**Characterization of a halotolerant lipase from the lactic acid
bacteria *Lactobacillus plantarum* useful in food fermentations**

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ABSTRACT

Lactobacillus plantarum is a lactic acid bacteria found in fermented foods on which high salt concentrations are present. So far, no salt-tolerant lipase has been described from lactic acid bacteria. In the present study, the gene *lp_3562* encoding a putative esterase/lipase was cloned and expressed in *Escherichia coli* BL21 (DE3) and the overproduced Lp_3562 protein has been biochemically characterized. Lp_3562 is a lipase active on tributyrin and also on other long chain substrates. Although the highest activity was observed at pH 7, the enzyme showed activity at pH 5.0-8.0. Lp_3562 exhibited optimal lipase activity at 40 °C and, showing more than 40% of maximal activity at refrigeration temperature (5 °C). Conversely, Lp_3562 presented good thermostability exhibiting more than 80% of its maximal activity after 20h incubation at 45 °C. More interestingly, Lp_3562 was active under NaCl concentrations higher than 20%. The biochemical properties exhibited by Lp_3562 make this halotolerant lipase attractive for its use in food fermentations.

Keywords

Esterase; Tributyrin; Halophilic; Lactic acid bacteria; Food fermentation

1. Introduction

Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly due to the versatility of their applied properties (Jaeger, & Eggert, 2002). Microbial lipases are highly diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial applications. Lipases are widely used in the food industry to modify flavour by synthesis of esters of short chain fatty acids and alcohols which are known flavour and fragrance compounds (McSweeney, & Sousa, 2000). Microbial lipases are widely applied in different food elaboration processes, including dairy and meat products, to produce a selective hydrolysis of fat triglycerides to release free fatty acids which can serve as either flavours or flavours precursors (Flores, & Toldrá, 2011).

Lipases performed an essential role during fermentation and ripening steps of food elaboration. In most of these processes a salting step is present (Bautista-Gallego, Rantsiou, Garrido-Fernández, Cocolin, & Arroyo-López, 2013; Johnson, Kapoor, McMahon, & McCoy, 2009). Lipases used during the elaboration of these fermented food products need to be active on the presence of high salt concentration, therefore halophilic or halotolerant enzymes are recommended. To date, limited research has been undertaken regarding halophilic lipases and their applications in industrial processes (Schreck, & Grunden, 2014).

Halophilic proteins are isolated from halophilic microorganisms, which require salt for growth. However, there are also numerous proteins that tolerate high salt concentrations, which are not isolated from halophilic sources. These proteins are defined as halotolerants (Graciano, & Merlino, 2014). Halotolerant enzymes remain active over a broad range of NaCl concentration, without any specific salt dependence. According to

salt tolerance, most food fermentations are carried out by non-halophilic microorganisms; however, specific proteins produced by these fermenting bacteria could be adapted to remain active under high salt concentrations. To date, some research on lipases from halophilic bacteria has been carried out (Schreck, & Grunden, 2014), but attention to halotolerant lipases from non-halophilic microorganisms should be also done.

Lactic acid bacteria are extensively used for the fermentation of food products. Among lactic acid bacteria, *Lactobacillus plantarum* is an industrially important species which can be found in numerous fermented foods, such as sourdoughs, olives, fermented vegetables, fermented sausages, and cheese (Kleerebezem et al, 2003) on which a high salt concentration is present. *L. plantarum* is a good source of esterase enzymes since lipolytic and esterase activity have been previously described in *L. plantarum* strains (Otherholm, Witter, & Ordal, 1967; Otrherholm, Ordal, & Witter, 1968; Oterholm, Witter, & Ordal, 1972; Silva Lopes et al., 1999). Moreover, lipase proteins have been partially purified, although their halotolerance have not been assayed (Andersen, Østdal, & Blom, 1995; Gobetti, Fox, Smacchi, Stepaniak, & Damiani, 1996; Gobetti, Fox, & Stepaniak, 1997; Silva Lopes et al., 2002). So far, hydrolytic proteins from *L. plantarum* possessing lipase activity have not been molecularly identified neither recombinantly produced.

Since *L. plantarum* is a non-halophilic bacterium, which would often find NaCl concentrations up to 20% in food fermentations, it seems likely that it would be a possible source of halotolerant enzymes active on high salt concentrations. To date, limited research has been undertaken regarding halophilic lipases and their applications in industrial processes (Schreck, & Grunden, 2014). This study represents an effort to dissect the complex array of lipolytic activities in *L. plantarum* whole cells by cloning, heterologous expression, purification and biochemical characterization of the lipase

Lp_3562 from *L. plantarum*. Lipase Lp_3562 is the first halotolerant lipase described in a lactic acid bacteria commonly used in food fermentations.

2. Materials and methods

2.1. Strains, plasmids, media and materials

L. plantarum WCFS1, kindly provided by M. Kleerebezem (NIZO Food Research, The Netherlands), was grown in MRS medium (Pronadisa, Spain) adjusted to pH 6.5 and incubated at 30 °C. *Escherichia coli* DH10B was used as host strain for all DNA manipulations. *E. coli* BL21 (DE3) was used for heterologous expression in the pURI3-TEV vector (Curiel, de las Rivas, Mancheño, & Muñoz, 2011). *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C and shaking at 200 rpm. When required, ampicillin and chloramphenicol were added to the medium at a concentration of 100 or 20 µg/ml, respectively.

Plasmid DNA was extracted by a High Pure plasmid isolation kit (Roche). PCR product was purified with a QIAquick gel extraction kit (Quiagen). Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). *DpnI* and HS Prime Star DNA polymerase were obtained from TaKaRa. His-tagged protein was purified by a Talon Superflow resin (Clontech).

2.2. Cloning of Lp_3562 lipase

Genomic DNA from *L. plantarum* WCFS1 was extracted. The gene encoding a putative lipase/esterase (*lp_3562*) in *L. plantarum* WCFS1 was amplified by PCR by using

the primers 575 (5'-GGTGAAAACCTGTATTTCCAGGG*Catggagatacgcacactagctttaa*) and 576 (5'-ATCGATAAGCTTAGTTAGCTATTACTA*aatttcagtactattcttgcta*) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *lp_3562* gene sequence are written in lowercase letters). Prime Star HS DNA polymerase (TaKaRa) was used for the PCR amplification. The 837-bp purified PCR product was inserted into the pURI3-TEV vector using a restriction enzyme- and ligation-free cloning strategy (Curiel et al., 2011). The vector produces recombinant proteins having a six-histidine affinity tag in their N-termini. *E. coli* DH10B cells were transformed, recombinant plasmids were isolated, and those containing the correct insert were identified by size, verified by DNA sequencing, and then transformed into *E. coli* BL21 (DE3) cells for expression.

2.3. Expression and purification of *Lp_3562* esterase

E. coli BL21(DE3) harbouring pGro7 (TaKaRa), a vector overexpressing GroES/GroEL chaperones, was transformed with the recombinant plasmid pURI3-TEV-3562. *E. coli* was grown in LB medium containing 100 µg/ml ampicillin, 20 µg/ml chloramphenicol, and 2 mg/ml arabinose, on a rotary shaker (200 rpm) at 37 °C until an optical density (OD) at 600 nm of 0.4 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM and protein induction was continued at 22 °C during 18 h.

The induced cells were harvested by centrifugation (8,000 g, 15 min, 4 °C), resuspended in phosphate buffer (50 mM, pH 7) containing 300 mM NaCl and disrupted by French Press passages (three times at 1,100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47,000 g for 30 min at 4 °C, and the supernatant was

filtered through a 0.2 μ m pore-size filter and then loaded onto a Talon Superflow resin (Clontech) equilibrated in phosphate buffer (50 mM, pH 7) containing 300 mM NaCl and 10 mM imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted using phosphate buffer (50 mM, pH 7) containing 300 mM NaCl and 150 mM imidazole. The purity of the enzyme was determined by SDS-PAGE in Tris-glycine buffer. Fractions containing the His6-tagged protein were pooled, dialyzed against phosphate buffer (50 mM, pH 7) containing 300 mM NaCl, and analyzed for esterase activity.

2.4. Enzyme assay

Esterase/lipase activity was determined by a spectrophotometric method using *p*-nitrophenyl acetate (Sigma-Aldrich) as the substrate. The rate of hydrolysis of *p*-nitrophenyl acetate for 10 min at 37 °C was measured in 50 mM sodium phosphate buffer pH 7.0 at 348 nm in a spectrophotometer (UVmini-1240 Shimadzu). The reaction was stopped by chilling on ice.

In order to carry out the reaction (1 ml), a stock solution of 25 mM of *p*-nitrophenyl acetate was prepared in acetonitrile/isopropanol (1:4, v/v) (Glogauer et al., 2011) and mixed with 50 mM sodium phosphate buffer (pH 7.0) to obtain a 1 mM substrate final concentration. Control reactions containing no enzyme were utilized to account for any spontaneous hydrolysis of the substrates tested. Enzyme assays were performed in triplicate.

2.5. Substrate specificity

To investigate the substrate specificity of Lp_3562, activity was determined using different *p*-nitrophenyl esters of various chain lengths (Sigma-Aldrich): *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl caprylate (C8), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14) and *p*-nitrophenyl palmitate (C16) as substrates as described previously (Brod, Vernal, Bertoldo, Terenzi, & Maissonave Arisi, 2010). A stock solution of each *p*-nitrophenyl ester was prepared in acetonitrile/isopropanol (1/4 v/v). Substrates were emulsified to a final concentration of 0.5 mM in 50 mM sodium phosphate buffer, pH 7.0, containing 1.1 mg/ml Arabic gum and 4.4 mg/ml Triton X-100. Reaction mix consisted of 990 μ l of emulsified substrate and 10 μ l of enzyme solution (50 μ g protein). Reactions were carried out at 37 °C in a spectrophotometer (UVmini-1240 Shimadzu) as described above.

The enzymatic substrate profile of purified protein was determined by using an ester library described previously (Liu et al., 2001). *p*-Nitrophenol was used as pH indicators to monitor ester hydrolysis colorimetrically. The screening was performed in a 96-well Flat Bottom plate (Sarstedt) where each well contains a different substrate (1 mM) in acetonitrile (1%). A buffer/indicator solution containing 0.44 mM of *p*-nitrophenol in 1 mM sodium phosphate buffer pH 7.2 was used as pH indicator. Esterase solution 10 μ g (20 μ L in 1 mM sodium phosphate buffer pH 7.2) was added to each well and reactions were followed by measuring the decrease in absorbance at 410 nm for 2 h at 37 °C in a Synergy HT BioTek microplate spectrophotometer. Blanks without enzyme were carried out for each substrate and data were collected in triplicate and the average activities were quantified. Results are shown as means $\pm$ standard deviations.

2.6. Determination of optimum pH, temperature and thermostability

Effect of pH was studied by assaying lipase activity in a range of pH values from 3.0 to 9.0. Buffers (100 mM) used were acetic acid-sodium acetate buffer (pH 3-5), sodium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8) and glycine-NaOH buffer (pH 9). In order to investigate temperature effect, reactions were performed in 50 mM sodium phosphate buffer (pH 7.0) at 5, 20, 30, 37, 40, 45, 55, and 65 °C. For temperature stability measurements, the lipase was incubated in 50 mM sodium phosphate buffer (pH 7.0) at 20, 30, 37, 45, 55 and 65 °C for 5 min, 15 min, 30 min, and 1, 2, 4, 6, and 20 h. After incubation, the residual activity was measured as described above.

2.7. Effects of metal ions and additives on Lp_3562 lipase activity

To test the effect of metals and ions on the activity of the lipase, the enzyme was incubated in the presence of different additives at a final concentration of 1 mM during 5 min at room temperature. Then, the substrate was added and the reaction was incubated at 37 °C. The metal ions and additives analyzed were MgCl₂, KCl, MnCl₂, CuCl₂, NiCl₂, CaCl₂, HgCl₂, ZnCl₂, DEPC, Cysteine, SDS, DTT, Triton-X-100, Urea, Tween 80, Tween 20, EDTA, DMSO, PMSF and β-mercaptoethanol. In all cases, each analysis was performed in triplicate.

2.8. Effect of salt on lipase activity

The effect of NaCl was determined by adding NaCl at concentrations ranging from 0 to 25% (w/v). Reaction mixtures were prepared as described for the temperature optimum experiments, but different volumes of 25% NaCl were added, and the volume of the buffer was adjusted accordingly to maintain the final reaction volume. Reactions

mixtures were pre-incubated for 5 min at room temperature before the enzyme was added.
After the reaction, the absorbance was measured.

3. Results

3.1. Identification of *Lp_3562* esterase from *L. plantarum*

When the published sequence of *L. plantarum* WCFS1 genome was analyzed, numerous ORFs encoding putative esterases/lipases were found. Apart from other structural features, salt-adapted enzymes revealed some structural traits, such as a large number of acidic residues (Asp and Glu), which correlates with a low *pI* value (4.3-6.8) (Graziano, & Merlino, 2014). Among the putative esterases encoded by the *L. plantarum* WCFS1 genome, *lp_3562* encodes a protein that exhibits these structural features. When the amino acid composition of *Lp_3562* was analyzed, the ratio of acidic/basic residues was found to be high (1.52) resulting in a low theoretical *pI* (5.17) for the enzyme. These characteristics may suggest that *Lp_3562* could be a halotolerant esterase.

3.2. Production of *Lp_3562* esterase

The *lp_3562* gene was cloned into the pURI3-TEV expression vector by a ligation-free cloning strategy described previously (Curiel et al., 2011). The vector incorporates the DNA sequence encoding hexa-histidine to create a His-tagged fusion enzyme for further purification step. The integrity of the construct was confirmed by DNA sequencing. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) and expressed under the control of an inducible IPTG promoter. Cell extracts were used to

detect the presence of overproduced proteins. SDS-PAGE analysis showed that there was one major band of protein, approximately 30 kDa, present as inclusion bodies in the insoluble fraction (data not shown). In order to get Lp_3562 soluble, plasmid pGro7, producing GroES/GroEL chaperones, was used. When pURI3-TEV-3562 and pGro7 plasmids were used simultaneously, Lp_3562 appeared in the intracellular soluble fraction of the cells (Fig.1). The molecular weight of the overproduced protein was consistent with the theoretical one expected for Lp_3562 (30 kDa). Since the cloning strategy would yield a His-tagged protein variant, Lp_3562 could be purified on an immobilized metal affinity chromatography (IMAC) resin. Elution of the recombinant protein from the resin was performed with phosphate buffer (50 mM, pH 7) containing 150 mM imidazole. SDS-PAGE of fractions from the eluate revealed that the protein was essentially homogeneous (Fig. 1). Routinely about 25 mg of purified protein from 1-liter culture was obtained.

3.3. Biochemical characterization of Lp_3562 esterase

Lp_3562 protein purified by the affinity resin was biochemically characterized. Substrate specificity was determined using *p*-nitrophenyl-linked esters of various acyl chain lengths (C2 to C16) at 20 °C (Fig. 2A). The enzyme was active on all the substrates assayed, exhibiting maximal activity with chain lengths of C2 and C14. The hydrolysis of long chain esters suggested lipase activity of Lp_3562. The results obtained by using the ester library revealed that Lp_3562 was unable to hydrolyze most of the substrates assayed (Fig. 2B). However, from the esters assayed, tributyrin was efficiently hydrolyzed. In addition, phenyl acetate was hydrolyzed to a lesser extend, and methyl hydroxyacetate, butyl acetate, chlorogenic acid, propyl acetate, ethyl acetate and trilaurin were minimally

hydrolyzed by Lp_3562. The hydrolysis of tributyrin and trilaurin confirmed lipase activity of Lp_3562.

The influence of pH, in the range 3.0 to 9.0, on esterase activity was studied (Fig. 3A). Although the enzyme showed activity at pH from 3.0 to 9.0, the highest activity was observed at pH 7. The influence of temperature on enzymatic activity was determined (Fig. 3B). Maximal activity was observed at 40 °C, although Lp_3562 showed more than 40% activity at 5 °C. Fig. 3C shows that Lp_3562 was fairly stable under temperatures up to 45 °C. The enzyme retained up to 80% activity after incubation during 20 h at 45 °C. In addition, Lp_3562 showed 40% of its maximal activity after 10 h incubation at 55 or 65 °C.

The effects of several ions and additives on Lp_3562 activity are shown in Table 1. Compared to the enzyme incubated in 50 mM phosphate buffer pH 7, the enzymatic activity was only significantly increased by MnCl₂, and, by contrast, HgCl₂, CuCl₂, and SDS greatly inhibited Lp_3562 activity.

3.4. Effect of NaCl on Lp_3562 activity

The influence of NaCl, a salt commonly added in food fermentations, on Lp_3562 activity was studied. As expected for a halotolerant enzyme, NaCl did not inhibit Lp_3562 activity. Moreover, the presence of 2-15% NaCl greatly increased enzymatic activity (Fig. 4). At all the NaCl concentrations assayed (up to 25%), NaCl increases Lp_3562 activity

4. Discussion

L. plantarum is a flexible and versatile bacterial species that is found in a variety of environmental niches, including some dairy, meat, and many vegetable or plant fermentations. The genome sequence of *L. plantarum* WCFS1 was published in 2003 (Kleerebezem et al., 2003) and more than twenty putative esterase or lipase genes were annotated on the basis of similarity searches. Although an operational distinction is made between esterases, which preferentially break the ester bonds of shorter chain acyl substrates at least partly soluble in water, and lipases, which display maximal activity toward water-insoluble long-chain triglycerides, there is no fundamental biochemical difference (Arpingy, & Jaeger, 1999; Bornscheuer, 2002; Kim, Jung, Choi, Ryu, Oh, & Lee, 2004).

Similarly, it is not possible to predict the behaviour of the proteins encoded by these *L. plantarum* ORFs under high salt conditions. However, some common features present in halophilic proteins from halophilic microorganisms have been described. When the overall distributions of residues in 15 pairs of halophilic (or halotolerant) and non-halophilic protein molecules was compared, the results obtained confirmed that halophilic proteins are characterized by higher frequency of acidic over basic residues, lower frequency of large nonpolar residues Val, Leu, Ile and Phe and higher frequency of Ser and Thr. It seems that halophilic protein present low hydrophobicity, over-representation of acidic residues, particularly Asp, a low content of large hydrophobic residues and Lys. These data correlated with the low *pI* values associated with halophilic proteins, which range from 4.3 to 6.8 (Graziano, & Merlino, 2014). Among the putative esterases/lipases encoded by the *L. plantarum* WCFS1 genome, *lp_3562* encodes a protein that exhibits some of these structural features. Thus, when the amino acid composition of *Lp_3562* was analyzed the acidic/basic amino acid ratio was found to be 1.52, and a high frequency of Ser and Thr residues was found. In other *L. plantarum* putative esterase/lipases, such as

Lp_1760 or Lp_2923 proteins, the ratio is significantly smaller (1.00 or 1.01). In addition, the *pI* of Lp_3562 was low (5.17). These characteristics may suggest that Lp_3562 could be a halotolerant lipase. However, Lp_3562 is not the only esterase or putative esterase from *L. plantarum* which presented the common features found in halophilic proteins, feruloyl esterase Lp_0796, carboxylesterase Lp_0973 or arylesterase Lp_1002 also exhibited these features. However, the halotolerance of these esterases has not been studied (Alvarez et al., 2014; Brod et al., 2010; Esteban-Torres, Reverón, Mancheño, de las Rivas, & Muñoz, 2013; Esteban-Torres, Barcenilla, Mancheño, de las Rivas, & Muñoz, 2014).

Lp_3562 is annotated as a putative esterase/lipase, since esterases and lipases could not be distinguished by sequence comparison (Kim et al., 2004), the activity of Lp_3562 against *p*-nitrophenyl esters possessing different acyl chain lengths was assayed. Lp_3562 showed activity on all the acyl esters assayed, exhibiting significant activity on *p*-nitrophenyl myristate (C14) (Figure 2A), confirming that Lp_3562 is a true lipase. As far as we know, Lp_3562 is the first enzyme possessing lipase activity among all the esterases that have been recombinantly produced and biochemically characterized from *L. plantarum*.

Lipase activity of Lp_3562 was confirmed when an ester library was assayed. Lp_3562 showed highest activity on tributyrin although activity on trilaurin was also observed. Tributyrin is a true fat and the simplest triglyceride occurring in natural fats and oils. Triglycerides are composed of glycerol and three long chain fatty acids. Tributyrin is a common constituent of lipase testing media as it is easily dispersed in water (Samad et al., 1989). Tributyrin esterases from *L. plantarum* have been isolated and partially purified previously (Andersen et al., 1995; Gobbetti et al., 1996; Gobbetti et al., 1997; Silva Lopes et al., 2002) however they have not been genetically identified so far. From these lipases,

tributylin esterases from *L. plantarum* 2739 strain (Gobbetti et al., 1996, 1997) exhibited a similar optimal pH (pH 7), whereas *L. plantarum* MF32 lipase showed a more alkaline optimum pH (pH 9.3) (Andersen et al., 1995). Food products fermented by lactic acid bacteria are characterized by a low pH. For example, a final pH around 5.5 could be found in meat fermentations, at this pH value Lp_3562 still exhibited half of its maximal activity.

Regarding temperature, maximal activity was observed at 40 °C, being similar to the optimal temperature exhibited by the *L. plantarum* lipases reported previously (35 °C) (Andersen et al., 1995; Gobbetti et al., 1996; Gobbetti et al., 1997; Silva Lópes et al., 2002). Interestingly from a technological point of view, Lp_3562 showed more than 40% activity at refrigeration temperatures (5 °C). This is an important property in food fermentations: e.g., during the ripening of cheese or fermented meat products, temperatures can be lower than 15 °C; moreover lipase activity could be important even at storage temperatures (Papon, & Talon, 1988). More interestingly, esterase Lp_3562 not only showed high activity levels at low temperature, but also exhibited high thermostability. Lp_3562 retained up to 80% activity after incubation during 20 h at 45 °C. In addition, Lp_3562 showed 40% of its maximal activity after 10 h incubation at 55 or 65 °C.

Regarding to the effects of several ions and additives, Lp_3562 activity was greatly increased by Mn^{2+} ions, and, by contrast, greatly inhibited by Hg^{2+} , Cu^{2+} ions and SDS (Table 1). The extracellular lipase produced by *L. plantarum* DSMZ 12028 was also greatly activated by Mn^{2+} ions (Silva Lopes et al., 2002) which do not affect significantly the activity of tributyrin esterase from *L. plantarum* 2739 (Gobbetti et al., 1997). This tributyrin esterase, similarly to Lp_3562, was greatly inhibited by Hg^{2+} ions.

Once the lipase activity of Lp_3562 was confirmed and characterized, its halotolerance was verified. *L. plantarum* strains are active in fermented foods, such as milk or meat fermentations, on which high salt concentrations are present (Ammor, & Mayo, 2007; Bautista-Gallego et al., 2013; Johnson et al., 2009). For example, salt is used in the manufacture and aging of good-quality cheese. During cheese-making, one of the final steps is the addition of salts. In dry salted cheese like cheddar, the salt is added directly to the curds just before hopping and pressing; some cheeses have salt rubbed on the outer side of the cheese. Finally, in other types of cheeses, the salt is added by submersing the cheese in brine (up to 20%) for an appropriate period of time (Johnson et al., 2009).

From the *L. plantarum* lipases partially purified, salt-tolerance was only studied on a lipase from *L. plantarum* MF32 which was slightly affected by salt concentrations up to 5% NaCl (Anderson et al., 1995). However, the results obtained in this study confirmed that lipase Lp_3562 is a true halotolerant protein since NaCl concentrations as high as 25% did not affect lipase activity. Moreover, NaCl concentrations up to 20% increased Lp_3562 activity. Therefore, under most cheese ripening conditions, Lp_3562 activity was more than two-fold increased. It seems that Lp_3562 under these harsh conditions maintains its fold and avoids aggregation. It has been suggested that to prevent protein aggregation and electrostatic interaction hindrance, halophilic enzymes have acidic amino acid residues and a reduced number of hydrophobic residues on exteriors of proteins (Schreck, & Grunden, 2014). When structural data from Lp_3562 were available, they could be used to confirm this suggestion. So far, no halotolerant lipase has been described from a lactic acid bacteria used in food fermentations, and in fact, only a few halophilic lipases and esterases have been discovered and characterized (Schreck, & Grunden, 2014). To date, limited research has been undertaken regarding halophilic lipases or esterase and their applications in industrial processes. Since the progressive improvement of

biotechnological applications demands novel enzymes halotolerant enzymes are currently gaining attraction.

In summary, in the present study the halotolerant lipase namely Lp_3562 from *L. plantarum*, an industrially important species of lactic acid bacteria which can be found in numerous fermented foods, was purified and biochemically characterized. Lp_3562 is a halotolerant lipase that is stable under 25% NaCl concentration, exhibited maximum activity at 40 °C, and more importantly, retains more than 40% activity at refrigeration temperatures. In addition, esterase Lp_3562 exhibited good activity at the acidic pH found in lactic food fermentations. Based on the findings reported in this study, it appears that Lp_3562 will retain adequate activity under food fermentation conditions. Further studies are needed to know the effect of the simultaneous presence of salt, acidic pH, and low temperature on Lp_3562 lipase activity. In spite that the demand for salt-tolerant enzymes in current manufacturing or related processes is limited, the biochemical characteristics showed by Lp_3562 make this halotolerant lipase especially attractive for its industrial use in food fermentations.

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Figure legends

Fig. 1. SDS-PAGE analysis of the purification of Lp_3562 lipase from *L. plantarum* WCFS1. Analysis by SDS-PAGE of soluble cell extracts of IPTG-induced *E. coli* BL21(DE3) (pGro7) (pURI3-TEV) (1) or *E. coli* BL21(DE3) (pGro7) (pURI3-TEV-3562) (2), flowthrough (3), or fractions eluted after His affinity resin (4-8). The arrow indicated

the overproduced and purified protein. Molecular mass markers are located at the left (SDS-PAGE Standards, Bio-Rad).

Fig. 2. Substrate profile of Lp_3562 lipase against chromogenic substrates (*p*-nitrophenyl esters) with different acyl chain lengths (C2, acetate; C4, butyrate; C8, caprylate; C12, laurate; C14, myristate; C16, palmitate) (A) or toward a general ester library (B). The error bars represent the standard deviation estimated from the three independent assays. The observed maximum activity was defined as 100%.

Fig. 3. Biochemical properties of Lp_3562 lipase. (A) pH-activity profile of Lp_3562. (B) Temperature-activity profile of Lp_3562. (C) Thermal stability profile for Lp_3562 after preincubation at 20 °C (circle), 30 °C (square), 37 °C (diamond), 45 °C (triangle up), 55 °C (triangle down), and 65 °C (star) in phosphate buffer (50 mM, pH 7), at indicated times, aliquots were withdrawn, and analyzed as described in the Materials and Methods section. The experiments were done in triplicate. The mean value and the standard error are showed. The percentage of residual activity was calculated by comparing with unincubated enzyme.

Fig. 4. Activity of Lp_3562 in the presence of NaCl. The activity of the enzyme in the absence of the compound was defined as 100%. The experiments were done in triplicate. The mean value and the standard errors are shown.

Table 1

Table 1. Effect of additives on Lp_3562 lipase activity

Additions (1 mM)	Relative activity (%)
Control	100
KCl	98
HgCl ₂	27
CaCl ₂	96
MgCl ₂	115
ZnCl ₂	64
CuCl ₂	27
NiCl ₂	132
MnCl ₂	152
Tween 20	94
Tween 80	103
Triton-X-100	114
SDS	11
Urea	111
DMSO	113
Cysteine	121
DTT	95
β-mercaptoethanol	111
EDTA	121
PMSF	92
DEPC	84

Figure 1

Figure 1

Figure 2

Figure 2

A

B

Figure 3

Figure 3

Figure 4

Figure 4