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Revealing the Potential of Xylanase from a New Halophilic *Microbulbifer* sp. CL37 with Paper De-Inking Ability

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Abstract
Paper de-inking is one of the critical processes in pulp and paper industry as it is ecofriendly and energy saving. This process requires microbial enzymes such as xylanases with ability to withstand harsh bioprocess conditions. *Microbulbifer* is a halophilic genus with ability to produce hydrolytic enzymes that could be applied in the biotechnological industry. So far, none of the xylanases from this genus have been studied, particularly in paper de-inking process. Therefore, in this study, the xylanase of a new halophilic bacterium, *Microbulbifer* sp. strain CL37, was characterized. Strain CL37 produced maximum amount of xylanase at 14th hour of incubation at 30 °C. The xylanase demonstrated optimal activity at 70 °C and pH 7. The xylanase was stable at wide range of NaCl (0–14%, w/v), in the presence of Al^{3+}, Ca^{2+}, Co^{2+}, Cu^{+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, acetone, chloroform, ethanol, sodium deoxycholate, Triton X-100, Tween 20, 40, 60, and 80, indicating that it is a halotolerant enzyme with high stability in various additives. The xylanase also demonstrated its ability to de-ink paper with considerably high efficiency (159%) as compared to other strains. The valuable characteristics possessed by xylanase of strain CL37 could potentially benefit to de-inking process in paper industry.

Keywords *Microbulbifer* · Xylanase · Paper de-inking · Salt tolerant enzyme · Bioprocess

1 Introduction

Halophiles are a group of microorganisms that inhabit saline environment such as mangrove, seawater and marine sediment [1–4]. They possess various strategies to overcome the stress caused by salty environment [5]. Properties of halophiles, including their ability to grow at extreme conditions, could produce negatively charged enzymes and bioactive compounds that are potentially beneficial to broad range of biotechnological applications such as agriculture and medicine [6, 7]. In recent years, mangrove areas that are rich in organic matter and lignocellulosic biomass represent one of the hunting hot-spots for bioprospecting industrial useful enzymes such as amylases and xylanases [8–10].

Xylanases are hydrolytic enzymes that belongs to enzyme class of glycoside hydrolases (GH) [11, 12]. This enzyme can hydrolyse xylan into xylose, xylobiose, and xylooligomers [13, 14], which can be used in wide range of industries such as paper and pulp, biofuel, animal feed, and textile industries [15–17]. In paper and pulp industry, wastepaper is the largest solid waste stream which has brought harmful effect to the environment. The recycling of wastepaper has become important to the industry as this process reduces the utilization of forest resources and waste disposal [18]. Furthermore, the wastepaper recycling process is estimated to save 28–60% of energy when compared to the new paper making process from wood [19]. Therefore, wastepaper recycling could be considered as a sustainable solution. Presently, paper de-inking is one of the essential steps in wastepaper recycling that uses physical method, chemicals or microbial enzymes to remove the ink [20]. Microbial enzymes such as xylanases could specifically hydrolyse the bonding region

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between fibre and ink to eliminate the hard-to-remove ink particles from the fibre [18], thus, improving the quality of de-inked laser printed paper [21]. Moreover, utilization of microbial enzymes for paper de-inking also offers several advantages such as environmental friendly and better efficiency when compared to other physical and chemical methods [19].

Microbulbifer is one of the bacterial genera which comprised of 25 valid published species up to date [22]. They are well-known polysaccharide degrading halophiles with abilities to produce a range of hydrolytic enzymes. Among these enzymes, cellulases, carrageenases, agarases and chitinases produced by *Microbulbifer* spp. have been characterized with excellent performance in withstanding various stress and conditions [23–27]. However, the potential of xylanase from genus *Microbulbifer* has yet to be reported. In this study, a xylanase-producing halophilic bacterium, *Microbulbifer* sp. strain CL37 was isolated from mangrove sediment, and its xylanase was characterized. The ability of xylanase from strain CL37 in paper de-inking was also evaluated. The xylanase produced by *Microbulbifer* sp. strain CL37 could potentially serve as a new sustainable option for paper de-inking process in paper and pulp industry.

2 Materials and Methods

2.1 Isolation and Screening of Xylanase-Producing Bacteria from Mangrove Sediment

Mangrove sediment samples were collected at Tanjung Piai National Park, Johor, Malaysia (permit reference: CJB G No. 887005). The bacteria in the sample were isolated following the previous methods as described [28]. In brief, a 0.1 g of the mangrove soil sample was diluted with 9 ml of sterile distilled water. The serially diluted sample (0.1 ml) was then plated onto marine agar 2216 (MA; BD Difco) and incubated at 30–35 °C for 1–14 days. The bacterial isolates were screened for extracellular xylanase production by incubating on MA supplemented with 0.5% (w/v) xylan from beechwood (Apollo Scientific). After 3 days of incubation, the MA-xylan plates were flooded with Lugol's iodine. The positive result was indicated by halozone formed surrounding the colonies. A strain, assigned as CL37, with xylan-degrading ability was selected for further investigation.

2.2 16S rRNA Gene Sequencing and Phylogenetic Analysis

Genomic DNA of strain CL37 was isolated using Wizard® Genomic DNA Purification kit (Promega) following the manual with modifications. The 16S rRNA gene was amplified by PCR using primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′) as stated [29]. PCR was performed according to the conditions as stated in Lam et al. [30]. The PCR products were purified by Wizard® SV Gel and PCR Clean-Up system (Promega) according to manufacturer's protocol with modifications and were sequenced at Apical Scientific Sdn. Bhd., Seri Kembangan, Malaysia, using Sanger method. The forward and reverse sequences were aligned by ClustalW, and noises of sequences were trimmed. The presence of chimeric sequences was checked by using DECIPHER v. 2.0 [31]. The nearly full length 16S rRNA was searched against NCBI (BLASTn) and EzBioCloud [32] databases for bacterial identification. The 16S rRNA genes of closely related taxa were downloaded from GenBank and aligned by Muscle. Phylogenetic tree was constructed by using MEGA v. 7.0 [33] with neighbour-joining method [34], Kimura 2-parameter model, and 1000 bootstrap value.

2.3 Nucleotide Sequence Accession Number

The 16S rRNA gene (1475 bp) of *Microbulbifer* sp. strain CL37 is available at GenBank under accession of MK256319.

2.4 Morphological and Biochemical Characterization

The colony morphology of strain CL37 on MA was observed after 48 h of incubation at 30 °C. Gram stain reaction and bacterial shape were observed under light microscope (Nikon Eclipse E200). Biochemical characteristics and enzyme activity were tested by using API 20 NE and API ZYM (bioMérieux, France), respectively. The oxidase reaction and catalase activity were determined using 1% (w/v) *N*,*N*,*N*,*N*,-tetramethyl-1,4-phenylenediamine reagent (Merck) and 3% (*v*/*v*) hydrogen peroxide accordingly. Bacterial motility, indole and urease production were tested by using MIU medium (Himedia). The ability of strain CL37 to hydrolyse starch, casein, and gelatin were examined following the protocols as described by Smibert, Krieg [35].

2.5 Inoculum Preparation and Effect of Time Course on Xylanase Production

A loopful of fresh colonies of strain CL37 was inoculated in a xylanase production medium containing Marine Broth 2216 (MB; BD Difco) supplemented with 0.5% (w/v) xylan from beechwood (Apollo Scientific) and incubated at 30 °C and 180 rpm. After 24 h, a 5% (*v*/*v*) of inoculum was transferred into the new xylanase production medium (with same components as stated) and cultured in the same condition for another 24 h. The effect of time course on microbial

growth and xylanase production was investigated every 2 h' time interval. At each time interval, 2 ml of culture was withdrawn, and the optical density at 600 nm (OD_{600}) was measured by using a GENESYS™ 10S UV–Vis spectrophotometer (Thermo Scientific). A dilution was performed when the OD_{600} was greater than 1.0, and the final cell density was obtained by multiplying OD_{600} reading with the dilution factor. The leftover broth culture was centrifuged at 4 °C and 10,000 rpm for 20 min. Culture-free supernatant was utilized as crude enzyme in xylanase assays.

2.6 Xylanase Assay

Xylanase assay was conducted by employing 3,5-dinitrosalicylic acid (DNS) method [36] with beechwood xylan (Apollo Scientific) as a substrate. A 0.5 ml of crude enzyme was added into 0.5 ml of 50-mM sodium phosphate buffer (pH 7.0) containing 0.5% (w/v) beechwood xylan and incubated at 37 °C for 30 min. Optical density at 540 nm (OD_{540}) was measured using a spectrophotometer. The amount of reducing sugar released was quantified using DNS with xylose as standard. One unit of xylanase activity (U/ml) was defined as amount of enzyme that released one μmol of reducing sugar per minute under assay conditions. All enzyme assays were performed in biological triplicates.

2.7 Effect of pH, Temperature and Salinity on Xylanase Activity and Stability

Effect of pH, temperature, and salinity on xylanase activity was studied by incubating reaction mixture at different pH (4–10), temperatures (4–80 °C), and salinity (0–14% NaCl, w/v), respectively. The stability of xylanase was investigated by pre-incubating the xylanase for 1 h in varying pH (4–10), temperatures (4–80 °C), and salinity (0–14% NaCl, w/v) before the residual activity (%) was determined by standard xylanase assay. The buffers used in this study were 50-mM citrate phosphate (pH 4–5), 50-mM sodium phosphate (pH 6–7), 50-mM glycine–NaOH (pH 8–9), and 50-mM NaOH (pH 10).

2.8 Effect of Various Additives on Xylanase Activity

The activity of xylanase in the presence of various additives including organic solvents (acetone, chloroform, methanol, dimethyl sulfoxide, ethanol, and isopropanol), metal ions (Al^{3+}, Ca^{2+}, Co^{2+}, Cu^{+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+}, and Zn^{2+}), and detergent components (sodium dodecyl sulphate, sodium carbonate, and sodium deoxycholate in w/v, Tween 20, Tween 40, Tween 60, Tween 80, Triton X-100, and hydrogen peroxide in *v*/*v*) were examined. The final concentration used in the investigation were 25% (*v*/*v*), 5 mM and 1% for organic solvents, metal ions, and detergent components, respectively. Xylanase was pre-incubated with aforementioned additives at 37 °C for 1 h (except the incubation time for stability test in the presence of organic solvents which was 4 h), and then the xylanase activity was measured by performing standard xylanase assay. Relative activity (%) was expressed by taking enzyme activity without any additives (control) as 100%. The experiments were conducted in biological triplicates.

2.9 Assessment of Paper De-inking Ability of Xylanase

The ability of the crude xylanase to de-ink paper was assessed by using black colour printed paper from laser jet printer. The printed paper was cut into 1 × 1 cm strip and incubated with crude xylanase at 37 °C and 180 rpm for 24 h. The absorbance of the released ink at 596 nm was measured by using a spectrophotometer, and the colour change on paper was examined visually [37]. A negative control was prepared by replacing the crude xylanase with heat-denatured crude xylanase (boiled at 100 °C for 15 min). All the experiments were performed in biological triplicates.

3 Results and Discussion

3.1 Identification and Phenotypic Characterization of Xylanase-Producing Halophilic *Microbulbifer* sp. CL37

A bacterial strain assigned as CL37 with positive result on MA-xylan plate was isolated from mangrove sediment. This extracellular xylanase-producing strain CL37 was then identified via the 16S rRNA gene analysis. Based on NCBI database search, the nearly full length 16S rRNA gene (1475 bp) of strain CL37 shares 98.77%, 98.75%, 97.74% and 97.67% similarities with *Microbulbifer maritimus* TF-17^{T} (NR025772), *Microbulbifer rhizosphaerae* Cs16b^{T} (NR148866), *Microbulbifer taiwanensis* CC-LN1-12^{T} (NR108519), and *Microbulbifer aggregans* CCB-MM1^{T} (NR158143), respectively. The phylogenetic analysis of strain CL37 with all the species under genus *Microbulbifer* demonstrated that strain CL37 forms a clade with three *Microbulbifer* spp., while shows a clear distant with *Microbulbifer maritimus* TF-17^{T} with 94% bootstrap of support (Fig. 1). Thus, it was identified as *Microbulbifer* sp. CL37 (MK256319).

The *Microbulbifer* sp. strain CL37 was characterized in terms of its morphology, biochemistry and physiology. Strain CL37 was determined as Gram-negative, non-endospore forming, rod-shaped bacterium, with ability to produce catalase and oxidase. These characteristics are in accordance with the description of the species under genus *Microbulbifer*

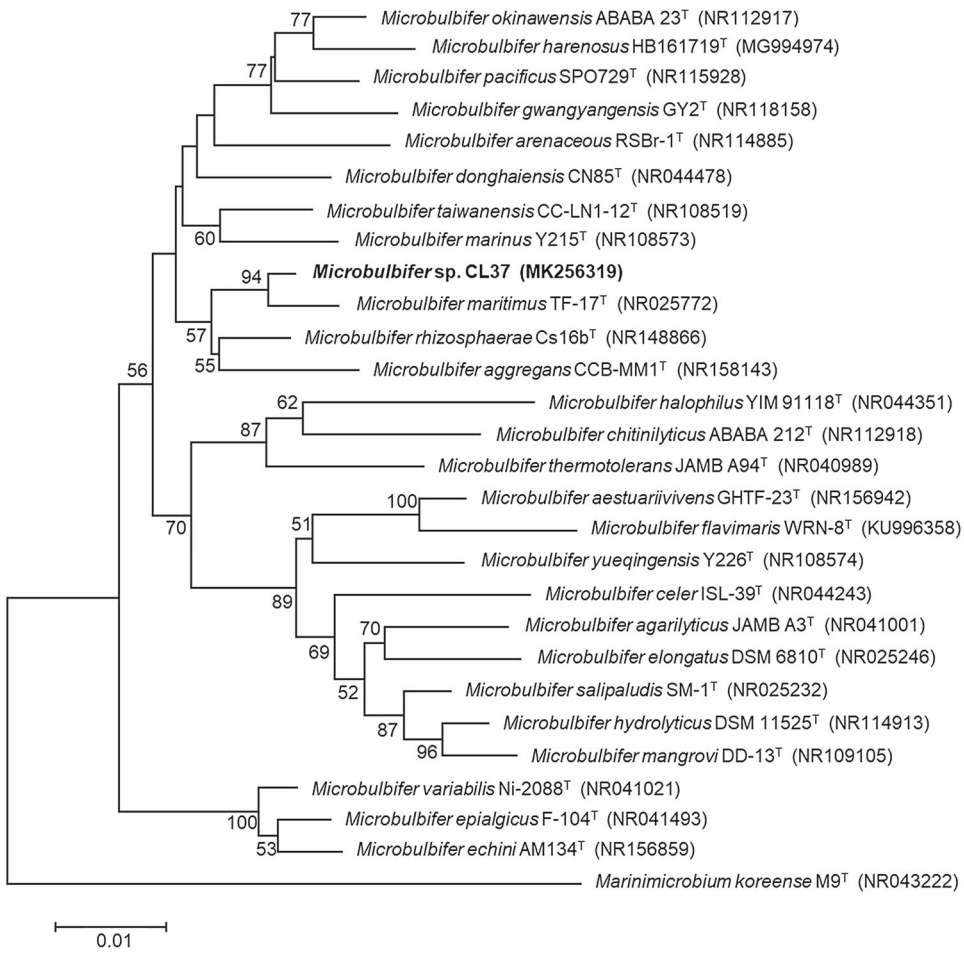

Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationship between strain CL37 with other members of genus *Microbulbifer* and other closely related bacteria. Percentages at nodes are levels of bootstrap support based on 1000 resampled dataset

[38]. When comparing strain CL37 with its closely related species [39–41], several differential phenotypical characteristics were detected (Table 1). In terms of morphology, strain CL37 was motile by gliding, while *M. maritimus* TF-17 ^{T}, *M. aggregans* CCB-MM1^{T} and *M. rhizosphaerae* Cs 16b^{T} are non-motile bacteria. In terms of biochemical capability, gelatin can be hydrolysed by strain CL37 and *M. maritimus* TF-17 ^{T} but were not hydrolysed by *M. aggregans* CCB-MM1^{T} and *M. rhizosphaerae* Cs 16b^{T} (Table 1), while for enzyme activity profile, lipase (C14), cystine arylamidase, and α-galactosidase were produced by strain CL37 but not by other closely related members of *Microbulbifer* (Table 1). Collectively, the differential phenotypic characteristics and phylogenetic analysis suggested that the strain CL37 is unique as compared to other closely related species of *Microbulbifer*.

According to Table 1, xylanase is produced by *Microbulbifer* sp. strain CL37 but not *M. aggregans* CCB-MM1^{T} and *M. maritimus* TF-17 ^{T}. Interestingly, through literature search, none of the xylanase from *Microbulbifer* genus has been reported so far. Therefore, the xylanase of strain CL37 was further characterized to reveal the potential and applicability of this enzyme in biotechnological industry.

3.2 Effect of Time Course on Microbial Growth and Xylanase Production

Strain CL37 was cultured in xylanase production medium, and the effect of time course on microbial growth and xylanase production were investigated. The growth curve of strain CL37 followed a sigmoid pattern, with 8 h of exponential phase, and remained at stationary phase for another 8 h (Fig. 2). The xylanase production increased gradually until it reached maximum production at 14th hour (Fig. 2). This indicates that strain CL37 could breakdown xylan (carbon source) that is available in the medium for growth starting from second hour of incubation. The maximal xylanase production at 14th hour is also the time point where the culture had been in stationary phase for 6 h. A similar trend was also observed for xylanase production of other halophilic bacteria such as *Chromohalobacter* sp. TPSV 101 [42] and *Gracilibacillus* sp. TSCPVG [43].

Table 1 Phenotypic characteristics of *Microbulbifer* sp. strain CL37 and other three closely related *Microbulbifer* species. Strains: 1, *Microbulbifer* sp. CL37; 2, *M. aggregans* CCB-MM1^T [39]; 3, *M. maritimus* TF-17 ^{T} [40]; 4, *M. rhizosphaerae* Cs 16b^T [41]. +, positive; -, negative; ND, not determined

Characteristics	1	2	3	4
Colony colour	Wood brown	White	Yellowish-brown	Sand yellow
Motility	+	–	v	–
Reduction of nitrate	–	–	+	ND
Hydrolysis of				
Xylan	+	–	–	ND
Starch	–	+	+	–
Casein	+	–	+	+
Gelatin	+	–	+	–
Acid production from				
D-Glucose	–	–	+	–
D-Mannose	–	+	+	+
D-Mannitol	–	–	+	+
D-Maltose	–	–	+	+
Enzyme activity (API ZYM)				
Alkaline phosphatase	+	+	+	–
Lipase (C14)	+	–	–	–
Valine arylamidase	+	–	–	+
Cystine arylamidase	+	–	–	–
α-galactosidase	+	–	–	–
β-galactosidase	–	+	–	+
N-acetyl-β-glucosaminidase	–	+	–	–

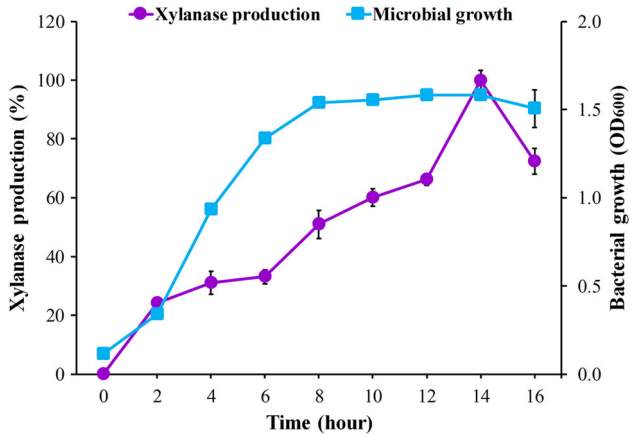

Fig. 2 Effect of time course on microbial growth and xylanase production of *Microbulbifer* sp. strain CL37 throughout 16 h of incubation. Mean value (n = 3) is expressed and standard deviations are indicated as error bars

3.3 Is Xylanase of Strain CL37 Constitutive or Inducible Enzyme?

To assess whether xylanase production is either constitutive or inducible, we cultured the strain CL37 in Marine Broth supplemented with and without xylan as substrate for 14th hour (where the production is maximum) and measured the xylanase production. Following Fig. 3, it could be seen that xylanase were produced in both media with and without xylan as substrate, which suggests the production of xylanase is constitutive [44]. A significantly higher xylanase production was also noted in the medium added with xylan as compared to medium without xylan as substrate (Fig. 3). This stipulates that the xylanase of strain CL37 is not only constitutively expressed, but the production of this enzyme was further enhanced by induction of substrate. Collectively, the medium supplemented with xylan was utilized to culture strain CL37 for 14 h to produce maximal amount of xylanase and was subsequently used in following experiments.

3.4 Examination of Xylanase Activity and Stability in Different Salinity, pH, and Temperature

The biochemical characteristics (salinity, pH, and temperature) of extracellular xylanase of strain CL37 was examined and shown in Fig. 4. In terms of salinity, the xylanase of strain CL37 was active at a broad range of NaCl, with up to 14% (w/v) (Fig. 4A). Xylanase of strain CL37 demonstrated optimal activity at 0% (w/v) NaCl, with more than 95% relative activity at 2% (w/v) NaCl (Fig. 4A), showing that it is a halotolerant enzyme. This result is comparable with the previous studies obtained from other halophilic bacteria such as xylanase activities of *Streptomyces viridochromogenes* M11 and *Alkalibacterium* sp. SL3 which have optimum at 0% (w/v) NaCl [45, 46]. With respect to stability in the presence of salt, the xylanase of strain CL37 was most stable at 0%

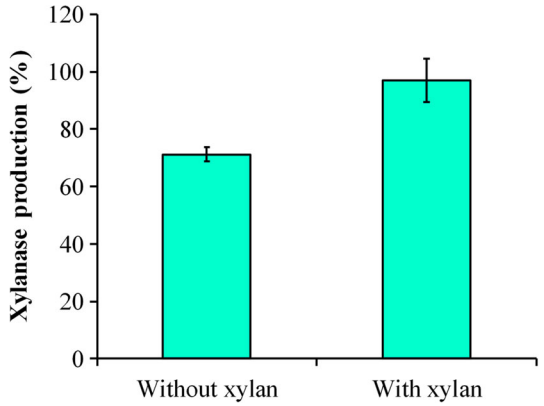

Fig. 3 Xylanase production of *Microbulbifer* sp. strain CL37 in the absence and presence of xylan as substrate at 14 h. Xylanase production (%) was calculated by relative to the case of reaction at which the maximum production value was taken as 100%. Mean value (n = 3) is expressed and standard deviations are indicated as error bars

(w/v) NaCl, with reduction in stability to 64% at 2% (w/v) NaCl (Fig. 4a), also indicating its halotolerant property. The xylanase of strain CL37 with salt tolerant property is possibly due to its survival strategy in natural habitat which is mangrove, an area located between marine and terrestrial environments with halophytes as lignocellulose material [47]. To adapt such saline environment with lignocellulosic biomass, the xylanase produced by strain CL37 has to possess the criteria of salt tolerant and at the same time could degrade lignocellulose and releasing simple sugar as nutrient for growth [48]. Moreover, the halotolerant property of xylanase of strain CL37 makes it an enzyme with flexibility to be utilized in industries work in condition without salt such as paper and pulp industry [17] and also condition with salt such as fish feed processing [49].

With respect to the effect of pH, the xylanase from strain CL37 was most active and stable at pH 7, a neutral condition (Fig. 4B). The result of optimal xylanase activity of strain CL37 at pH 7 is in accordance with the xylanase from other halophilic bacteria, including *Saccharophagus degradans* 2-40 ^{T} [50], *Glaciecola mesophila* KMM 241 [51], *Flammeovirga pacifica* WPAGA1 [52], *Luteimonas abyssi* XH031^{T} [53], and *Marinimicrobium* sp. LS-A18 [54]. Xylanase of *Microbulbifer* sp. strain CL37 that possessed neutral optimum pH condition can potentially be useful and cost-effective production in various industries such as baking, animal feed, and paper and pulp industries [55, 56]. Normally, paper and pulp industry uses harsh alkali chemicals in paper de-inking process, and the harsh alkali chemicals are not environmental friendly [19, 57]. Xylanase optimum at neutral pH has potential in de-inking process because no alkali chemicals are needed to increase the de-inking condition to alkaline pH. Hence, the industry not only

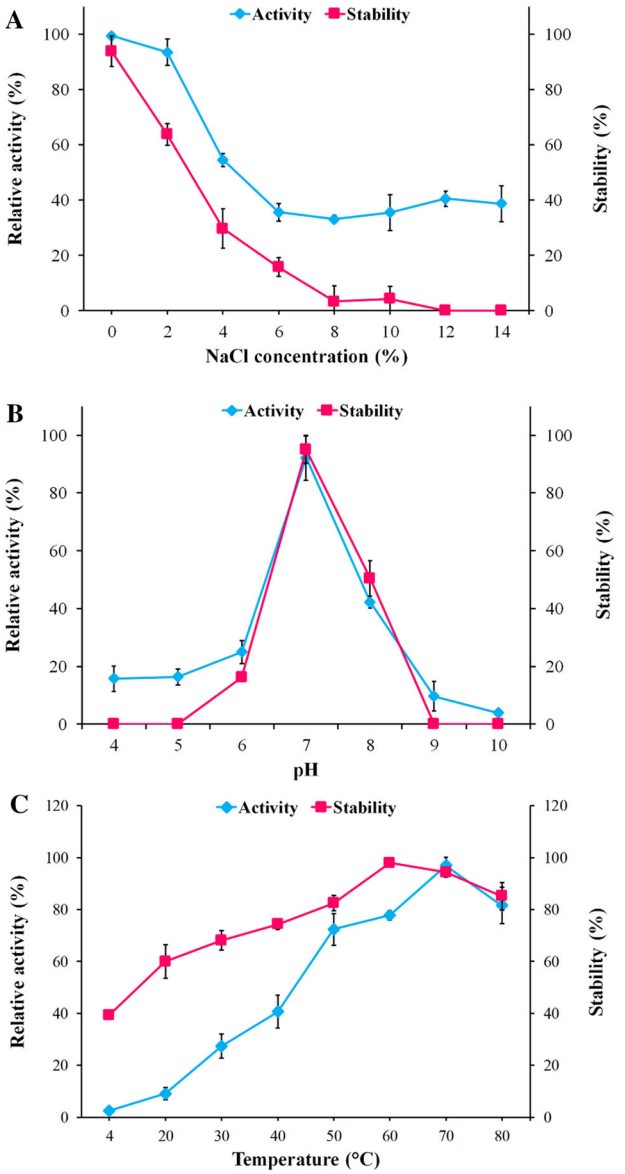

Fig. 4 Effect of different pH (**a**), salinity (**b**), and temperature (**c**) on activity and stability of xylanase from *Microbulbifer* sp. strain CL37. Relative activity (%) and stability (%) were calculated by relative to the case of reaction at which maximum activity was taken as 100%. Mean value (n = 3) is expressed and standard deviations are indicated as error bars

can reduce the operating cost, but also minimize the chemical usage, thus protect the environment [19].

While in terms of temperature, xylanase activity of strain CL37 increased with elevated temperature and reached its optimum at 70 °C, with more than 70% relative activity from 50 °C to 80 °C (Fig. 4C), indicating it is a thermoactive enzyme. This feature is distinctive as many of the xylanases produced by halophilic bacteria such as *Flammeovirga pacifica* WPAGA1, *Luteimonas abyssi* XH031^{T}, *Saccharophagus degradans* 2-40 ^{T}, *Streptomyces olivaceus*,

Zunongwangia profunda, *Alkalibacterium* sp. SL3, and *Marinimicrobium* sp. LS-A18 have lower optimal temperature (20–40 °C) [46, 52–54, 58–60]. Furthermore, xylanase of strain CL37 was also stable at wide range of temperatures from 40 to 80 °C (>70% stability; Fig. 4c). The thermostability of xylanase from strain CL37 was up to 60 °C and still retained 85% stability at 80 °C after 1 h of pre-incubation (Fig. 4c). The thermostable property of xylanase from strain CL37 makes it suitable to be applied in biofuel production and paper and pulp industry. These industries normally operate under temperature range of 40 °C to 60 °C in order to achieve the optimum performance [61, 62]. Xylanase from strain CL37 has the potential as it meets the industrial criteria due to its thermostable characteristic to give optimum performance at wide temperature range and high temperature.

3.5 Effect of Metal Ions, Organic Solvents, and Detergent Components on Xylanase Activity

A total of 13 metal ions, six organic solvents, and nine detergent constituents were used to test the effect of these additives on xylanase activity of strain CL37. According to the results summarized in Table 2, xylanase of strain CL37 demonstrated high stability in the presence of Na^+, K^+, and Ni^{2+} (83.9–91.4%; Table 2) and increment in activity in the presence of most of the metal ions, including Al^{3+}, Ca^{2+}, Co^{2+}, Cu^+, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, and Zn^{2+} (112.3–175.1%; Table 2). Only Mg^{2+} showed inhibitory effect to xylanase of strain CL37. As a halotolerant xylanase, the activity remained high or enhanced in the presence of Na^+, K^+, Ca^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+} and Zn^{2+} in which the findings are expected as these metal ions are present in natural habitat of strain CL37 (mangrove sediment) [63]. Unlike xylanase of strain CL37, Co^{2+} and Zn^{2+} caused reduction in xylanase activity of many halophilic bacteria, including *Bacillus* sp. SN5 [64], *Gracilibacillus* sp. TSCPVG [43], *Glaciecola mesophila* KMM 241 [51], *Thermoanaerobacterium saccharolyticum* NTOU1 [65], *Streptomyces viridochromogenes* M11 [45], and *Flammeovirga pacifica* WPAGA1 [52]. The uniqueness and flexibility of xylanase of strain CL37 stimulated by various metal ions could be discerned in this case.

In relation to the effect of organic solvents, reduction in xylanase activity was observed in the presence of ethanol, isopropanol, and dimethyl sulfoxide (36.9–52.3%; Table 2). Apart from that, xylanase of strain CL37 remained stable in the presence of chloroform and methanol (71.1–71.7%; Table 2), while enhancement effect was observed in the presence of acetone (127%; Table 2). The increase in xylanase activity in the presence of acetone was also displayed by the xylanase of halophilic *Halomonas meridiana* APCMST-KS4 [66]. Xylanases that retained good stability and activity in the presence of organic solvents are beneficial to industry in terms of economical process [67, 68]. Therefore, the search of organic solvent-stable xylanase is one of the important aspects.

Table 2 Effect of various additives on xylanase activity of *Microbulbifer* sp. strain CL37 after pre-incubation at 37 °C

Additives	Concentration	Relative activity (%)
Control	–	100
Metal ions		
Mn^{2+}	5 mM	175.1 ± 1.7
Ca^{2+}	5 mM	165.8 ± 5.2
Co^{2+}	5 mM	155.9 ± 4.9
Cu^+	5 mM	126.2 ± 2.0
Cu^{2+}	5 mM	123.6 ± 6.1
Fe^{2+}	5 mM	117.5 ± 10.3
Al^{3+}	5 mM	115.6 ± 14.4
Zn^{2+}	5 mM	114.5 ± 2.6
Fe^{3+}	5 mM	112.3 ± 2.4
Na^+	5 mM	91.4 ± 0.8
K^+	5 mM	88.1 ± 3.7
Ni^{2+}	5 mM	83.9 ± 10.2
Mg^{2+}	5 mM	59.7 ± 7.2
Organic solvents		
Acetone	25% (*v*/*v*)	127.0 ± 12.7
Chloroform	25% (*v*/*v*)	71.7 ± 6.9
Methanol	25% (*v*/*v*)	71.1 ± 8.0
Ethanol	25% (*v*/*v*)	52.3 ± 3.7
Dimethyl sulfoxide	25% (*v*/*v*)	50.1 ± 12.2
Isopropanol	25% (*v*/*v*)	36.9 ± 10.8
Detergent components		
Tween 20	1% (*v*/*v*)	98.2 ± 4.6
Sodium deoxycholate	1% (w/v)	83.4 ± 9.4
Triton X-100	1% (*v*/*v*)	83.3 ± 2.5
Tween 60	1% (*v*/*v*)	77.3 ± 4.2
Tween 40	1% (*v*/*v*)	71.6 ± 9.5
Tween 80	1% (*v*/*v*)	71.6 ± 3.3
Sodium carbonate	1% (w/v)	40.3 ± 4.0
Hydrogen peroxide	1% (*v*/*v*)	36.9 ± 5.0
Sodium dodecyl sulphate	1% (w/v)	3.8 ± 1.2

Similar to organic solvent, detergent constituents such as surfactants have substantial roles in industries. Hydrolytic enzymes could combine with surfactants to be utilized in paper de-inking process, lignocellulosic biomass bioconversion, detergents, and personal care products formulations [69–71]. Xylanase of strain CL37 that have considerably good stability in the presence of various surfactants such as sodium deoxycholate, Triton X-100, Tween 20, Tween 40, Tween 60, and Tween 80 (71.6–98.2% relative activity; Table 2) could be suitable for related applications. The retention of xylanase activity by the non-ionic surfactants such as

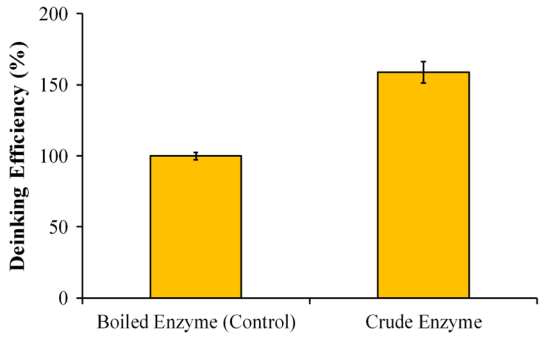

Fig. 5 De-inking efficiency of crude enzyme from *Microbulbifer* sp. strain CL37. De-inking efficiency in boiled enzyme was taken as 100%. Mean values (n = 3) were reported and standard deviations are indicated as error bars

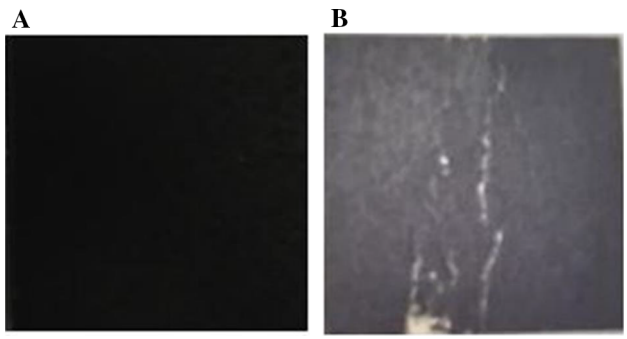

Fig. 6 Paper de-inking activity of crude xylanase from *Microbulbifer* sp. strain CL37. A, treated with boiled enzyme; B, treated with crude enzyme

Triton X-100, Tween 20, and Tween 80 were also seen in the case of *Gracilibacillu*s sp. TSCPVG [43].

3.6 Applicability of Xylanase in Paper De-inking Process

The ability of xylanase of strain CL37 in paper de-inking process was evaluated on a laser-inked paper. This non-impact ink of laser-inked paper is very difficult to remove as compared to newspaper [20, 72]. Xylanase of strain CL37 was able to detach the adsorbed ink particle from the surface of paper, with 159% de-inking efficiency when compared to the control (Fig. 5). This result is relatively similar to the laser-inked paper de-inking efficiency of crude xylanase from *Bacillus pumilus* (166%) [37] and even better than commercial enzymes produced by *Aspergillus niger* [20]. Furthermore, the crude xylanase of strain CL37 treated paper showed a significant colour change on the surface as compared to the control (Fig. 6), and further demonstrated that xylanase of strain CL37 was able to decolourize the laser-inked paper.

4 Conclusion

Collectively, to the extent of our knowledge, this is the first study to report the characterization of xylanase from the genus *Microbulbifer*. The characteristics of halotolerant xylanase of strain CL37 with excellent thermostability, optimal activity in neutral pH, high stability in the presence of various organic solvents, and detergents with considerably high paper de-inking efficiency make this strain a promising candidate to be applied in paper de-inking process as a sustainable solution in paper and pulp industry.

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Author's Contributions Ming Hui Mah performed the experiment, analysed the data, and drafted the manuscript. Ming Quan Lam designed the experiment, assisted in performing the experiment, drafted the manuscript, and provided the expertise. Lili Tokiman, Mohd Farizal Kamaroddin, Zaharah Ibrahim, and Shafinaz Shahir provided the expertise. Chun Shiong Chong conceived the presented idea, designed the experiment, and provided the expertise. All authors read, edited, and approved the final manuscript.

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Availability of Data and Material The 16S rRNA gene (1475 bp) of *Microbulbifer* sp. strain CL37 is available at GenBank under accession of MK256319.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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