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CATALYTIC CONDITIONS OF FUCOIDANASE FROM *VASTICARDIUM FLAVUM*

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Abstract. Fucoidanases are widely distributed in both marine microorganisms and marine invertebrates, however the data on the properties of this enzyme are scarce. In the present study, we isolated the fucoidanase from gastrointestinal tracts of the marine shell *Vasticardium flavum* and determined its enzymatic properties. The fucoidanase cleaved 1→3-α-L-fucan link of fucoidan extracted from sea cucumbers *Stichopus variegatus*, *Holothuria spinifera*, did not cleave fucoidans from *F. evanescens* and *F. vesiculosus* including rotational α-1→4 and α-1→3 glycoside chains. This enzyme did neither catalyze the hydrolysis of fucoidans from *U. pinnatifida*, *S. mcclurei,* which belongs to the galactofucan group. The fucoidanase showed the best activity at pH 3-4 and 24 hours of incubation. The enzyme activity was enhanced by Ca^{2+}, Ba^{2+}, Co^{2+} and Mg^{2+} cations, but it was inhibited by the Cu^{2+}, Sn^{2+}, Fe^{2+} and Al^{3+} cations. After incubation at 65 °C for 5 min, the enzyme activity was completely disappeared.

Keywords: fucoidanase, *Vasticardium flavum*, fucoidan, enzyme.

Classification numbers: 1.5.1, 1.5.4.

1. INTRODUCTION

Fucoidans are a family of polysaccharides found in brown seaweeds and some other marine organisms. These polysaccharides exhibit a lot of biological activities, such as anticoagulant, antithrombotic, anticancer, anti-inflammatory and immunomodulatory. For these reasons, they are interesting to scientists around the world [1, 2]. In general, fucoidan from brown seaweeds of Ectocarpales and Laminariales orders, has been shown to be a sulfated fucan with 1→3-α-L-Fucp in the backbone [2, 3, 4]. The structure of alternating 1→3- and 1→4-linked α-L-fucosyl residues was described for fucoidan from brown seaweeds of Fucales order (Fucaceae family) [2, 3, 5]. As the length of backbone and complicated structure affect the bioactivity of fucoidan, the low molecular fucoidan becomes attracted by increased researches.

There are different methods for preparing oligofucoidans including chemical, physical or enzymatic tools to get biomaterials containing bioactivities similar to those of original fucoidan.

The unspecific hydrolysis property is one of disadvantage issue of the chemical tool. Additionally, the types of sulfation or the structure of polysaccharides may be broken up by the high acid concentrations. Oppositely, the enzymes of degrading fucoidan, including fucoidanase or α-L-fucosidases, are able to modify fucoidans, while the position of sulfate groups or the main physicochemical characteristics of these polysaccharide are remained [6].

Enzymes are substances which act as a catalyst to bring about a specific biochemical reaction. Enzymes have actually the ability to separate specifically on one kind of bonds in the polymer molecules. Enzymatic hydrolysis provides an indispensable tool for both the structural studies of fucoidans and the production of their oligomers [7]. There are sources of fucoidanases that have been found in marine organisms, such as marine bacteria [8, 9, 10], invertebrates [11, 12, 13] and some fungi [14]. However, the data on the specificity of fucoidanases such as the type of cleaved glycoside bond, the relation between catalytic activity and the degree of substrate sulphation, are scarce compared to those of other enzymes, including laminarinase, cellulase, or another glycosidase [7].

In this paper, we report on the characterizations of a fucoidanase from marine shell *Vasticardium flavum*, which degrades fucoidan from sea cucumbers *Stichopus variegatus*, *Holothuria spinifera* containing α-1→3 glycoside bonds.

2. MATERIALS AND METHODS

2.1. Materials

Crude fucoidans from the brown seaweed *Sargassum mcclurei* and from sea cucumbers *Stichopus variegatus*, *Holothuria spinifera* were prepared as described by Zvyagintseva et al. and after that fucoidans were purified by ion-exchange chromatography [15, 16]. The structural characteristics of fucoidans from the brown seaweed *Sargassum mcclurei* were reported before by our colleagues [17]. Fucoidans from the brown seaweeds *Undaria pinnatifida*, *Fucus evanescens*, *Fucus vesiculosus* were purchased from Sigma-Aldrich (USA).

2.2. Enzyme activity assay

2.2.1. Activity of fucoidanase measured by Nelson method [18]

Fucose was used as a sugar standard. The substrate was completely dissolved in the buffer solution just prior to do the hydrolysis reaction. A reaction mixture was composed of the following ingredients: 200 µl of 0.1 % substrate solution and 50 µl of an enzyme solution in 0.025 M succinic buffer, pH 5.2. These mixtures were incubated at 37 ^{o}C for 4 h to perform the hydrolysis reaction. The increase in the amount of reducing sugars is a measure of enzyme activity [18]. The amount of the enzyme that catalyzed the formation of 1 mole of α-L-fucopyranose per minute was adopted as a unit of activity (U).

2.2.2. The electrophoresis method for exploring the enzyme activity

We used the carbohydrate polyacrylamide gel electrophoresis (C-PAGE) as described earlier for discovery of the fucoidanase activity [9]. Fucoidan and oligo fucoidan after degradation of the fucoidan were found by electrophoresis into 23% acrylamide gel. Gel staining was indicated with a solution consisting of 0.01 % O-toluidine blue in EtOH, AcOH and H_2O with a volume ratio of 2:1:1.

2.2.3. Protein concentration

The determination of protein concentration was done using Bradford method [19] and quantifying protein using absorbance at 280 nm.

2.3. Extraction of fucoidanases

Marine shell (*Vasticardium flavum*) samples were used in this research which were collected in December 2016 on the 49th voyage aboard the R/V “Akademil Oparin” in the territorial waters of the Socialist Republic of Vietnam. 154 g of the mollusk gastrointestinal tracts were crushed to a homogenized mixture and extracted with 0.025 M succinic buffer, pH 5.2 at a ratio digestive glands: buffer = 1:3 (w/v). This mixture was centrifuged as 9,000 g for 20 min at 4 ◦C to remove the insoluble material, and the supernatant was mixed with ammonium sulphate to 80 % saturation. The precipitate fractions separated by centrifugation were continuesly dissolved in 2 M ammonium sulphate with the minimum of volume. The next steps were dialysis of the solution against 0.025 M succinic buffer, pH 5.2 and concentration with a 10 kDa cut-off ultrafiltration membrane (Amicon, USA). The enzyme solution after concentration was used for the following investigation.

2.4. Determination of substrates specificity

The reaction mixture consisting of 100 µl of enzyme solution, 200 µg of fucoidan (from brown seaweed *U. pinnatifida*, *S. mcclurei*, *F. evanescens*, *F. vesiculosus* and from sea cucumbers *Stichopus variegatus*, *Holothuria spinifera*, 4 mg/ml) was incubated for 24 h at 37 ^{o}C. The Nelson method and C-PAGE method as described above were carried out for detection of fucoidanase activity.

2.5. Determination of the optimal incubation time

The mixtures were composed of posterior elements, 100 µl of enzyme solution, 200 µg of fucoidan from sea cucumber *Stichopus variegatus* (4 mg/ml) as the reaction amalgation. The incubation at 37 ^{o}C for 0, 1, 4, 7, 17, 24, 30 and 48 hours were executed with above mixture. The fucoidanase activity was measured by Nelson method and C-PAGE method as presented above.

2.6. Determination of thermal stability

Thermal stability of fucoidanase was studied. At first, the enzyme solution was denatured at various temperatures (20, 37, 45, 50, 55 and 65 ^{o}C) for 20 min. Samples of enzyme after preincubation were cooled at 4 ^{o}C and the substrate then was added. The C-PAGE method was applied for determination of thermal stability.

2.7. Determination of the optimum pH

We studied the effect of different pH values on the fucoidanase activity, so that ten of pH values were researched (0.2 M succinic buffers with pH range 3.0 - 7.0 and pH range 5.0 - 9.0 with Tris buffers). The mixtures including 50 µl of enzyme solution, 200 µg of fucoidan from sea cucumber *Stichopus variegatus* (4 mg/ml) and 50 µl of buffers with different values of pH were incubated for 24 h at 37 ^{o}C. Activity was detected by Nelson method and C-PAGE method.

2.8. Influence of bivalent metals

The influence of bivalent metal ions was verified as the following: the incubation at 37^oC for 24 hours with solution: 100 µl of enzyme, 20 µl of 0.1 M solution of bivalent metal salt ($MgCl_2$, $BaCl_2$, $SnCl_2$, $CaCl_2$, $CoCl_2$, $FeCl_2$, $AlCl_3$, $CuSO_4$) and 200 µg of the fucoidan from sea cucumber *Stichopus variegatus* (4 mg/ml). Both of the Nelson method and C-PAGE method were also used for measuring the enzyme activity.

3. RESULTS AND DISCUSSION

3.1. Screening fucoidanase from marine invertebrates

The distribution of fucoidanases in 86 species of marine invertebrates in Vietnam was studied. Fucoidanases were found to be distributed widely and quite diversely in Vietnamese marine invertebrates. In samples belonging to Class Gastropoda, 44.2 % of them are able to degrade fucoidan from *F. evanescens*, that bring both of α-1→4 and α-1→3 glycoside links in the backbone and 30.2 % are able to degrade fucoidan from *S. mcclurei*, a kind of galactofucan. Meanwhile, the percentages of samples having hydrolytic activity fucoidan from *F. evanescens* and fucoidan from *S. mcclurei* in samples belonging to Class Bivalvia were 79.3 % and 65.5 %, respectively (Fig. 1).

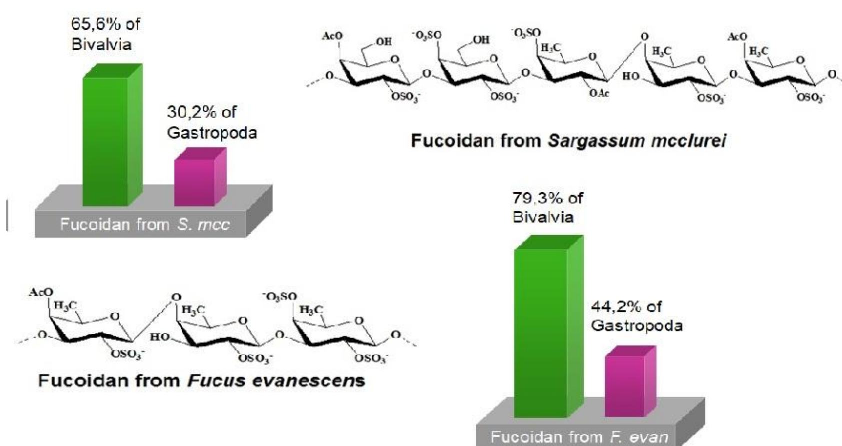

Figure 1. Distribution of fucoidanases with different specificity in marine invertebrates. Fucoidan from *S. mcclurei* (galactofucan); Fucoidan from *F. evanescens* (1→4; 1→3-α-L-fucan).

3.2. Catalytic conditions of fucoidanase from *Vasticardium flavum*

Based on the screening results, we chose the marine shell *Vasticardium flavum* as the fucoidanase producer.

3.2.1. Specificity of enzyme action on different substrates

Mode of action and specificity of the fucoidanases are less studied, the catalytic organization of these enzymes is nearly unknown, especially. The substrates employed in this investigation were six kinds of fucoidans distinct on the features both of the major chains and

the branches frame. These were fucoidans were extracted from the brown seaweed, *Undaria pinnatifida*, *Sargassum mcclurei*, *Fucus evanescens*, *Fucus vesiculosus* and from sea cucumbers *Stichopus variegatus*, *Holothuria spinifera*.

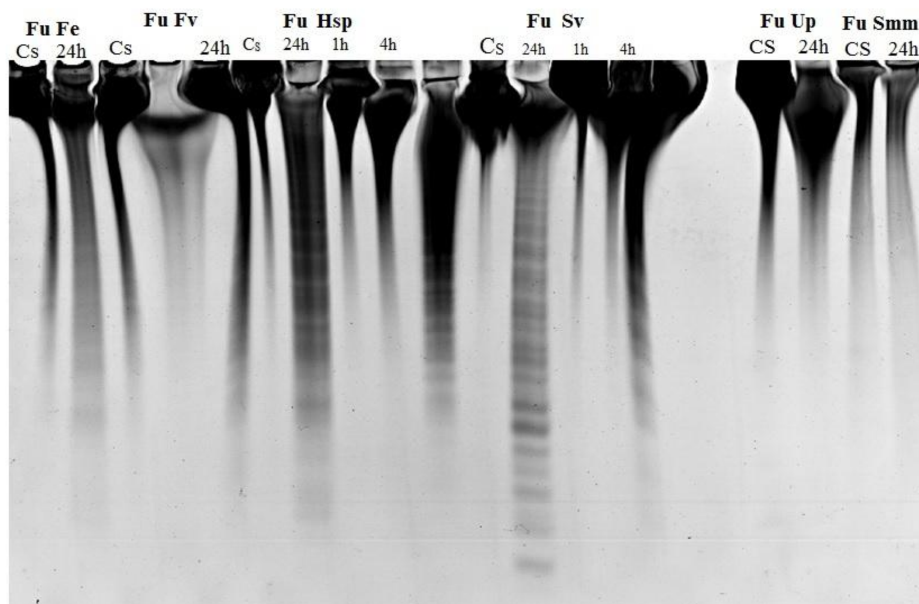

Figure 2. Description of oligo fucoidans produced by enzyme action on different substrates. Fu Fe: fucoidan from *Fucus evanescens*; Fu Fv: fucoidan from *Fucus vesiculosus*; Fu Up: fucoidan from *Undaria pinnatifida*; Fu Smm: fucoidan from *Sargassum mcclurei*; Fu Hsp: fucoidan from sea cucumbers *Holothuria spinifera*; Fu Sv: fucoidan from sea cucumbers *Stichopus variegatus*. Cs: control substrate (unhydrolyzed fucoidan); 1h, 4h, 24h: fucoidan fragments produced by enzyme after 1 hour, 4 hours, 24 hours of incubation.

Table 1. Specificity of enzyme action on different fucoidan substrates as monitored by Nelson method.

Substrates	Structure	Sources, references	Relative activity, %
Fucoidan from *Undaria pinnatifida* (Fu Up)	galactofucan	Sigma, purchase	0
Fucoidan from *Sargassum mcclurei* (Fu Smm)	galactofucan	NITRA, [19]	0
Fucoidan from *Fucus evanescens* (Fu Fe)	1→3;1→4-α-L-fucan	Sigma, purchase	0
Fucoidan from *Fucus vesiculosus* (Fu Fv)	1→3;1→4-α-L-fucan	Sigma, purchase	0
Fucoidan from sea cucumber *Holothuria spinifera* (Fu Hsp)	1→3-α-L-fucan	NITRA, not yet published	95 ± 0.87
Fucoidan from sea cucumber *Stichopus variegatus* (Fu Sv)	1→3-α-L-fucan	NITRA, not yet published	100

The research results indicated that the fucoidanase was not active for the hydrolysis of fucoidan from *F. evanescens* and *F. vesiculosus*, that consisting of α-1→4 and α-1→3 glycoside bonds alternating in the main chains. This enzyme did neither degrade fucoidan from *U. pinnatifida*, *S. mcclurei,* which belong to the galactofucan group. Fucoidan including only α-

1→3 glycoside links from sea cucumbers *Stichopus variegatus*, *Holothuria spinifera* were hydrolysed by the enzyme. From these data, we can conclude that the fucoidanase from *Vasticardium flavum* is specific for the α-1→3 glycosidic bonds (Fig. 2, Table 1).

3.2.2. Optimal incubation time

The optimal incubation times for fucoidan hydrolysis of enzyme were studied by the Nelson and electrophoresis methods. The oligofucoidans were detected after 4 hours of reaction, and the full amount of products of hydrolysis was seen after 24 hours incubation (Fig. 3A and Fig. 3B).

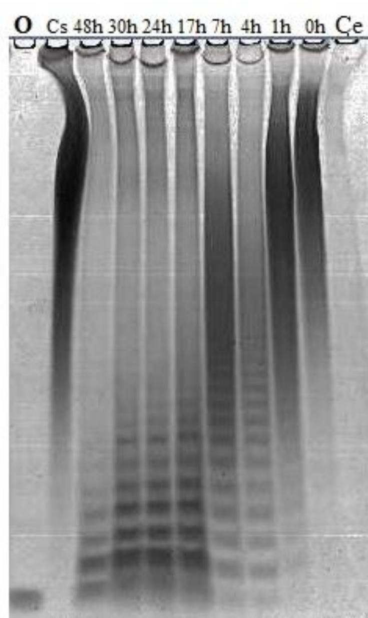

*Figure 3*A. Description of fucoidan degradation using enzyme from *V. flavum* at various reaction times. The incubation times: 0, 1, 4, 7, 17, 24, 30 and 48 hour. Cs: Polysaccharide fucoidan; Ce: enzyme solution; O: standard tetraride.

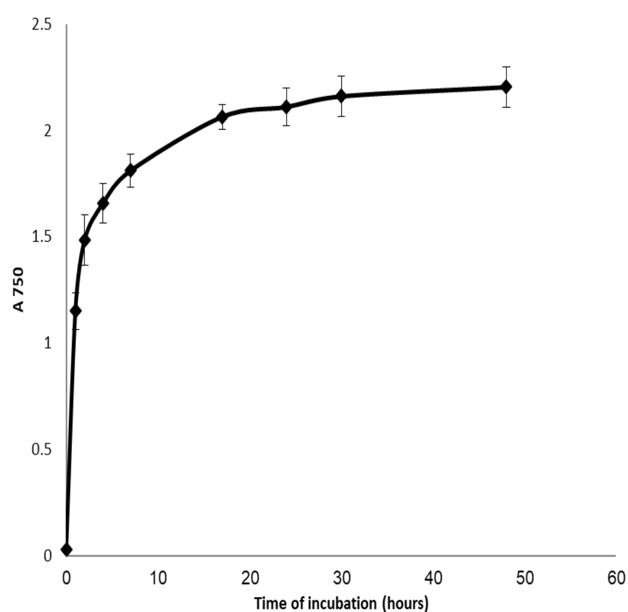

Figure 3B. Effects of incubation time on enzyme activity.

3.2.3. Optimal pH

In most of previous studies, the acidic pH conditions were the pH optimum for fucoidanases isolated from marine invertebrates. With an exception case of enzyme were found from the gastrointestinal tracts of the marine animals *Littorina kurila,* this enzyme had the optimal pH at base condition [7]. In this issue, fucoidanases were also detected that had an acidic pH optimum (around 3-4) (Fig. 4A and Fig. 4B), the pH range was often observed for the fucoidanases of marine invertebrates.

Figure 4A. Electropherogram of fucoidan after hydrolysis by enzyme at different pH values.

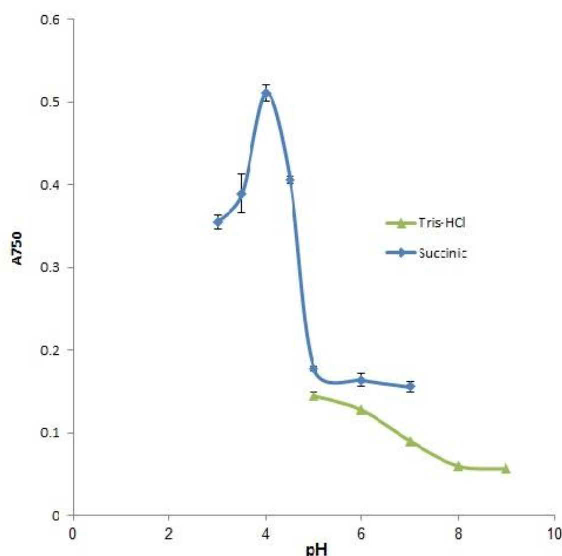

Figure 4B. Effects of pH on enzyme activity.

3.2.4. Influence of metal ion for fucoidanase activity

The studied results on the effects of metal ions for fucoidan hydrolysis by enzyme from marine shell *V. flavum* were displayed on the Fig. 5 and Table 2.

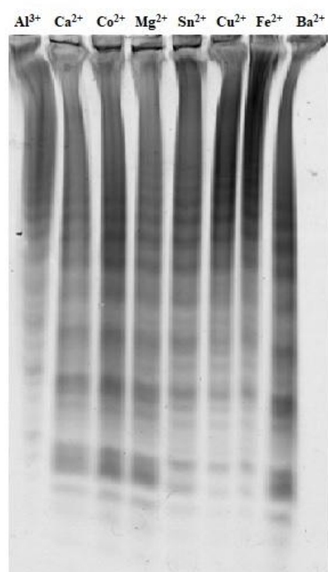

Figure 5. The effect of various metal ions on fucoidanase as monitored by C-PAGE. The metal ions are shown over the line.

Table 2. The effect of various metalions on the enzyme activity as monitored by Nelson method.

Metal ions	Relative activity (%)
Control	100
Al^{3+}	20,70 ± 0,77
Ca^{2+}	106,69 ± 0,1
Co^{2+}	107,36 ± 0,73
Mg^{2+}	116,49 ± 1,49
Sn^{2+}	26,84 ± 1,03
Cu^{2+}	31,74 ± 0,90
Fe^{2+}	19,42 ± 1,73
Ba^{2+}	110,59 ± 1,10

Whenever there was the attendance of the Ca^{2+}, Ba^{2+}, Co^{2+} or Mg^{2+} ions, the enzyme activity was slightly increased. Oppositely, the enzymes were significantly inactivated if there was the attendance of one of following cations Cu^{2+}, Sn^{2+}, Fe^{2+} or Al^{3+}. In the before report of Artem et al. [13], the fucoidanase from the marine invertebrates, *Lambis* sp., was not metal-dependent; however, this enzyme activity was affected by the presence of some of metal cations such as the Ca^{2+}, Ba^{2+} and Mg^{2+} cations weakly activated the fucoidanase, while the Zn^{2+}, Cu^{2+} and Hg^{2+} ions had an repressive influence on the operation mechanism of enzyme.

3.2.5. The fucoidanase stability at different temperatures

The specificity and formula of working are the most basic characterizations when studying on enzyme, they are important data for the further investigation in structural studies and biotechnological processes. The enzyme stability from marine shell *V. flavum* at different temperatures was reported in this article. After 5 min of the incubation, the catalysis activity of fucoidanase was completely out of order. And the enzymatic activity was greatly reduced after 60 min of the denaturation at 45 ^{o}C.

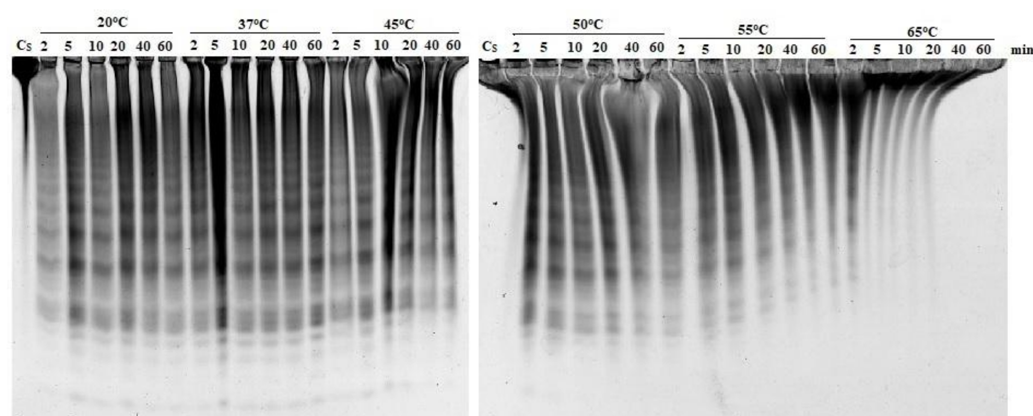

Figure 6. Description of the fucoidanase stability at different temperatures. 2, 5, 10, 20, 40, 60 min were various period of enzyme preincubation time before experiment. The preincubation temperature is shown over the brackets. Cs: polysaccharide fucoidan.

4. CONCLUSION

The catalytic conditions of fucoidanase from the gastrointestinal tracts of the marine shell *Vasticardium flavum* was studied. We have shown that the fucoidanase is specific for the α-1→3 glycosidic chains because there are only fucoidan from sea cucumbers *Stichopus variegatus*, *Holothuria spinifera* (1→3-α-L-fucan) were hydrolyzed. Meantime the fucoidanase from *Vasticardium flavum* did not cleave fucoidan from *F. evanescens* and *F. vesiculosus* (1→3; 1→4-α–L-fucan) and the enzyme also did not cleave fucoidan from *U. pinnatifida*, *S. mcclurei* (galactofucan). Optima of pH, incubation time, stability temperature and the influence of metal ion for fucoidanase activity have been investigated.

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