Characterization of L-fucose isomerase from *Paenibacillus rhizosphaerae* to produce L-fuculose from hydrolyzed fucoidan and commercial fucose

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Research

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Abstract

Background

L-fuculose is an expensive and rare sugar used against different kinds of diseases such as HIV, anti-cancer, anti-viral, Hepatitis-B, human lysosomal disease (fucosidosis), and cardio-protective drugs. The enzymatic way of converting L-fucose into L-fuculose would be an effective method with great industrial applications. The purpose of this research is to introduce a high production of L-fuculose from cheap and natural sources (fucoidan) and commercially source (Sigma-Aldrich) by a recombinant enzyme L-fucose isomerase from *Paenibacillus rhizosphaerae* (Pa-LFI).

Results

Fucose containing polysaccharide (FPs) called fucoidan was extracted, hydrolyzed and characterized by *U. pinnatifida* for enzymatic production of L-fuculose. The FPs provide 35.9% of fucose along with few other monosaccharides. Pa-LFI was characterized and purified with a single band at 65 kDa. It showed an activity of 104.5 U mg -1 and exhibited as a hexamer with native molecular mass 396 kDa. The maximum activity for recombinant Pa-LFI was detected at pH 6.5 and 50 °C in 1 mM of Mn 2+ . The melting temperature observed 75 °C and half-life at 50 °C was 12.6 h. The isomerizing activity of Pa-LFI with aldose substrate (L-fucose) was higher exposing K m , k cat and k cat / K m 86.2 mM, 32831 min -1 and 335 min -1 mM -1 respectively. The conversion ratio of L-fuculose from 100 g L -1 of FPs and commercial fucose after the equilibrium state was about 6% (5.6 g L -1) and 30% (30.2 g L -1) respectively.

Conclusion

Pa-LFI catalyzed the reaction to convert L-fucose into L-fuculose. The enzyme will be helpful in the production of L-fuculose with an efficient and simple method without producing any by-product.

Background

Fucoidan, fucose containing polysaccharides (FPs) belongs to a heterogeneous polysaccharide group obtained mainly from the extracellular medium with cellulose, hemicellulose, and alginate in brown seaweeds and primarily segregated by Kylin in 1993 [1, 2]. In recent years FPs has acquired much curiosity in functional food and pharmaceutical industries because of its abundant bioactive properties such as anti-cancer/anti-tumor [3, 4], anti-viral, anti-oxidant, anti-coagulant, anti-diabetic, anti-obesity, anti-bacterial [5] and therapeutic potential against injuries [6, 7]. Different studies exhibited that the structure of FPs varies from each other due to the position of glycosidic bonds, branches of the chain, relative molecular weight and sulfate group substitutions, which are caused by extraction methods, type of species, region of growth, time of harvesting and other environmental and geographical factors [8, 9]. FPs is a water-soluble polysaccharide that contains several monosaccharide components, including fucose, glucose, galactose, mannose, glucuronic acid, xylose, lactose, rhamnose and arabinose [10].

The brown seaweed named Undaria pinnatifida mainly grown in seas of China, Japan, and Korea also native to cold temperature regions such as French Mediterranean, New Zealand, European Atlantic, and Australia [11]. It is an extremely invasive species of seaweed having a high capacity to tolerate temperature, light, salinity, and very fertile with rapid growth rate and substantial propagative output. It releases spores throughout the year and provides seaweed food for Chinese and Japanese people [12]. An excessive amount of FPs could be extracted from U. pinnatifida and used for its biological activities such as for the extraction of monosaccharide (mono sugars). The FPs is normally extracted from the sporophyll part of U. pinnatifida [13].

Fucose is one of the industrially relevant and useful rare sugar having high economic potential isolated from U. pinnatifida [14, 15]. Especially, it has occupied high demand in pharmaceutical and cosmetics industries for the liability and invention of anti-aging, anti-cancer and anti-allergic r cosmetics and drugs [14, 15]. Furthermore, it was reported to be relatively useful to produce fuculose by the isomerization process, which is the most expensive and rare sugar [16, 17]. Various enzymes (isomerases) have been used for the isomerization of sugars and their phosphate, having an inclusive range of substrate specificity for the conversion of monosaccharides [18]. Each sugar isomerase has its novelty and specificity of reaction that convert to mono sugars more efficiently. L-Fucose isomerase (E.C.5.3.1.25) is an isomerizing enzyme that catalyzes the L-fucose to L-fuculose by the isomerization process. L-Fucose isomerase (L-FI) not only specific to isomerize L-fucose to L-fuculose but also converts D-arabinose to D-ribulose. L-FI and D-arabinose isomerase (D-FI), both are similar kinds of enzymes with identical reaction mechanisms [19].

According to the previous enzyme database (BRENDA), L-FI was generally produced from Caldanaerobius polysaccharolyticus (C. polysaccharolyticus) [17], Dictyoglomus turgidum (D. turgidum) [19], Caldicellulosiruptor saccharolyticus (C. saccharolyticus) [18] and Escherichia coli (E. coli) [20, 21]. Paenibacillus rhizosphaerae (P. rhizosphaerae) is a gram positive, sporulating, rod shaped, motile, and strictly aerobic bacteria, isolated from the soil of rhizosphere legume (Cicer arietinum) plants at flowering stage in Chaco Arido, Argentina. The colonies of P. rhizosphaerae (Pa) were opaque, cream colored, round, and convex [22]. P. rhizosphaerae was used to extract enzyme (L-FI) in this research. This is the thermostable enzyme having plenty of uses and benefits such as resistant to denaturation during chemical and biochemical reaction, stable against high temperature, increase the substrate solubility, enhanced reaction velocities, reduce the risk of by-products and contamination [16]. There were only a few strains have been discovered for the characterization of L-FI, but L-FI from P. rhizosphaerae (Pa-LFI) were reported as the thermostable enzyme having the ability to isomerize L-fucose from natural source (fucoidan) and from commercial source (Sigma-Aldrich) to L-fuculose through opening the ring structure, which is naturally very rare sugar (Fig. 1). L-Fuculose used in food, pharmaceutical, and cosmetic industries. It is also applied against different kinds of diseases such as HIV, anti-cancer, anti-viral, Hepatitis-B, human lysosomal disease (fucosidosis), and cardio-protective drugs [16, 19]. Although, our focus was to produce rare sugar (L-fuculose) from natural and commercial sources by simple method which is very economical and easily available.

Recently, different new techniques have been introduced to produce rare sugars in which chemical and biochemical are included but enzymatic conversion taking economical and having a better potential for industrial application than formers. The purpose of this research is to introduce a high production of L-fuculose from cheap and natural sources (fucoidan) and commercially source (Sigma-Aldrich) by a recombinant enzyme (Pa-LFI). For this purpose, U. pinnatifida, the most famous and abundant seaweed in China and South Korea was preferred as a preliminary material to produce fucoidan (fucose containing seaweed) and the enzymatic isomerization was carried out on this fucoidan as well as on commercial fucose obtained from Sigma-Aldrich. From the chosen seaweed, FPs was isolated, and optimized yield and hydrolysis conditions were examined. Then the basic structure of hydrolyzed (HF) and non-hydrolyzed (NHF) FPs were determined by FTIR spectrophotometer (FTIR), the compositional analysis was performed by High Performance Anion Exchange Chromatography equipped with Pulsed Amperometric Detection (HPAEC-PAD), and the molecular mass was analyzed by Gel permeation chromatography equipped with MALLS (GPC-MALLS).

Moreover, Pa-LFI was identified from gene encoding of a hypothetical protein in P. rhizosphaerae, which was induced, cloned, overexpressed in E. coli (BL21) and purified. Then enzymatic biochemical properties such as molecular mass (subunit, native), optimum pH, optimum temperature, thermostability, and metal ion were studied. Kinetic parameters and activity showed that recombinant Pa-LFI exhibited the great potential to produce L-fuculose for the downstream industry.

Results And Discussion

Extraction and M_w determination of fucoidan

The fucoidan (FPs) was extracted by the acidic method in 0.1 and 0.2 M HCl to achieve the maximum yield of fucoidan. Figure 3A showed that different ranges of temperatures (50, 70, and 90 °C) and time (1, 3, and 5 h), applied with 0.1 and 0.2 M HCl. The extracted fucoidan was reacted with 2 M TFA to further hydrolyze the fucoidan. According to the plot as mentioned earlier, an optimum yield (6.5% DW) was attained by using 0.1 M HCl at 90 °C after 3 h, while 4.0% of yield was obtained on 0.2 M HCl at the same processing conditions. The results also elaborated that the yield was decreased as the time interval increase at the same temperature, so the duration of hydrolysis also have a significant impact on yield (Fig. 3A). Previous reports mentioned that about 7.1% DW of fucoidan was extracted by using acid hydrolysis at 90 °C and after 3 h [23] while, about 3.4, 1.8, and 2.8% of fucoidan were reported from U. pinnatifida by using different extraction methods [24–26].

The M_w of HF was determined by GPC, and the results revealed that the polysaccharide had an M_w of 1,109 kDa (Table 1). The M_w obtained was higher than the fucoidan extracted previously by chemical (1,035.5 kDa) [27] and hot water (262 kDa) [28] treatments.

Compositional and structural analysis of fucoidan

The composition of monosaccharides present in fucoidan was analyzed by HPAEC-PAD, and the results demonstrated that there were various proportion of different monosaccharides (Table 1) such as galactose (21.8%), mannose (6.7%), fucose (35.9%), arabinose (1.5%), glucuronic acid (26.2%), and rhamnose (0.05%). The fucoidan extracted from the same species have different compositions; the difference might be due to growing conditions, environmental changes, region, analytical process, and extraction methods [27–29]. The literature exposed that fucose was the main monosaccharide present in fucoidan, however other monosaccharides were also reported, but the composition of fucose vary among the same species (Table 1). This variation was due to the extraction methods that have a significant influence on yield and compositional properties [24–26, 28, 30].

The structural changes of hydrolyzed and non-hydrolysed polysaccharides can be observed by FTIR spectrum (Fig. 3B), it is an imperative technique to describe and classify the functional or chemical groups existing in different samples [31]. A characteristic band of non-hydrolysed fucoidan (NHF) was shown at 3,419.3 cm^{-1} which corresponds to O-H deformation [11], while for hydrolyzed fucoidan (HF) the O-H band shifted at higher wavenumber (3453.8 cm^{-1}). The small peaks at 2,927.3 and 2,925.3 cm^{-1} were assigned as stretching vibration of C-H bonds of NHF and HF, respectively [11, 32]. The fractions appeared in HF at 1,667.4–1,704.2 cm^{-1} may indicate the occurrence of acylamino groups [33]. A small vibrational peak at 2,927.3 cm^{-1} was assigned to O-H frequency for NHF although this peak was shifted to low number at 2,925.3 cm^{-1} for HF [32, 34]. The occurrence of CH_2 and CH_3 was indicated by finding the peaks at 1,442.8 and 1,236.3 cm^{-1} (Fig. 3B) [32]. The peaks (HF) observed at 3,418 and 2,875 confirmed the existence of OH and CH group of fucose at the C-6 position [34].

Table 1. Comparison of molecular weight and composition of monosaccharides in fucoidan extracted from *U. pinnatifida* by different methods

Extraction method	Yield (%)	Monosaccharide composition								M_w (kDa)	References
		Fucose (%)	Galactose (%)	Glucose (%)	Mannose (%)	Xylose (%)	Arabinose (%)	Glucuronic acid (%)	Rhamnose (%)		
Acid hydrolysis	6.5	35.9	21.8	4.1	6.7	3.7	1.5	26.2	0.05	1109.7	Present study
HCl extraction	3.4	53.0	38.0	2.0	5.0	2.0	NR	NR	ND	NR	[24]
Hot water extraction	NR	NR	NR	23.81	4.26	64.0	5.90	NR	NR	1035.52	[27]
Anion exchange chromatography	0.5 – 1.8	59.0 – 51.0	30.0 – 48.0	1.0	8.0 – 1.0	2.0	NR	NR	ND	NR	[25]
Chemical extraction	16.6 – 43.4	52.0 – 72.5	27.5 – 39.0	NR	3.6 – 8.9	NR	NR	NR	NR	262	[28]
Ethanol extraction	NR	78.8	21.	NR	NR	NR	NR	NR	NR	23600 - 5200	[30]
$CaCl_2$ extraction	2.8	16.4	NR	NR	NR	NR	NR	NR	NR	171	[26]

NR: not reported; ND: not displayed

Amino acid sequence analysis of recombinant Pa-LFI

The whole-genome from P. rhizosphaerae was examined and assigned the GenBank accession No. WP_076167696.1 with 596 number of amino acids having isoelectric point 5.06 and characterized as L-fucose isomerase (L-FI). The hypothetical amino acids sequence of recombinant Pa-LFI exhibited 76% identity with A. pallidus (accession No. WP_063389244.1), 73% with both T. toyohensis (accession No. WP_084665866.1) and C. polysaccharolyticus (accession No. WP_035172025.1), while 69% and 64% with D. turgidum DSM6724 (accession No. YP_002352328.1) and E. coli W (accession No. ADT76410.1), respectively.

To investigate the amino acids sequence of recombinant Pa-LFI, the sequence alignment tool ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was applied to relate the recombinant Pa-LFI from earlier reported bacteria. The secondary structure of A. pallidus (WP_063389244.1) with PDB ID: 3A9T was determined by the ESPript server (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) [35] and verified by (http://services.mbi.ucla.edu/SAVES/) server.

The comparison between the sequence alignment of different previously characterized L-fucose isomerases (L-FIs) was mentioned in Figure S1. The multiple sequence alignment of P. rhizosphaerae (WP_076167696.1) was compared with A. pallidus (WP_063389244.1), C. polysaccharolyticus (WP_035172025.1), D. turgidum DSM6724 (YP_002352328.1), T. toyohensis (WP_084665866.1) and E. coli W (ADT76410.1). All of these sequences displayed approximately 65% similarity among themselves. The secondary structure premeditated from the previously discovered 3D structure of A. pallidus (WP_063389244.1) with PDB ID: 3A9T using ESPript server [35]. The figure S1 represents strictly conserved residues with a red background, the highly conserved residues epitomize with red type in blue boxes, while the symbol stars above the residues show secondary structure of recombinant Pa-LFI which attained from L-FI from A. pallidus (PDB ID: 3A9T).

Expression and purification of the recombinant Pa-LFI

The truncated Pa-LFI sequence was commercially synthesized with restriction endonuclease sites NdeI and XhoI and an in-frame 6 × histidine tag sequence, which cloned into the pET-22b(+) an expression vector. The recombinant plasmid was emulated and expressed into E. coli BL21 (DE3) cells. The non-characterized recombinant Pa-LFI protein was purified by single-step nickel affinity chromatography through electrophoretic homogeneity. The purified protein of recombinant Pa-LFI exhibited 33-folds of purification with 104.5 U mg^{-1} of specific activity and 86% yield for L-fucose. The recombinant Pa-LFI presumed expression yield in E. coli cell culture 4,695 U L^{-1} for L-Fucose, which is higher than 2,360 U L^{-1} from T. toyohensis [16]. The specific activity of recombinant Pa-LFI was sophisticated than that of 85.5, 93 and 76 U mg^{-1} from T. toyohensis, D. turgidum and C. saccharolyticus, respectively [16, 18, 19].

The recombinant purified Pa-LFI protein formed a single thick band of protein around 65 kDa on SDS-PAGE, called subunit molecular mass, which is equal to theoretical value 6,5439.90 Da analyzed by using a server of ExPASy-Compute pI/M_w tools (Fig. 4A). The molecular mass was consistent with the predicted values of recombinant Pa-LFI, which revealed that the recombinant Pa-LFI was successfully overexpressed. The native molecular mass was deliberated by using HPLC with a single peak at a retention time of 6.87 min in elution profile possessed a molecular mass of around 396 kDa under nondenaturing conditions by comparing with various reference standards (Fig. 4B). The theoretical molecular mass of one unit of recombinant Pa-LFI is around 65 kDa, and the native molecular mass was estimated to be 396 kDa, corresponding to six subunits. The results elaborate that recombinant Pa-LFI is a homohexamer, parallel to the previously characterized L-fucose isomerase from D. turgidum [19].

Effect of pH, temperature, and metal ions on the activity of recombinant Pa-LFI

The influences of pH on the activity of recombinant Pa-LFI was examined at 50 °C, and different pH ranges from 5.0 to 10.0, using three buffers system: Na-phosphate (pH 5.0–7.0), Tris-HCl (pH 7.0–9.0) and glycine-NaOH (pH 9.0–10.). The recombinant Pa-LFI exhibited optimum activity on pH 6.5 in Na-phosphate buffer (Fig. 5A). The activity increased in Na-phosphate buffer pH 6.5, and it decreased rapidly as the pH increased from 6.5 to 10.0 in Tris-HCl and glycine-NaOH buffers. While in Tris-HCl buffer, the pH behavior for the enzyme was first raised to 7.5 and then suddenly decreased to 9.0. Glycine-NaOH showed the lowest activity in decreasing pattern, presenting that recombinant Pa-LFI was more sensitive to higher pH than lower pH. Lower pH reactions are favorable to industrial application because they inhibit the browning reactions and diminish unwanted by-products [36]. The enzyme activity maintained higher level under weak acidic conditions at pH 6.5, which is consistent with other L-FIs such as C. saccharolyticus (7.0) [18], D. turgidum (7.0) [19] and lower than T. toyohensis (9.0) [16].

The enzyme stability was assessed by incubating purified recombinant Pa-LFI at 4 °C for 24 h (Fig. 5B), and 50 °C for 1 h (Fig. 5C) with different pH ranges from 5.0–10.0 in above three buffers. The recombinant Pa-LFI fully sustained its activity from pH 5.0–10.0 which revealed no significant effect on its activity at 4 °C for 24 h, while incubating at 50 °C for 1 h the activity increased from the optimum which shows the thermal effect of pH on enzyme stability.

The influences of temperature on the activity of recombinant Pa-LFI was evaluated in Na-phosphate buffer at pH 6.5, temperature ranging from 35–70 °C (Fig. 5D). The optimum temperature for recombinant Pa-LFI was determined as 50 °C. More than 85% of relative activity was maintained between 40–55 °C. This result indicates that 50 °C is a favorable temperature for the isomerization of L-fucose which is higher than E. coli B/r (37 °C) [37], lower than C. saccharolyticus (75 °C) [18], T. toyohensis (75 °C) [16] and D. turgidum (80 °C) [19]. Generally, higher temperatures are required for industrial applications, to produce functional sweeteners because high temperature can reduce the risk of microbial contaminations, enhance the speed of reaction, convert the reaction equilibrium towards products and increase solubility [38].

The influences of metal ions on recombinant Pa-LFI activity were studied after removal of previously existing metal ions from the purified protein with the help of EDTA to achieve precise results. Afterward, the EDTA was removed with Tris-HCl buffer (pH 7.0) by subsequently dialyzing the enzyme. The recombinant Pa-LFI is metalloenzyme in which metals ions entertain as a cofactor for the isomerization process of rare sugars. The enzyme was incubated with ten different divalent metals ions (EDTA, Ni^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, and Cu^{2+}) at a final concentration of 1 mM to examine their effects on the enzyme activity at pH 6.5 and 50 °C (Fig. 5E). The optimum metal ion for recombinant Pa-LFI was Mn^{2+} (450%) while Co^{2+} (261%) also enhanced the activity after Mn^{2+}. Numerous divalent metal ions, including Ni^{2+}, Zn^{2+}, Ba^{2+}, Ca^{2+}, and Mg^{2+} enhanced more than 100% activity of recombinant Pa-LFI. In contrast, EDTA, Fe^{2+}, and Cu^{2+} exert a negative effect on the catalytic activity of the recombinant enzyme.

Thermostability and melting temperature (T_m) of recombinant Pa-LFI

The thermostability of recombinant Pa-LFI was measured at different ranges of temperatures from 30, 40, 50, 60 and 70 °C for 0, 4, 8, 12, 16, and 20 h by determining the residual activity (Fig. 5F). The recombinant enzyme revealed good thermostability at a temperature under 40 °C and retained more than 60% of residual activity than that of 50 °C when incubated for 20 h. Nevertheless, the residual activity of recombinant Pa-LFI was significantly decreased when the incubated temperature increases from 40 to 70 °C. However, the residual activity decreased to 4% and 7% at 60 and 70 °C after 12 h and 8 h respectively (Fig. 5F). The recombinant Pa-LFI was more than 50% active after 12 h on its optimum temperature (50 °C), which is the useful property of this enzyme. According to the first-order kinetics, the half-lives ($t_{1/2}$) at different temperatures were measured to be 70 h (30 °C), 25.4 h (40 °C), 12.6 h (50 °C), 5.3 h (60 °C) and 2.1 h (70 °C), which is quite higher than previously reported C. saccharolyticus having 62 h, 13 h, 6 h, 2 h and 1 h at 60, 65, 70, 75 and 85 °C [18] and for D. turgidum 20 h, 12 h, 7 h, 5 h and 2 h at 65, 70, 75, 80 and 85 °C [19].

DSC delivers another significant property for the structural stability of recombinant Pa-LFI, which provides a differential heat flow as a transformation of temperature. The melting temperature (T_m) is a distinguished property to analyze the structural stability of the protein. The T_m of recombinant Pa-LFI examined by nano-DSC was measured to be 75 °C (Figure S2), which is higher than the optimum temperature (50 °C) and valuable for industrial application. The T_m of L-FI from C. polysaccharolyticus was 80.3 °C having 55 °C of optimum temperature, which was sustained with the present findings [17].

Specificity of substrates and kinetic parameters of recombinant Pa-LFI

The specific activities of recombinant Pa-LFI were inquired for L-fucose, D-arabinose, D-altrose, L-galactose, and L-xylose (Table 2). The recombinant Pa-LFI isomerization reaction displayed only a single product against each substrate and L-fucose was found to the optimum substrate with 104.5 ± 1.15 U mg^{-1} of the specific activity. The specific activities for D-arabinose, D-altrose, and L-galactose were exhibited to be 91.9 ± 1.77, 26.8 ± 0.76 and 2.3 ± 0.29 U mg^{-1}, respectively followed by L-fucose (Table 2). There were lowest activities detected against L-galactose, and no activity was for L-xylose because hydroxyl groups existing in aldose substrates on the left-handed configuration at C2 and right-handed C3 and C4 positions such as L-fucose, D-arabinose, D-altrose, and L-galactose. All these results specify that L-fucose was the optimum substrate for recombinant Pa-LFI. These results further verified that recombinant Pa-LFI belongs to L-FI family similar to D. turgidum (93 U mg^{-1}) [19], higher than T. toyohensis (85.5 U mg^{-1}) [16], C. saccharolyticus (76 U mg^{-1}) [18], E. coli B/r (64 U mg^{-1}) and E. coli K-2 (63 U mg^{-1}) [37].

Table 2
Substrate specificity and Kinetic parameters of recombinant Pa-LFI

Substrate	Product	Specific Activity (U mg^{-1})	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m (mM^{-1} min^{-1})
L-Fucose	L-Fuculose	104.5 ± 1.15	86.2 ± 2.3	32831 ± 20	335 ± 3.5
D-Arabinose	D-Ribulose	91.9 ± 1.77	67.5 ± 2.1	19782 ± 43	293 ± 1.7
D-Altrose	D-Allulose	26.8 ± 0.76	57.3 ± 1.8	2275 ± 9	40 ± 2.8
L-Galactose	L-Tagatose	2.3 ± 0.29	ND	ND	ND
L-Xylose	L-Xylulose	0.0 ± 0.00	ND	ND	ND
ND: not displayed					

The kinetic parameters of recombinant Pa-LFI for L-fucose, D-arabinose, and D-altrose were investigated by the nonlinear-regression method under optimum conditions of reaction (pH 6.5 and 50 °C) (Table 2). L-fucose was the optimum substrate for recombinant Pa-LFI, and it showed K_m 86.2 ± 2.3 mM, which is higher than D-arabinose, D-altrose because of the isomerization rate higher for L-fucose. The K_{cat}/K_m values were 335 ± 3.5, 293 ± 1.7 and 40 ± 2.8 (mM^{-1} min^{-1}) for L-fucose, D-arabinose and D-altrose, respectively (Table 2). The results elaborate that L-fucose from recombinant Pa-LFI showed lower affinity but higher activity than that of D. turgidum [19]. A comparison of enzymatic characteristics and kinetic parameters of recombinant Pa-LFI with other microorganisms are given in Table 3.

Table 3
Comparison of enzymatic properties and kinetic parameters of recombinant Pa-LFI

Microorganisms	Molecular weight (kDa)	Temperature (°C)	Optimum pH	Specific Activity (U mg^{-1})	Metal ions	K_m (mM)	V_{max} (U mL^{-1})	Reference
P. rhizosphaerae	66	50	6.5	104.5	Mn^{2+}	86.2	9.62	Present work
T. toyohensis	66	75	9.0	85.5	Mn^{2+}	81.2	3.7	[16]
C. polysaccharolyticus	65	55	6.5	108.2	Mn^{2+}	94.2	9.6	[17]
D. turgidum	68	80	7.0	93	Mn^{2+}	90	2.3	[19]
E. coli B/r	65	37	8.0	64	Co^{2+}	42	0.25	[37]
E. coli K-2	65	37	8.0	63	Co^{2+}	45	0.26	[37]
C. saccharolyticus	68	75	7.0	76	Mn^{2+}	141	0.25	[18]

Production of L-fuculose commercial fucose and fucoidan by recombinant Pa-LFI

L-fuculose was produced from commercial L-fucose (Sigma, St. Louis, MO, USA) and hydrolyzed FPs by recombinant Pa-LFI in a reaction mixture of 0.5 mL at pH 6.5 and 50 °C. Upon the addition of 30, 50 and 100 g L^{-1} of commercial L-fucose (Sigma-Aldrich) (Fig. 6A) and HF (Fig. 6B), the enzymatic reaction attained its equilibrium state at 4, 5 and 6 h, demonstrating conversion rates of 30.32, 30.27, and 30.29% and 5.80, 5.80, and 5.66% respectively. During the high level of L-fuculose production by recombinant Pa-LFI, 9.0, 15.13 and 30.27 g L^{-1} and 1.74, 2.90 and 5.7 g L^{-1} L-fuculose was obtained from 30, 50 and 100 g L^{-1} of L-fucose and HF respectively. As compare to the previously described bacteria, C. polysaccharolyticus (28%) pH 6.5 at 55 °C [17], T. toyohensis (24.9%) pH 9.0 at 75 °C [16], C. saccharolyticus (24%) pH 7.0 at 70 °C [19] and K. pneumonia (10%) pH 9.0 at 40 °C [39] produced L-fuculose. Although, we used a different concentration of L-fucose and HF to produce L-fuculose still the conversion rates were similar and closed to 30% and 5.66% respectively.

After a comparison of all other L-fuculose producing bacteria, it exhibited that recombinant Pa-LFI produced a higher conversion ratio (30%) of L-fuculose from L-fucose with no byproducts and 5.8% from HF, which was a natural and cheap source from seaweed of U. pinnatifida. It was well distinguished and convenient for the industrial processes of recombinant Pa-LFI for the production of highly valuable L-fuculose. It could simplify the process of purification method and reduced the cost of downstream industry.

Conclusions

We have purified and characterized a novel recombinant L-fucose isomerase from P. rhizosphaerae which displays promising activity in the production of L-fuculose from fucoidan, fucose containing polysaccharide (FPs) extracted from abundantly available seaweed (U. pinnatifida) and commercial fucose. Pa-LFI efficiently produced L-fuculose from Hydrolyzed fucoidan (HF) as well as from commercial fucose. HF is a cheap source of fucose, and when recombinant Pa-LFI was applied to HF, it efficiently produced L-fuculose. At optimal reaction conditions, the conversion ratio of L-fuculose from 100 g L^{-1} of

FPs and commercial fucose after the equilibrium state was about 6% (5.6 g L^{-1}) and 30% (30.2 g L^{-1}) respectively. Due to these characteristics, the recombinant Pa-LFI has an auspicious future for industrial production of L-fuculose.

Materials And Methods

Chemicals, bacterial strains and plasmid

U. pinnatifida was procured from Shanghai, China, trifluoracetic acid (TFA), NaOH, Isopropyl-β-D-thiogalactoside (IPTG), ampicillin, monosaccharide standards (glucose, mannose, fucose, rhamnose, galactose, glucuronic acid, and xylose), and all other chemicals and reagents were bought from Sigma-Aldrich (St. Louis, MO, USA). The bacterial strain P. rhizosphaerae in the form of genomic DNA used as a source of Pa-LFI, plasmid pET-22b(+) and E. coli BL21 (DE3) used as a host cell were obtained Sangon Biotech. (Shanghai, China, and Novagen, USA). The His-Trap Nickle affinity resin column for purification of protein was purchased from GE Healthcare (Uppsala, Sweden). The cells of E. coli were cultured in Luria–Brentani (LB) medium.

Extraction of fucoidan

Sampling and processing

U. pinnatifida (1,000 g) was washed with ultra-pure water to make the sample free from dirt, debris, and other impurities than the sample was stored at 4 °C until further processing. The sample was dried out in an oven at 65 °C for 72 h till a persistent weight of the sample was attained. The dried sample was subjected to grinding so that a fine powder was obtained and this powder was kept in air-tight jar prior to storage for further analysis. The sample (25 g) was pre-treated with 250 mL of solvent methanol (MeOH): chloroform ($CHCl_3$): distilled water (H_2O) (4:2:1) at room temperature for 3 h, this step was repeated 4–5 times and it was necessary to eradicate impurities (lipids, colorants, and low molecular weight compounds). The cells were collected from solution by centrifugation (8,000 × g for 25 min) and desiccated at room temperature overnight. These desiccated samples were processed for acid hydrolysis following the method described previously [40]. A schematic illustration of the extraction and hydrolysis of fucoidan is given in Fig. 2.

Hydrolysis of fucoidan

The fucoidan was extracted by using 0.1 M and 0.2 M HCl, 1 g of sample was reacted with HCl (0.1 and 0.2 M) at various temperatures (50, 70, and 90 °C) for 1, 3 and 5 h. The quantitative analysis of extracted fucoidan was analyzed by Eq. 1. After set time intervals, the reaction mixtures were precipitated with two volumes of EtOH and kept at a temperature of 4 °C for 16 h. The precipitates were collected by using centrifugation (8,000 × g for 20 min), washed three times with double distilled water and dialyzed against ultra-pure water for 24 h. Then the dialyzed samples were lyophilized overnight to get powdered fucoidan for acidic hydrolysis. The acid hydrolysis was achieved by adding 2 M TFA in 50 mg of fucoidan at 100 °C for 2.5 h, after hydrolysis, the sample was freeze dried and stored in a desiccator for further analysis [23].

$$\text{Yield of fucoidan (\% dry weight)} = \frac{\text{Mass of fucoidan (g)}}{\text{Mass of dried ground seaweed (g)}} \times 100 \qquad (1)$$

HPAEC, GPC, and FTIR analysis of fucoidan

High Performance Anion Exchange Chromatography attached with Pulsed Amperometric Detection (HPAEC-PAD) analysis was used to detect the composition of different sugars in the hydrolyzed polysaccharide. The lyophilized samples were dissolved in ultrapure water (200 µL) and analyzed on HPAEC-PAD, various monosaccharides (glucose, manse, fucose, rhamnose, galactose, glucuronic acid, and xylose) were used as standards. The separation was carried out by the CarboPacTM PA20 column (150 mm × 3 mm) and data was obtained and analyzed by Chromeleon software version 6.80.

The molecular weight (M_w) of fucoidan was examined by following the technique mentioned by You et al. [28]. Gel permeation chromatography equipped with MALLS (Wyatt Technology, Santa Barbara, CA, USA) was used to find the M_w of HF, 100 µL sample (2 mg mL^{-1}) was applied onto the system. The mobile phase was 0.15 M $NaNO_3$ with the flow rate of 0.5 mL min^{-1}, and the M_w was investigated using Astra software. The structural analysis of polysaccharide analyzed using the FTIR spectrophotometer (IR Affinity-IS Shimadzu Co. Japan). The dried samples of NHF and HF were assorted with KBr, and the spectrum was noted between 4,000 cm^{-1} and 500 cm^{-1}.

Characterization of recombinant Pa-LFI

Gene cloning, expression and purification

A gene encoding recombinant Pa-LFI attained from the GenBank public database by applying the NCBI-BLAST tool (accession No. WP_076167696.1). The gene sequence used for expression of the protein was synthesized by the company Sangon Biotech. (Shanghai, China, and Novagen, USA). The 6 × histidine-tag at 3′-terminus accompanying with NdeI and 5′- and 3′-terminus are linked with XhoI restriction sites to construct an in-frame fusion. The constructed gene was injected into the pET-22b(+) an expression vector and transformed into cells of E. coli BL21 (DE3) for overexpression [16]. The cells were cultivated

into LB medium (10 g L^{-1} tryptone, 10 g L^{-1} NaCl and 5 g L^{-1} yeast extract) with the final concentration of ampicillin 100 µg mL^{-1} and incubated at 37 °C. When the absorbance on spectrophotometer was reached at 0.6–0.8 at 600 nm called optical density (OD_{600}), then 1 mM L^{-1} of IPTG was inserted for induction of recombinant Pa-LFI at 28 °C for additional 6 h with 200 rpm of shaking velocity.

The purification of recombinant Pa-LFI was achieved by the method described in previously reported work [16]. The sub-unit molecular mass of purified recombinant Pa-LFI was examined by SDS-PAGE analysis. The SDS-PAGE gel contained 12% resolving gels and 5% stacking gels, performed under denaturing circumstances with different kinds of reference proteins. The native enzyme molecular mass was examined by using high performance liquid chromatography (HPLC, TSK G3000SW) with light scattering and UV detectors (Dawn Heleos II, CA, USA), (Waters 2489, USA), respectively [17].

Determination of protein concentration and enzyme assay

The concentration of recombinant protein (Pa-LFI) was estimated with the method of Bradford, using bovine serum albumin (BSA) protein standard, [41]. The recombinant Pa-LFI activity was calculated by observing the amount of L-fuculose from L-fucose. The reaction mixture of 0.5 mL was prepared in 50 mM Na-phosphate buffer, pH 6.5 at 50 °C with 10 mM L-fucose as a substrate and 0.09 U mL^{-1} of purified recombinant Pa-LFI in the existence of 1 mM of Mn^{2+} for 15 min. By the addition of Pa-LFI the reaction ongoing for 15 min and then boiled for 5 min to stop it. The experiment was performed by using HPLC (Waters Corporation, MA, USA) attached with an Infrared detector (Shodex, RI-101) and column Sugar-Pak I (6.5 mm × 300 mm, with flow rate 0.4 mL min^{-1} Waters, MA, USA) at 85 °C. Double distilled water and 10% methanol was used as mobile phase and stationary phases, respectively. “One unit of enzyme activity was defined as the amount of enzyme required to increase 1 µmol of L-fuculose min^{-1} at 50 °C and pH 6.5” [17]

Effects of pH, temperature and metal ions on recombinant Pa-LFI activity

The pH influences were investigated by three buffer system with different pH values (5.0–10.0) at 50 °C in 50 mM of Na-phosphate buffer (pH 5.0–7.0), Tris-HCl (pH 7.0–9.0) and glycine-NaOH (pH 9.0–10.0). The pH stability was measured in the same ranges of buffers at 4 °C and 50 °C for 24 and 1 h, respectively [16]. To evaluate the effects of temperatures, L-fuculose production was measured at pH 6.5 over a range of 35–70 °C. The thermostability of recombinant Pa-LFI was determined at 30, 40, 50, 60 and 70 °C within regular intervals of times. The melting temperature (T_m) was examined through differential scanning calorimetry (Nano DSC III, TA Instruments, New Castle, PA) to assess the structural stability of the purified enzyme. The results were corrected using baseline and fitted with the help of NanoAnalyze software [42].

To inspect the role of different divalent metal ions on the activity of recombinant Pa-LFI, the enzyme was dialyzed against Tris-HCl (50 mM, pH 7.0) with 10 mM of EDTA at 4 °C for 24 h. To eradicate the influence of EDTA again dialyzed against Tris-HCl (50 mM, pH 7.0) 4–5 times at 4 °C for different intervals of time. The recombinant Pa-LFI activity was determined in different metals ions such as EDTA, Ni^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+} and Cu^{2+} with 1 mM of final concentration. The reaction without accumulation of metal ions was set as control (100%). All of the experiments, as mentioned above, were performed in triplicate.

Substrate specificity and kinetic parameters of recombinant Pa-LFI

The substrate specificity of recombinant Pa-LFI was determined with L-fucose, D-arabinose, D-altrose, L-galactose, and L-xylose at 50 mM of final concentration. The standard reaction conditions were applied to carry out reactions of different substrates. The reactions were executed in triplicate and calculate the specific activities of applied substrates.

The kinetic parameters of recombinant Pa-LFI were examined by detecting the activity of the enzyme at different concentrations (5-300 mM) of L-fucose, D-arabinose, D-altrose, L-galactose, and L-xylose. The reactions were implemented in 50 mM of Na-phosphate buffer, pH 6.5 at 50 °C for 15 min. Boiling water was used to stop the reaction and analyzed the production of L-fuculose, D-ribulose, D-allulose, L-tagatose, and L-xylulose sugars. The kinetic parameters; Michaelis-Menten constant (K_m, mM), turnover number (k_{cat}, min^{-1}) and catalytic efficiency (K_{cat}/K_m, mM^{-1} min^{-1}) for all the substrates were intended by fitting the data into Michaelis-Menten equation by a nonlinear-regression method using a software GraphPad Prism.

Enzymatic production of L-fuculose by recombinant Pa-LFI

The biological synthesis of L-fuculose was obtained in 50 mM of Na-phosphate buffer, pH 6.5 at 50 °C for different time intervals (1–6 h). Various concentrations 30, 50 and 100 g L^{-1} of FPs and commercial L-fucose from Sigma-Aldrich were used to analyze the enzymatic production of L-fuculose. The products were detected by HPLC with a sugar column at 85 °C temperature and 0.4 mL min^{-1} of flow rate equipped with RI detector (Waters Sugar-Pak 1, Waters Corp., Milford, MA, USA), using deionized water as mobile phase. The L-fuculose concentration was examined until the equilibrium state.

Abbreviations

FPs: Fucoidan, fucose containing polysaccharide; Pa-LFI: L-Fucose isomerase from Paenibacillus rhizosphaerae; L-FI: L-Fucose isomerase; D-FI: D-arabinose isomerase; HIV: human immunodeficiency viruses; HF: Hydrolyzed FPs; NHF: Non-hydrolyzed FPs; HPAEC-PAD: High-performance Anion-Exchange chromatography with Pulsed Amperometric Detection; GPC-MALLS: Gel permeation chromatography equipped with MALLS; FTIR: Fourier-transform Infrared Spectroscopy; TFA: trifluoracetic acid; IPTG: Isopropyl-β-D-thiogalactoside; LB: Luria–Brentani; M_W: Molecular weight; NCBI-BLAST: National Center for Biotechnology Information- Basic Local Alignment Search Tool; OD: Optical density; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

BSA: Bovine serum albumin; HPLC: High performance liquid chromatography; T_m: Melting temperature; NaOH: Sodium hydroxide; Tris-HCl: Tris-Hydrochloric acid; EDTA: Ethylenediaminetetraacetic acid; K_m; Michaelis-Menten constant; k_{cat}: Turnover number (Catalytic constant); DW: Dry weight; PDB; Protein data bank; DSC: Differential scanning calorimetry

Declarations

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Author contributions

M.W. Iqbal: conceptualization, experimentation, investigation. M.W. Iqbal and T. Riaz: wrote the original manuscript and data processing. S. Mahmood and I.M. Khan: reviewed and edited the draft. Y. Zhu and D. Ni: booked the instrument and did formal analysis. W. Zhang: investigation and validation. W. Mu: supervise the project, gave major comments, and reviewed the manuscript.

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Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Ethical approval and consent to participate
Not applicable.
Consent for publication
Not applicable.
Competing interests
The authors declare that they have no competing interests.

Conflict of interest

The authors declare that they do not have any conflict of interest.

Ethical statement

This study does not involve any human testing.

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Supplementary Information

Figure S1. Amino acid multiple sequence alignment of recombinant Pa-LFI. The GenBank accession No. of the L-fucose isomerases were Ae-LFI: *A. pallidus* (WP_063389244.1); Pa-LFI: *P. rhizosphaerae* (WP_076167696.1); Ca-LFI: *C. polysaccharolyticus* (WP_035172025.1); Di-LFI: *D. turgidum* DSM 6724 (YP_002352328.1); Th-LFI: *T. toyohensis* (WP_084665866.1); Es-LFI: *E. coli* W (ADT76410.1). The red background represents strictly conserved residues, and the red type in blue boxes represent highly conserved residues. The symbol stars above the residues indicate secondary structure elements by the template *A. pallidus* with PDB ID: 3A9T [35]. The alignment was performed by using ESPript server [43].

Figure S2: Nano-DSC was performed to measure the melting temperature (T_m) of Pa-LFI, the fitted curve (red line) were determined by TwostateScaled method from the raw data (blue line).

Figures

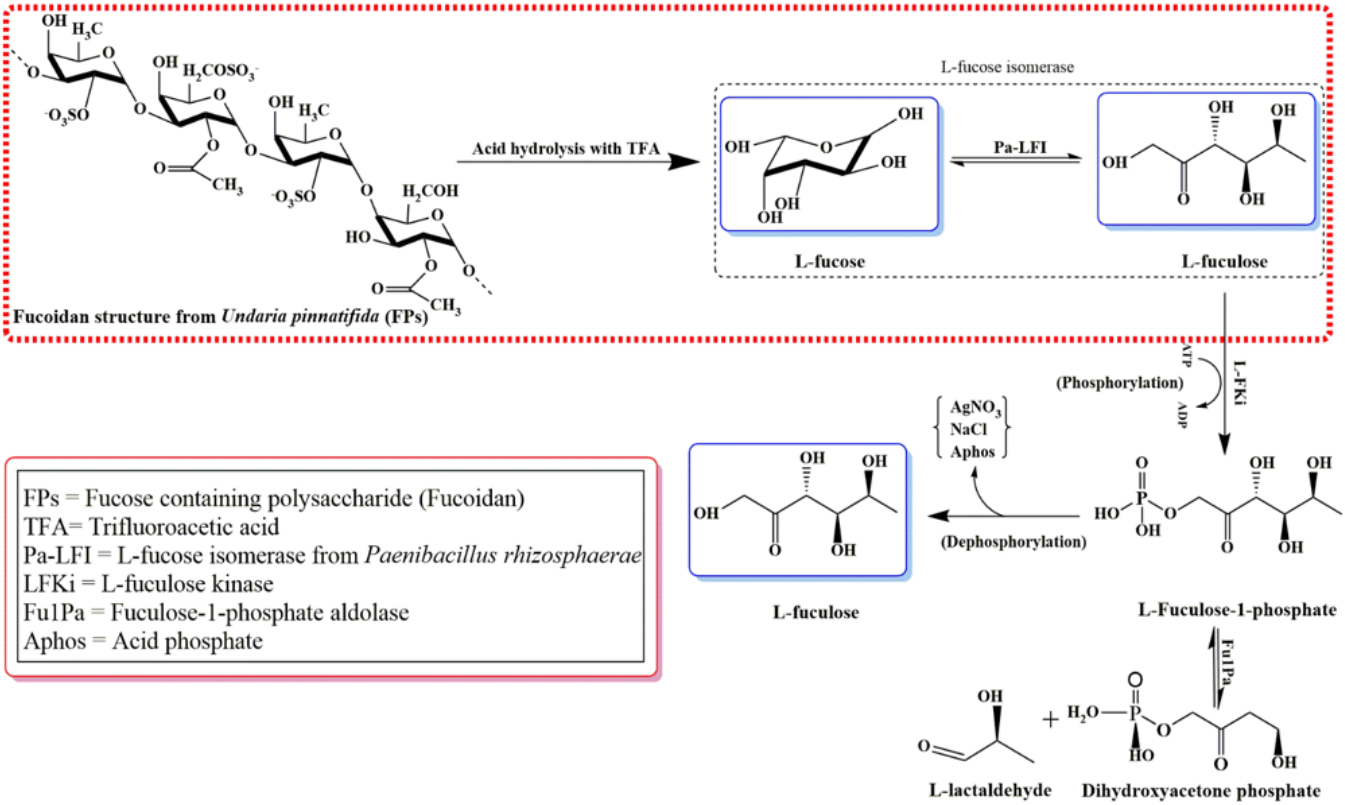


Figure 1

Figure 1

The schematic expression for the enzymatic production of L-fuculose from L-fucose of FPs and commercial fucose of Sigma-Aldrich by recombinant Pa-LFI.

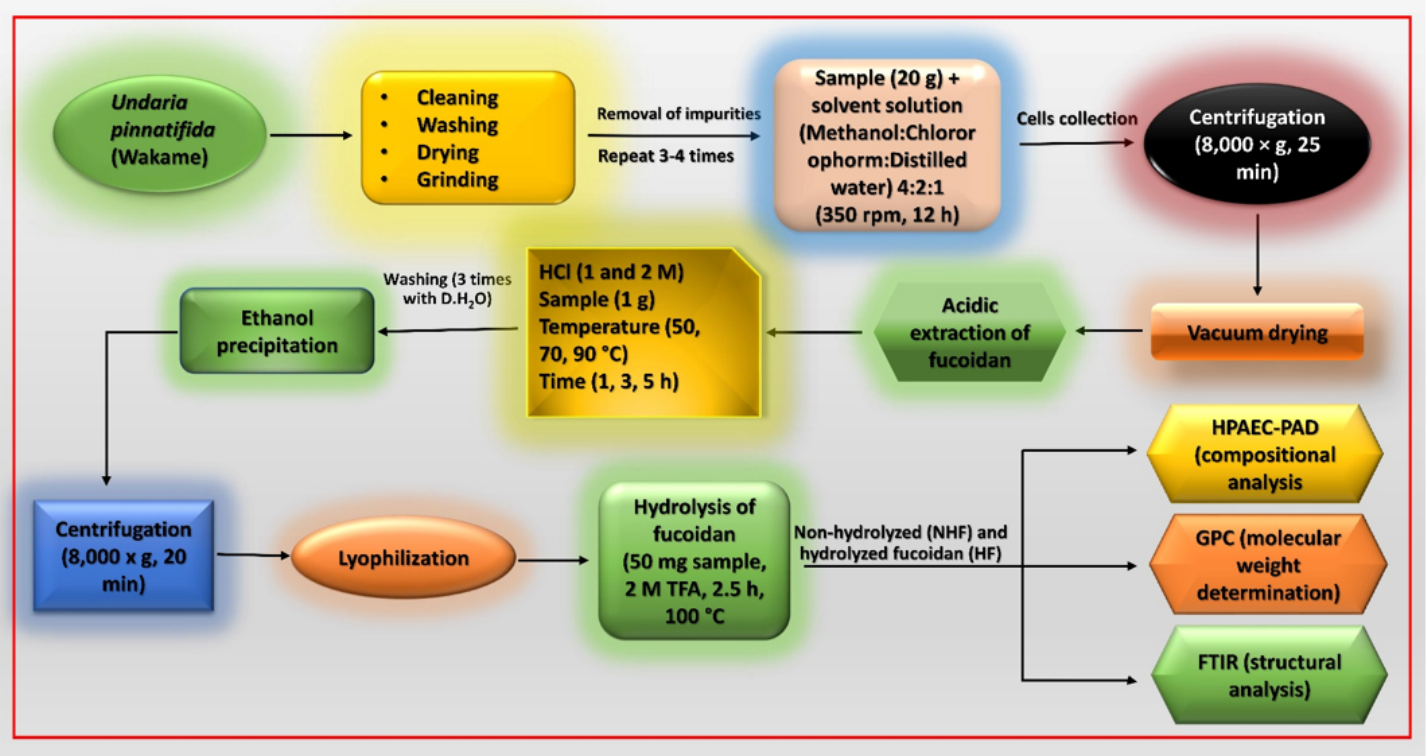
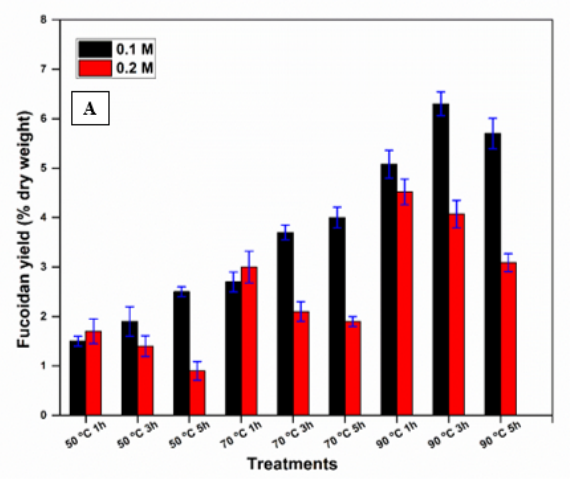


Figure 2

Figure 2

The schematic diagram for the extraction of fucoidan; fucose containing polysaccharide (FPs).



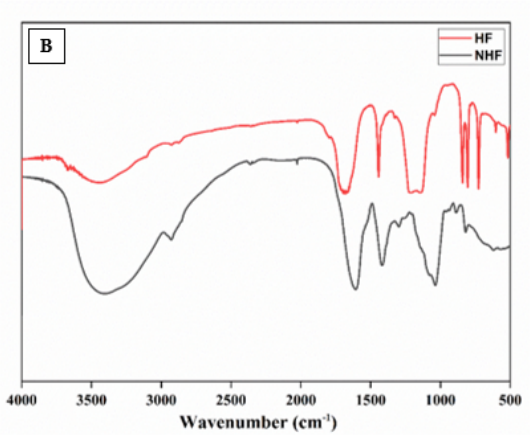


Figure 3

Figure 3

(A) FPs yield (% dry weight) from U. pinnatifida using 0.1 and 0.2 M HCl for 1, 3 and 5 h at 50, 70 and 90 °C. Data represent the mean of three replicates. (B) FTIR spectra of non-hydrolysed fucoidan (NHF) and hydrolyzed fucoidan (HF) from U. pinnatifida.

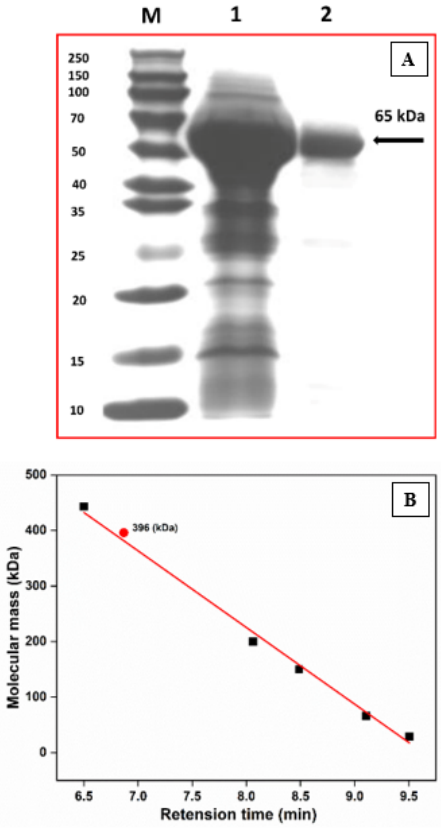


Figure 4

Figure 4

(A) The subunit molecular mass of crude and purified recombinant Pa-LFI was observed by SDS-PAGE analysis under denaturing conditions; lane M representing protein marker, lane 2 designates crude enzyme and lane 3 is purified enzyme by His-Trap affinity chromatography. (B) The native molecular mass of recombinant Pa-LFI was examined by HPLC with light scattering and UV detectors using anhydrase of 29 kDa, albumin of 66 kDa, alcohol dehydrogenase of 150 kDa, β-amylase of 200 kDa and apoferritin of 443 kDa as a reference protein standards. The red circle at 396 kDa was exhibiting the native molecular mass of recombinant Pa-LFI.

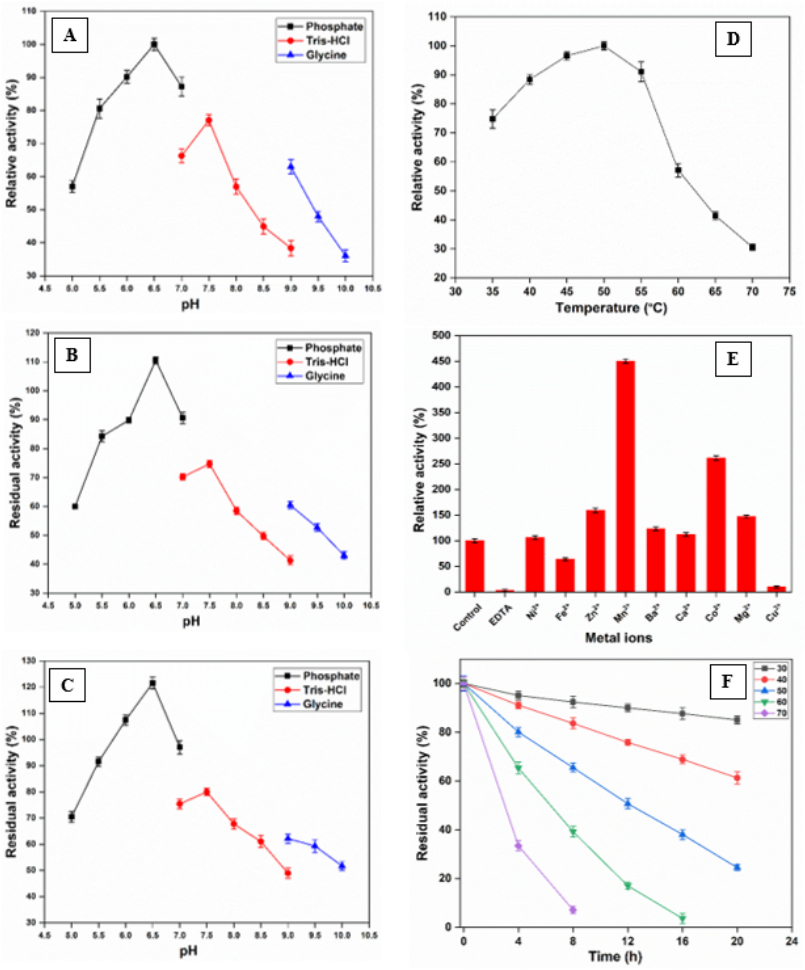


Figure 5

Figure 5

Influences of pH, temperature and metal ions on the activity of recombinant Pa-LFI. (A) The optimum pH effects were examined by using three different kinds of buffers [phosphate (pH 5.0-7.0), Tris-HCl (pH 7.0-9.0) and glycine (pH 9.0-10.0)] at 50 mM with recombinant Pa-LFI (enzyme) and L-fucose (substrate). (B) The effects of pH stability at 4 °C for 24 h were evaluated by incubating the recombinant Pa-LFI at pH ranges 5.0-10.0 and calculate the residual activities. (C) The effect of pH stability at optimum temperature 50 °C for 1 h was examined by incubating the enzyme at different pH ranges (5.0-10.0). (D) Influences of temperature on the activity of recombinant Pa-LFI for L-fucose; 50 mM phosphate with pH 6.5 hydrolyzed at different ranges of temperatures (35-70 °C). (E) Metal ion effects on the activity of recombinant Pa-LFI for L-fucose. (F) Effects of thermal stability of recombinant Pa-LFI for L-fucose. The recombinant Pa-LFI was incubated at 30, 40, 50, 60 and 70 °C for different intervals of times (0-20 h) to evaluate the residual activity. The error bars represent the mean of three replicate values. The enzyme was incubated with 1 mM of each metal ion after EDTA treatment for 1 h and control showing purified enzyme without metal ion. Error bars represent the mean of three replicates.

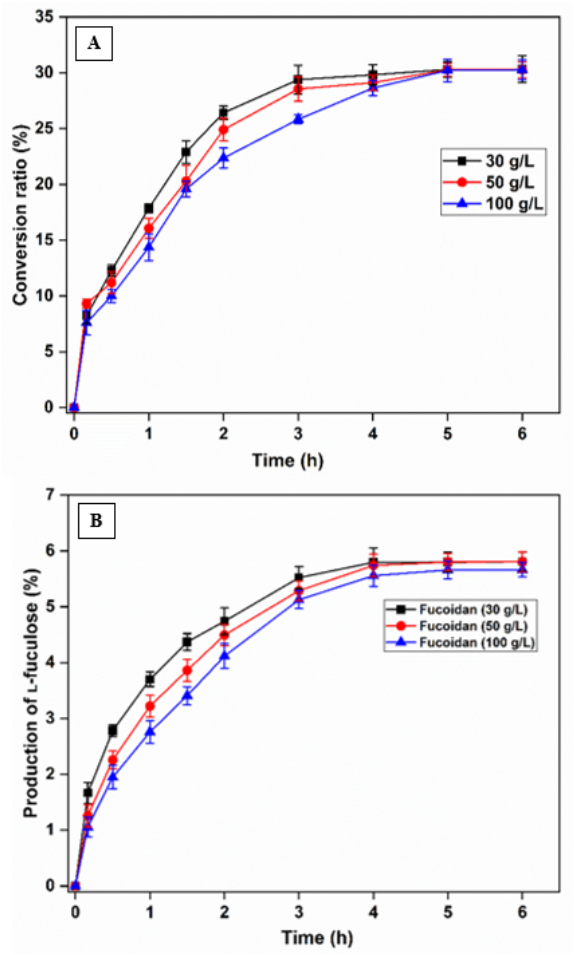


Figure 6

Figure 6

(A) L-Fuculose production from L-fucose by recombinant Pa-LFI at different concentrations (36, 50 and 100 g L-1) of L-fucose from commercially available Sigma-Aldrich. Error bars represent the mean of three replicates. (B) The production of L-Fuculose from L-fucose of FPs by recombinant Pa-LFI at different concentrations (30, 50 and 100 g L-1) of HF.

Supplementary Files

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