maximal activity was exhibited at 0.5 and 1 M with no significant difference (p > 0.05), which was approximately 60% higher than that obtained without the addition of NaCl. At concentrations higher than 1 M, the enzyme activity gradually decreased, but was still retained at a higher (at 2.5 M) or similar (at 5.0 M) level compared to the control without the addition of NaCl. Based on these results, HoFucI can be classified as a halophilic enzyme that remains active in molar concentrations of the salt.
Figure 3. Effect of NaCl concentration on the L-fuculose-isomerizing activity of HoFucI. The enzyme reactions were performed with 7 μg of HoFucI and 10 mM L-fuculose at 60 °C for 5 min in 20 mM sodium phosphate (pH 7.0) buffer in the presence of 1 mM Mn²⁺. The reaction solution contained various NaCl concentrations ranging from 0 to 5 M. The experimental data represent the means ± standard deviations of three replicates. Relative activity (%) represents the enzyme activity relative to that obtained without supplementation of NaCl, set as 100%.

3.3. Effect of Temperature and pH on HoFucI Activity

When the temperature effect on the activity of HoFucI on L-fuculose was investigated in the presence of 0.5 M NaCl (Figure 4a), the maximum activity was observed at 50 °C and 60 °C with no significant difference (p > 0.05), and the enzyme activity sharply decreased at temperatures higher than 60 °C, showing around 60% of relative activity (RA) at 70 °C, but only around 10% activity at 80–100 °C. At 30 and 40 °C, 65% of RA was maintained. This result indicates that HoFucI is a thermophilic enzyme that correlates well with the optimal growth conditions of H. orenii at 60 °C, with a range of 42–70 °C [18,19].

When the influence of pH on the enzyme activity of L-fuculose was examined (Figure 4b), the optimal pH was found to be pH 7, and more than 60% of RA was retained at a pH of 8 and 9. At pH values lower than 7 and higher than 9, the activity decreased sharply, showing only approximately 20% of RA at a pH of 6 and 10, and less than 10% of RA at a pH of 4, 5, and 11.

Figure 4. (a) Effect of temperature on the l-fuculose-isomerizing activity of HoFucI. Enzyme reactions were performed at various temperatures ranging from 30 to 100 °C with 7 μg of HoFucI and
10 mM L-fuculose for 5 min in 20 mM sodium phosphate (pH 7.0) buffer in the presence of 1 mM Mn\(^{2+}\) and 0.5 M NaCl. The experimental data represent the means ± standard deviations. Relative activity (%) represents the enzyme activity relative to that obtained at 60 °C, set as 100%. (b) Effect of pH on the L-fuculose-isomerizing activity of HoFucI. Enzyme reactions were performed at various pH ranging from 4 to 11 with 7 μg of HoFucI and 10 mM L-fuculose at 60 °C for 5 min in the presence of 1 mM Mn\(^{2+}\) and 0.5 M NaCl. The buffers used were 100 mM sodium acetate (pH 4, 5, and 6), 100 mM sodium phosphate (pH 7 and 8), and 100 mM glycine-NaOH (pH 9, 10, and 11). The experimental data represent the means ± standard deviations. Relative activity (%) represents the enzyme activity relative to that obtained at pH 7, set as 100%.

3.4. Effect of Metal Ions on HoFucI Activity

L-FucIs have a conserved metal-binding site (Figure S1 in Supplementary Material) and commonly require divalent cations, such as Mn\(^{2+}\), Mg\(^{2+}\), and Co\(^{2+}\), for their catalytic activities [2,5,6]. In particular, Mn\(^{2+}\) plays a crucial role in the thermal stability and catalytic activity of L-FucI [3]. In this study, we evaluated the effects of two different concentrations (1 and 10 mM) of Mn\(^{2+}\), Mg\(^{2+}\), and Co\(^{2+}\) on the activity of HoFucI. Without any metal supplementation (control), very low activity was observed, while the presence of metal ions boosted the enzyme activity (Table 1). Specifically, upon the addition of 1 mM Mn\(^{2+}\) and Co\(^{2+}\), the enzyme activities increased ~5 fold compared to that of the control, but the enzyme activities were lower at 10 mM Mn\(^{2+}\) and Co\(^{2+}\). In the case of Mg\(^{2+}\), higher activity was observed at 10 mM Mg\(^{2+}\) than at 1 mM, with an RA of 488%. However, EDTA, which is used as a metal chelator, had little effect on enzyme activity.

Table 1. Effect of metal ions on the L-fuculose-isomerizing activity of L-fucose isomerase (L-FucI) from Halothermothrix orenii (HoFucI) 1.

<table>
<thead>
<tr>
<th>Metal Ions</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>69.7 ± 24.6</td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>555.7 ± 78.7</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>100.0 ± 5.1 1,2</td>
</tr>
<tr>
<td>Co(^{2+})</td>
<td>523.1 ± 74.2</td>
</tr>
</tbody>
</table>

1 Relative activity (%) represents the enzyme activity relative to that obtained without any supplementation of metal ions, set as 100%. 2 Experimental data represent the means ± standard deviations of three replicates.

3.5. Amino Acid Composition of HoFucI

Halophiles are generally classified into two types depending on their adaptation strategies to external high salt conditions: salt-in and salt-out strategies [24], which are discussed in more detail in the Discussion section. Proteins derived from halophiles that employ the salt-in strategy tend to have a biased amino acid composition, particularly showing a high frequency of acidic residues, such as aspartate and glutamate, and small hydrophobic residues, such as alanine [25,26]. This is often accompanied by a low frequency of basic residues, such as lysine. Accordingly, an acidic signature is a good indicator of the enzymatic properties of halophiles. In this study, the amino acid composition of HoFucI was analyzed and compared with those of RdFucI and TfFucI from non-halophiles, as well as typical proteomes from salt-in halophiles (Table 2). As a result, most of the residues known to be crucial for halostability of salt-in proteins (e.g., alanine, aspartic acid, glutamine, and threonine) were not pronouncedly abundant in HoFucI when compared to the non-halophile-derived RdFucI and TfFucI. In addition, the contents of those residues of HoFucI were quite different and low compared to the proteins of salt-in-halophiles. In contrast, its lysine content, which is generally present in reduced frequency in salt-in halophiles, was much higher than that of salt-in proteins. Taken together, these results indicate that the amino acid composition of HoFucI is distinct from that of typical halophilic proteins.
Table 2. The content of several amino acid residues related to halophilic properties in HoFucI, l-FucI from Raoultella sp. (RdFucI), l-FucI from Thermanaeromonas toyohensis (TtFucI), and proteomes from general salt-in halophiles and thermophiles.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>HoFucI (%)</th>
<th>RdFucI (%)</th>
<th>TtFucI (%)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>8.5</td>
<td>11.0</td>
<td>9.7</td>
<td>High frequency in halophiles</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.3</td>
<td>5.4</td>
<td>5.0</td>
<td>Important for halostability</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.8</td>
<td>2.7</td>
<td>1.7</td>
<td>Low frequency in thermophiles</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.3</td>
<td>4.2</td>
<td>4.3</td>
<td>Important for halostability</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3.0</td>
<td>4.1</td>
<td>2.8</td>
<td>Important for halostability, but low frequency in thermophiles</td>
</tr>
<tr>
<td>Glutamate</td>
<td>7.1</td>
<td>6.9</td>
<td>7.0</td>
<td>Important for halostability</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.5</td>
<td>5.4</td>
<td>5.7</td>
<td>Important for halostability, but low frequency in thermophiles</td>
</tr>
</tbody>
</table>

1 Amino acid compositions of proteomes from general halophiles and thermophiles were adopted from studies by Mavromatis et al. and Bhattacharya et al. [18,19]. 2 The content of each amino acid is expressed as a percentage of the total number of amino acids.

3.6. Phylogenetic Tree of l-FucI

To date, no phylogenetic tree has been constructed for l-FucI. The phylogenetic tree built in our study showed that many separate clusters of l-FucI sequences were widely distributed among bacteria (Figure 5). Among the characterized l-FucI s, l-FucI from E. coli (EcFucI), l-FucI from Klebsiella pneumoniae (KpFucI), and RdFucI belonging to the Enterobacteriaceae family clustered together. The known thermophilic l-FucI s, such as DtFucI, CsFucI, and TtFucI, were also clustered together as disparate groups from EcFucI, KpFucI, and RdFucI, displaying high phylogenetic relatedness, which indicates their potential functional similarities. Notably, HoFucI was positioned in the same cluster as thermophilic l-FucI s, implying that its molecular properties might be similar to those of thermophilic l-FucI s.
Figure 5. Phylogenetic tree of the L-FucI family. The identities of individual L-FucI protein sequences are indicated by their UniProt accession numbers (https://www.uniprot.org (accessed on 30 March 2022)). The sequences belonging to the Enterobacteriaceae family, clustered together with characterized L-FucI from Escherichia coli (EcFucI), L-FucI from Klebsiella pneumoniae (KpFucI), and L-FucI from Raoultella sp. (RdFucI), are highlighted in orange. The sequences from thermophiles, including L-FucI from Caldicellulosiruptor saccharolyticus (CsFucI), L-FucI from Dictyoglomus turgidum (DtFucI), L-FucI from Thermaanaerobacter toyohensis (TtFucI), and HoFucI, are highlighted in blue. Functionally characterized L-FucI enzymes were labeled with black circles.

3.7. Comparison of the Specific Activities of L-FucIs against L-Fuculose

The specific activity of HoFucI was compared to those of RdFucI and TfFucI, the previously reported L-FucIs were investigated using L-fuculose as the substrate (Table 3). The specific activity of HoFucI obtained without any NaCl (106.2 U/mg) was lower than that of TfFucI (199.8 U/mg) by 1.9 fold, but only 1.3 fold lower in the presence of 0.5 M NaCl (151.1 U/mg). HoFucI activity, both in the absence and presence of the salt, was higher than that of RdFucI (64.0 U/mg). Although its activity was lower than that of TfFucI, the feature of HoFucI that handles the two harsh conditions simultaneously is unique. The key factor leading to the differences in the activities of L-FucIs could be related to the
binding affinity and/or geometry of the coordinated metal cofactor complex with the substrate. In addition, the architecture of the substrate recognition pocket and loop confirmation could lead to the difference in L-FucI’s activity and substrate specificity [2,3].

Table 3. Comparison of the specific activities of L-Fucls against l-fuculose 

<table>
<thead>
<tr>
<th>Specific Activity (U/mg)</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HoFuc1 [this study]</td>
<td>106.2 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>151.1 ± 11.9</td>
</tr>
<tr>
<td>RdFuc1 [2]</td>
<td>64.0 ± 3.4</td>
</tr>
<tr>
<td>TfFuc1 [3]</td>
<td>199.8 ± 0.3</td>
</tr>
</tbody>
</table>

1 Various concentrations of l-fuculose (10 mM) were incubated with 7 μg HoFuc1 in 20 mM sodium phosphate (pH 7) buffer at 60°C for 5 min in the presence of Mn²⁺. 2 Experimental data represent the means ± standard deviations in at least three replicates.

4. Discussion

L-FucI-encoding genes are distributed throughout a wide range of l-fucose-metabolizing bacteria, many of which are found in the phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Figure 5). L-FucI enzymes originate not only from typical mesophilic bacteria, such as E. coli [1,23], but also from unique extremophiles [3,5]. Among extremophiles, H. orenii represents one of the rarest cases owing to its dual ability to tolerate both high temperature and salinity [18,19]. Organisms that grow optimally at temperatures higher than 50°C and 1.5 M NaCl are defined as thermophiles and halophiles, respectively. In this regard, H. orenii that shows optimal growth at 60°C and 1.7 M NaCl is classified as a thermohalophile [18,19]. As enzymes derived from such organisms often have distinct features (i.e., high thermostability and halostability) that are beneficial for biotechnological applications [3,5], it is of key interest to investigate their biochemical properties.

Halophiles have evolved to adapt to hypersaline environments by equilibrating intracellular osmotic pressure with their external conditions based on two strategies [24]. One is the “salt-in strategy” that increases the intracellular ion strength up to 3–6 M in response to the external environment; hence, high concentrations of ions like Na⁺, K⁺, and Cl⁻ are accumulated in the cytoplasm [27]. Generally, proteins experience disruption of electrostatic interactions at intracellular molar concentrations of salt, losing their functional conformation [24,28]. In contrast, proteins from organisms utilizing the salt-in strategy are able to maintain their active conformation in the hypersaline cytoplasm. This may be due to the presence of excessive acidic amino acids and negatively charged acidic surfaces, which allow proteins to be sufficiently solvated in limited conditions of water availability by forming electrostatic interactions between the residues and salts [24,26,29]. Consequently, the presence of excessive acidic amino acids (e.g., glutamate and aspartate) over basic amino acids (e.g., lysine and arginine) has been regarded as a unique feature of such halophilic proteins [25,30]. In contrast to the salt-in strategy that increases the intracellular inorganic ions, the “salt-out strategy” exports salts back while accumulating organic solutes in the cytoplasm by synthesizing or acquiring them from the environment to counterbalance the intracellular osmotic pressure [31]. These organic solutes are represented by glycine, betaine, and ectoine, which are compatible with protein functionality and cellular metabolism [32]. Proteins from halophilic organisms that rely on salt-out strategies do not display acidic signatures.

From the NaCl and temperature profiles in this study, HoFuc1 was revealed as a polyextremozyme, evidenced by its therophilic and halophilic properties in synthesizing L-fuculose, with optimal activity at 50–60°C and 0.5–1 M NaCl (Figures 3 and 4a). This correlates well with the growth behavior of the original organism, H. orenii, which has adapted to multi-extreme environments (i.e., hot and salty conditions) [18,19]. It is noteworthy that
despite its maximized activity in the presence of a molar salt concentration, HoFucI retained considerable activity even with no or low salt concentrations. Keeping in mind that the halophilic proteins from organisms employing the salt-in strategy tend to be deactivated by low salt concentrations caused by the repulsive electrostatic forces among the excessive acidic amino acids [26], such a phenomenon with HoFucI provides evidence that H. orenii does not adopt the salt-in strategy. This is also consistent with the behavior of AmyA (an amylase), one of the few characterized enzymes from the same bacterium, which still exerts 40% activity without salt, while optimal activity is observed at 2 M NaCl [33]. Furthermore, HoFucI is active in a broad range of salinities from 0 to 5 M, which is also consistent with the results of AmyA [33]. Such behaviors of these enzymes could be associated with the extreme seasonal fluctuations of salt in the habitat of H. orenii (i.e., Tunisian salt lake of the Sahara desert) [18,19]. Since organisms dependent on the salt-out strategy are generally known to rapidly adjust to fluctuating salinity, the broad activity of HoFucI in saline conditions suggests that H. orenii may adopt a salt-out strategy [19].

Our bioinformatics analyses, carried out to better understand the halophilic properties of HoFucI, showed that HoFucI does not strictly follow the general trend of halophilic proteins that have been adapted to high ionic strength (i.e., salt-in strategy). First, there were no pronounced acidic residues, which is an exclusive feature of general halophilic proteins, in HoFucI when the amino acid composition was analyzed (Table 2). Next, because the negative surface electrostatic potentials are characteristic of proteins from organisms that rely on salt-in adaptation [23,34], surface electrostatic potentials were investigated by comparing the model structure of HoFucI with those of its non-halophilic counterparts with known structures, EcFucI and RdFucI (Figure S2 in Supplementary Material). No distinctive differences in surface electrostatic potentials of the three L-FucI (model) structures were observed, which further proves that the behavior of HoFucI is distinct from those of general halophilic enzymes. AmyA from H. orenii also did not show an acidic signature in either the amino acid composition or on its structural surface [33]. Nevertheless, it is too early to conclude that H. orenii relies on a salt-out strategy instead of a salt-in strategy because of the lack of related evidence, such as the identification of organic solute-synthetic enzymes [18,19]. Therefore, further studies are required to elucidate the halophilic mechanisms of this bacterium and its enzymes, such as HoFucI. It is noteworthy that proteins from several halophilic organisms belonging to the same order as H. orenii (Halanaerobiales) are also very halophilic in enzymatic functioning, but do not follow the general rule regarding the primary and tertiary structures of haloenzymes, similar to HoFucI in this study [35].

Interestingly, a thermophilic signature was clearly observed in the amino acid composition of HoFucI. Briefly, there was a reduced frequency of thermolabile amino acids, such as histidine, glutamine, and threonine, whose behaviors were quite similar to those of thermophilic TtFucI and proteins from general thermophiles. Further analysis based on the phylogenetic tree also showed that HoFucI is highly evolutionarily related to thermophilic enzymes (Figure 5).

Divalent metal ions are essential cofactors for L-FucIs [2,3,5,6]. These metal ions, in particular Mn²⁺, coordinated by the three conserved metal-binding residues (Glu337, Asp361, and His528, labeled with EcFucI) in the catalytic center of enzymes, can be platforms for substrate interaction [2,23]. More specifically, the metal cofactors can form a coordination complex with the ligands of the substrate at the O1 and O2 positions, by which L-fucose-L-fuculose interconversion occurs via the enediol intermediate [23]. Sequence alignment showed that HoFucI also contains a conserved metal-binding site (Figure S1 in Supplementary Material), proving its dependence on metal ions (Table 1). In the cases of Mn²⁺ and Co²⁺, 1 mM was sufficient to show the maximum activities while a higher concentration of Mg²⁺ (10 mM) was required to obtain a comparable value (Table 1). This could be because although divalent metal ions are expected to be coordinated by metal-binding sites in a similar manner, they might interact with the substrate with different affinities and/or geometries, resulting in the difference in activities. Despite their essential
role in the catalytic machinery of L-FucI, it is necessary to supply additional metal ions to obtain maximal enzyme activity [2,3]. This is well explained by our structural analysis with RdFuCl, in which the occupancy of metal ions in the apoenzyme is low, but can be increased by the addition of metal ions [2]. EDTA treatment did not cause any significant difference in the enzyme activity ($p > 0.05$), which further verifies this phenomenon. This was also verified by the fact that the isolated L-FucIs (i.e., apo-form) did not possess any detectable metal ions [5].

5. Conclusions

To the best of our knowledge, the HoFuCl studied here represents the first halophilic and thermophilic L-FucCl to be identified and characterized. The dual tolerance of HoFuCl for heat and salinity, together with its ability to function in a broad range of salt concentrations, is unique and offers attractive biotechnological applications in various industries that often employ harsh conditions, such as food, chemical, and pharmaceutical industries. In addition, since only a few enzymes from *H. orenii* have been reported to date, our study will aid in the further identification and development of useful polyextremozymes from this organism, which is an interesting microbial source for both academic and biotechnological purposes.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/app12084029/s1, Figure S1: Multiple sequence alignment of L-Fucose isomerase (L-FucI) from *Halothermothrix orenii* (HoFuCl) and previously characterized L-FucIs, including L-FucI from *Escherichia coli* (EcFuCl), L-FucI from *Klebsiella pneumoniae* (KpFuCl), L-FucI from *Raoultella* sp. (RdFuCl), SpFuCl, L-FucI from *Dictyoglomus turgidum* (DtFuCl), L-FucI from *Caldicellulosiruptor saccharolyticus* (CsFuCl), L-FucI from *Aerobicillus pallidus* (ApFuCl), and L-FucI from *Thermanaeromonas toyohensis* (TyFuCl); Figure S2: Electrostatic surface potentials of L-FucI from *Halothermothrix orenii* (HoFuCl) (homology model), EcFuCl (PDB code: 1FU1), and RdFuCl (PDB code: 6K1F).

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References
