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INDUCED FORMATION AND CHARACTERIZATION OF A LIPASE PRODUCED FROM *Lactobacillus plantarum* AND ITS USES FOR HYDROLYSIS OF SOME OILS AND FATS.

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ABSTRACT

Lactobacillus plantarum was used for lipase (Glycerol-ester hydrolase EC 3.1.1.3) production and the results revealed that: Maximum enzyme productivity was obtained in the third day of incubation, with glucose, peptone and olive oil as carbon, nitrogen and lipid sources, which induced the enzyme biosynthesis. pH 6.0 and 30 and 35 °C were found as the best environmental conditions used for maximum enzyme production. pH 7.5, 30 and 35 °C were found as the pH and temperature optima for enzyme activity. The enzyme protein showed highest or completely stable in the pH range between 5.5 to 9.5. Also, the enzyme showed completely stable up to 75 °C. This means that this enzyme is a thermostable one and alkaline in its nature. K^{+}, Ca^{+2} and Mg^{+2} ions activated the enzyme protein which stimulated its activity, but, Hg^{+2} and Cu^{+2} inactivated it. Bile salts reduced the enzyme activity especially in their higher concentration. The enzyme was successfully to hydrolyzed all tested substrates, but with different extent. The enzyme hydrolyzed vegetable oil with highest degree of hydrolysis than other lipids. The enzyme also successfully to hydrolyzing olive oil, coconut oil, palm oil and butter oil with highest degree of hydrolysis reached 66, 68.5, 26.4 and 58.9 %, respectively. This means that the preparations of this enzyme are commercially available in food processing especially, cheese ripening such as cheddar cheese.

Keywords: *Lactobacillus plantarum*, Lipase, production, properties, substrate specificity, lipid, bile salts, degree of hydrolysis.

INTRODUCTION

Enzyme catalysis is now widely used commercially. One aria of application of enzymes is in the biotechnology processes, which has a potential interest for the food industry, clinical and industrial chemistry. Such esters can be produced by an alcohol /acid mix reaction using either inorganic acid or enzymes as catalysts. One of these enzymes are lipases which used for the production of short – chain terpenyl esters. Also lipase is used for the chemical redesign of milk – fat for improving physical, chemical and / or nutrional properties. The enzyme is of great industrial importance, for it is widely used in pharmaceutical preparation, synthetic detergents, oil, fat hydrolysis, as well as dairy products such as development of characteristic flavor of blue cheese and other industries (Garcia – lepe *et al.*, 1997; Balcao & Malcata, 1998; Laboret & Perraud, 1999; Shady & Ramadan, 2001 and Rabie 2002)`

Lipases or glucerol ester hydrolases (EC, 3.1.1.3) are enzymes hydrolyzing oils and fats (glycerol esters) into di- mono-glycerids and fatty acids. Thus, these enzymes are of great industrial importances. Microorganisms abundant in nature, from economic and industrial standpoints, are preferable to animal and plants lipases as enzyme sources. *Aspergillus orizae, A. niger, Bacillus subtilis, B. licheniforms, B. stearothermophilus* are the important lipase producers as well as good

producers, since their enzymes are produced extracellularly. Very few studies have been done on the lipolytic and esterolytic system of lactic acid bacteria. Lipases have been characterized in crude preparations of lactic acid bacteria and several food – related microorganisms such as *Lactobacillus plantarum, Bacillus acidocaldarius, B. stearothermophilus, penicillium roqueforti* and others, which, these enzymes have only been partially purified (Andersen *et al.*, 1995; Gobbetti *et al.*, 1996b; Abd-Alla, 1999; Koops *et al.*, 1999; Palmo *et al.*, 2000; Shady & Ramadan, 2001 and Rabie, 2002).

Therefore, this study was aimed to give some light on the production and characterization of lipase produced from *Lactobacillus plantarum* and its uses for some oils and fats hydrolysis.

MATERIALS AND METHODS

Microorganism and fermentation media:

The strain of *Lactobacillus plantarum* was kindly obtained from Dairy Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt. The culture was maintained on MRS agar slants and incubated for 16 hrs at 30 °C (Gobbetti *et al.*, 1996 b). Stock cultures were stored at 4 °C. MRS broth was also used for *Lactobacillus plantarum* lipase production, the pH of the production media was adjusted to 6.0. 50 ml portions of the medium in 250 ml Erlenmyer flasks were each inoculated with 1 ml cell suspension and incubated at 30°C. The cells were harvested at 10000 g for 10 min. at 4 °C after incubation for different time ranging from 1 to 7 day at one day interval. The cultures were centrifuged and the clear supernatant solution was used as a source of enzyme used for enzyme assay.

Lipase assay:

Lipase activity was determined by the method of Oi *et al.*, (1969) with some modification by shady (1993). The reaction mixture consisted of 5.0 ml of 5% olive oil emulsion in 9% gum acacia in distilled water, 5 ml of 0.2 M Tris HCl buffer (pH 7.5), 2 ml 0.2M $CaCl_2$, 1 ml enzyme solution and 2.0 ml glass distilled water. The tubes containing reaction mixtures were incubated in a reciprocating shaking water bath at 40 °C for 10 min. The total amount of liberated free fatty acids (FFA) was titrated against 0.01N NaoH. The boiled enzyme solution in the reaction mixture was used as control. One unit of lipase was defined as the amount of enzyme which liberated one μ moles of FFA under the assay conditions.

Properties of lipase:

1- pH and temperature optima:

The effect of pH on enzyme activity was examined at 40 °C in the pH range 3.0 to10.0, using a universal buffers composed of boric acid (57 mM), citric acid (33 mM), NaH_2PO_4 (33 mM), NaOH (1 M) and varying amounts of 0.1 HCl. The temperature dependence of the lipolytic activity was determined in 50 mM phosphate buffer, pH 7.5, in the range 5 to 55 °C (Gobbetti *et al.*, 1996 b).

2- pH stability:

For studying pH-stability of this enzyme, 10 units from enzyme solutions were adjusted to pH levels from 3.0 to 10.0 using citrate,

phosphate, Tris–HCl and glycine buffers. These enyme solutions were incubated at the optimum temperature for 120 min, then, the residual activity of these preincubated samples was measured (Shady and Rammadan, 2001).

3- Heat stability:

Portions (40 units) of the enzyme solution was dissolved in 50 mM phosphate buffer, pH 7.5, were heated in glass tubes at different temperatures (20 to 90 °C) for 30 min (Gobbetti *et al.*, 1996 b). After heating, samples were cooled on ice and the remaining activity was measured at 30 °C using the procedure described previously (Oi *et al.*, 1969).

Effect of metal activators and inhibitors:

Mixture containing 40 units of the enzyme solution, 1 ml 0.002 of metal activators or inhibitors and phosphate buffer, pH 7.5 were incubated for 30 min at 35 °C. Reaction was initiated by adding 500 µL of the olive oil – gum acacia emulsion, activity was measured after incubation for 4 hrs at 35 °C (Gobbetti *et al.*, 1996 b).

Measurement of substrate specificity:

The substrate specificity of lipase was measured by replacing olive oil with other triglycerides or natural fats and oils according to the assay method as described above (wang *et al.*, 1995).

Hydrolysis of oils:

The degree of hydrolysis (DH%) of oils used was determined according to the method described by linfield *et al.*, (1984). Which DH% was calculated as: $DH\% = (AV - AVO/SV - SVO) \times 100$

Whereas: AV= acid value after hydrolysis.
AVO= acid value in the substrate fat.
SV= saponification value in the substrate fat.

Acid value and saponification number were determined according to the A.O.A.C. (1975) of official method of analysis.

RESULTS AND DISCUSSION

Fermentation conditions for maximization of enzyme production:

1- Effect of time-course:

The results shown in Fig. (1) indicate that the enzyme was synthesized at the beginning of the fermentation time, which lipase activity appeared clearly during the first day of incubation, and reached its maximum in the third day of *Lactobacillus plantarum* growth. Thereafter, decreased gradually. This results could be attributed to the over production of acidity and the effect of liberated constituents resulted in a marked decrease in the pH and the effect of eluted enzymes which degradiated the lipase molecule (Abd-Alla, 1999). These results are in agreement with those obtained by El-Sawah *et al.*, (1995), Pabia *et al.*, (1995) and Shady & Ramadan (2001).

2- Effect of carbon sources:

Different carbon sources (1% concentration) were investigated for lipase production by *Lactobacillus plantarum*. The results in Table (1) show that the enzyme was synthesized constitutively, which produced with all carbon

sources with much more amount. But, some of these carbon sources were induced the enzyme productivity. Glucose and maltose were served as superior carbon sources for lipase production, which gave highest amount of enzyme. Other carbon sources recorded reduction on enzyme activity. Generally, these results indicated that this enzyme was constitutive and inducible one. These findings are similar to those reported by El-Sawah *et al.*, (1995), Hauka *et al.*, (1999) and Shady & Ramdan (2001).

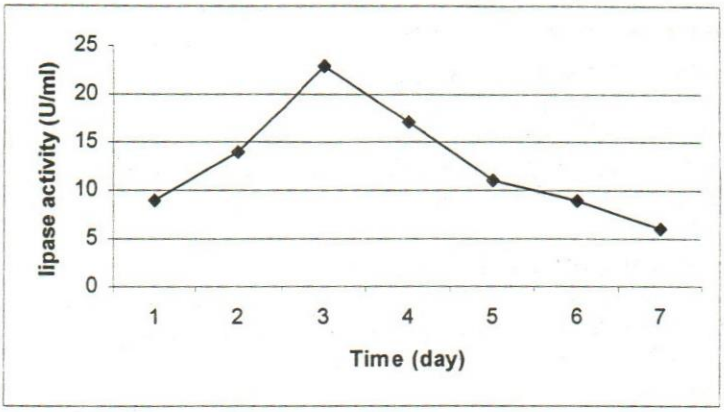

Fig. (1): Time-course of lipase production by *Lactobacillus plantarum* grown at 30° C and pH 6.0.

Table (1): Effect of different carbon sources on enzyme biosynthesis.

Carbon source	Enzyme activity (U/ml)
Glucose	27.9
Fructose	15.6
Galactose	22.3
Maltose	26.8
Glycerol	25.5
Sucrose	23.0
Molasses (sugar cane)	14.5
Starch	17.0
Pectin	12.0

3- Effect of nitrogen sources:

The results in Table (2) also revealed that all nitrogen sources induced the enzyme biosynthesis. Peptone exhibited maximum enzyme production (29.5) as compared with other nitrogen sources. This is may be due to the high content of amino acids in peptone stimulated the enzyme synthesis. Several investigators reported that, the addition of peptone to the fermentation media gave maximum lipase yield by many microorganisms (Abd-Alla, 1999; Hauka *et al.*, 1999 and Shady & Ramdan, 2001). Also, casein and ammonium sulphat allowed a high production of enzyme. Other nitrogen sources reduced the enzyme activity compared with peptone. These results also indicated that the biosynthesis of this enzyme is dependent upon the source of nitrogen and not the nitrogen content of the fermentation media (Hauka *et al.*, 1999 and Shady & Ramdan, 2001).

Table (2): Effect of different nitrogen sources on enzyme production.

N. source	Enzyme activity (U/ml)
Control	27.9
Casein	28.3
Peptone	29.5
Urea	25.0
Gelatin	19.9
Sodium nitrate	24.8
Ammonium sulphate	22.0
Potassium nitrate	15.6
Ammonium chloride	18.7

N. sources provided equalization of N. levels in all treatment.
Control= the initial N. sources of the medium.

4- Effect of different oils and fats:

The effect of several kinds of fats and oils and related substances on lipase production by *Lactobacillus plantarum* was studied. From the results (Table, 3) it could be concluded that: lipase from the above bacterium was constitutive, which produced with much more amount without inducers (fat, oil and other lipid substances). At the same time, some of these lipid substances were induced the enzyme biosynthesis with much lowest amount compared with control. This means that generally this enzyme was constitutive in its nature and induced one in some cases. Olive oil, soybean oil and butter oil gave highest productivity. Other substances reduced it's biosynthesis. El-Sawah *et al.* (1995), Hauka *et al.* (1999), Gao *et al.* (2000) and Shady & Ramdan (2001) reported similar results.

Table (3): Effect of different oils and fats on lipase production.

Oils and fats	Lipase activity (U/ml)
Control	29.5
Olive oil	31.6
Soybean oil	30.5
Coconut oil	28.6
Sunflower oil	24.8
Corn oil	29.7
Cotton seed oil	27.9
Stearin	23.7
Olein	18.5
Butter oil	30.3
Tallow fat	21.6

Control= without any lipid substances.
All oils and fats were added in the growth medium at 1% concentration.

5- Effect of pH on enzyme production:

Results illustrated in Fig. (2) show that the bacterium produced relatively high yield of lipase at narrow range of pH 5.0 – 7.0 and reached its maximum at pH 6.0. Above or below this pH level, enzyme activity decreased sharply. This means that, the initial pH of the fermentation medium has a great influence on enzyme production. These results are similar to those reported by Abou-Hamed (1996), Abd-Alla (1999) and Shady &

Ramdan(2001).

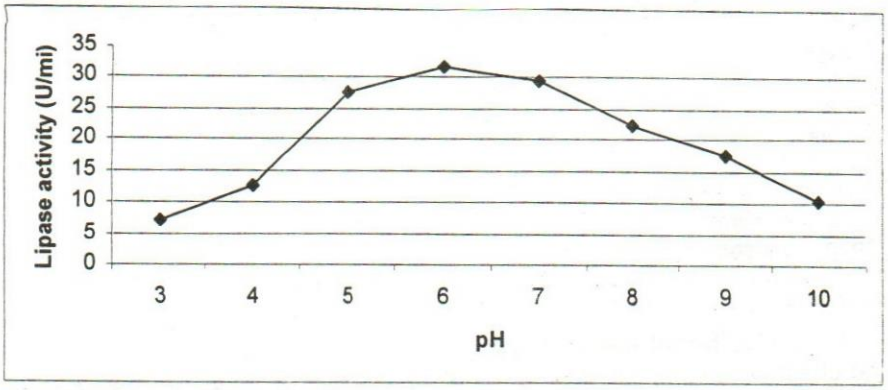

Fig. (2): Effect of initial pH value of the fermentation medium on enzyme production.

6- Effect of incubation temperature:

Data in Fig. (3) revealed that the yield of lipase is profoundly affected by the cultivation temperature. Also, the results indicated that 30 and 35 °C were the optimum fermentation temperature for enzyme production, which gave highest enzyme yield. Enzyme formation was decreased sharply at other temperature degrees. These results are in harmony with those obtained by Hauka *et al.* (1999).

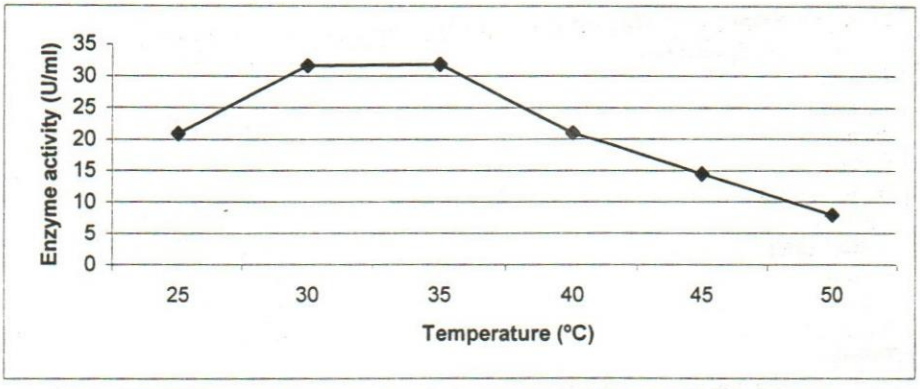

Fig. (3): Effect of incubation temperature on lipase formation.

Enzyme properties:

1-pH and temperature optima:

The pH optimum for the hydrolysis of olive oil (as substrate) was 7.5. Enzyme activity was decline greatly at other pH levels, which show about 45% from its maximum activity at pH 5.0 (Fig. 4). The retention of about 45% of maximal activity at pH 5.0 indicates the potential significance of this enzyme in the ripening of some dairy products especially cheese (Lee & Lee, 1990 and Gao *et al.*, 2000). Wang *et al.* (1995) and Goa *et al.* (2000) found

that the optimum pH of lipase activity is 9.5. Similar observations were reported by Khalid & Marth (1990), Lee & Lee (1990) and Gao *et al.* (2000).

The optimum temperature of enzyme activity was 30 and 35 °C. 43 and 47% from its maximum activity was determined at 15 °C and 50 °C, respectively (Fig. 5). Like several crude preparations of lipase from lactic acid bacteria especially *Lactobacillus plantarum* showed maximum enzyme activity at 35 °C (Andersen *et al.*, 1995; Gobbetti *et al.*, 1996 a & b).

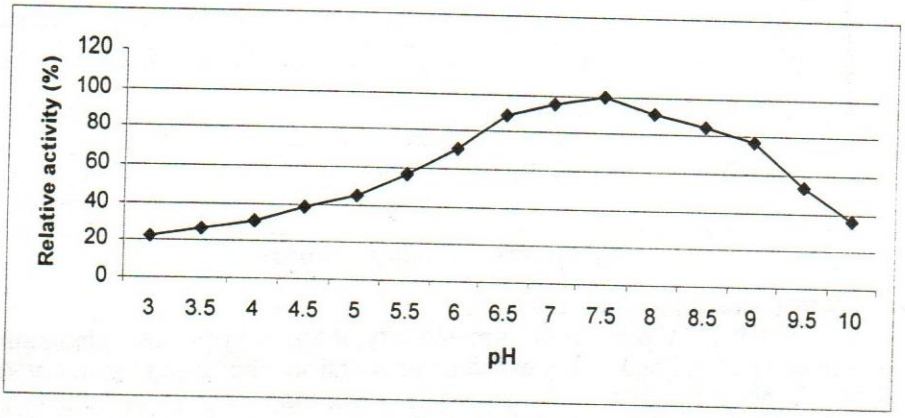

Fig. (4): pH optimum of lipase activity.

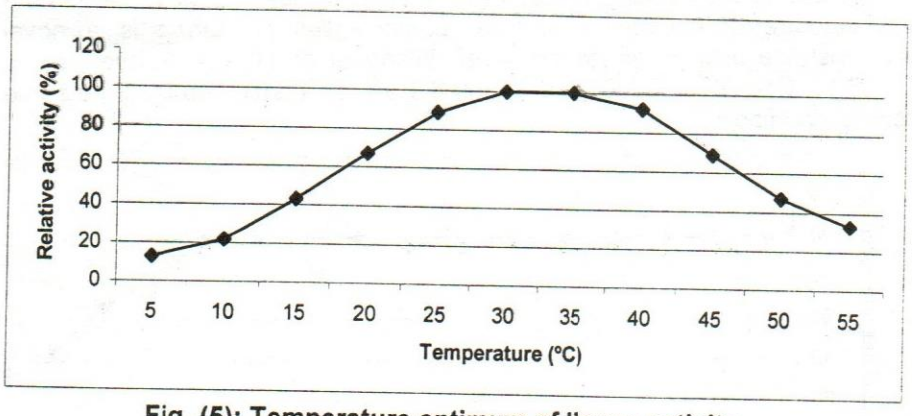

Fig. (5): Temperature optimum of lipase activity.

2- pH – stability:

The results illustrated in Fig. (6) showed that, enzyme activity was highly stable at the pH range between 5.5 to 9.5, which completely stable at pH 8.5 to 9.5, then it was gradually inactivated at pH values above or below 9.5 and 8.5, respectively. The enzyme retained 90 – 95% of the original activity after it was incubated at a pH from 5.5 to 9.5 for 120 min. in the absence of substrate. The results also indicated that the enzyme is stable at an alkaline pH. Thus it has potential application in fat hydrolysis processes.

These results are similar to these reported by Sugihara *et al.* (1995) and Wang *et al.* (1995).

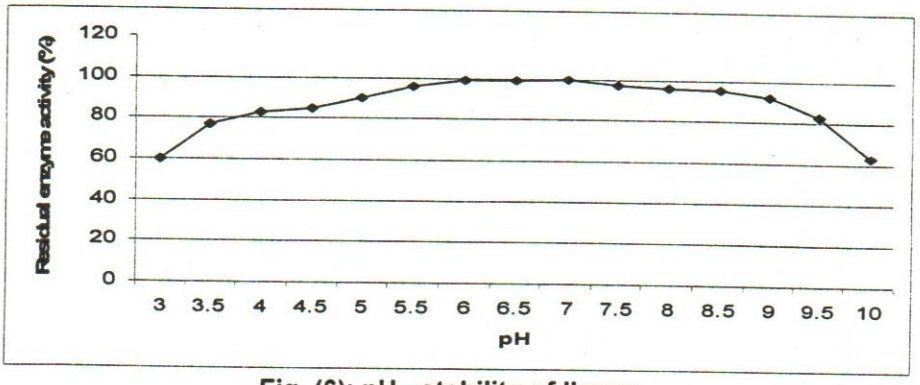

Fig. (6): pH - stability of lipase.

3- Thermal stability of the enzyme:

The thermal stability or heat–stability of this enzyme was remarkably high (Fig. 7). It retained 100% of the original activity after being heated at 75 °C for 30 min in the absence of substrate. Also, the results revealed that no loss of activity up to 75 °C. At 80 °C only 20% of its maximum activity was lost. This means that the enzyme is resistant both to the pasteurization of cheese – milk and to the cooking process (54 – 56 °C for few minutes) used in the manufacture of cheese. Therefore, *Lactobacillus plantarum* is a novel thermostable alkaline lipase producer. Wang *et al.* (1995); Gobbetti *et al.* (1996 b); Gao *et al.* (2000) and Shady & Ramdan (2001) reported the same results and findings.

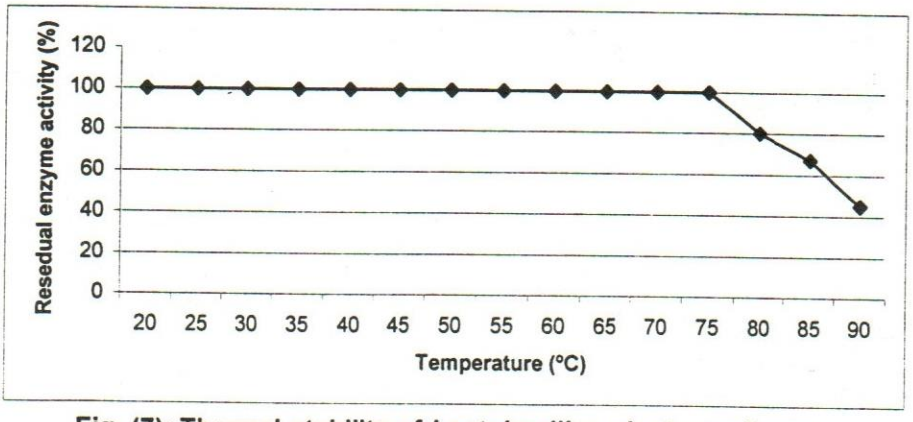

Fig. (7): Thermal-stability of *Lactobacillus plantarum* lipase.

4- Effect of metal ions on lipase activity:

The effect of various inorganic salts and metal chelators on the lipase activity is presented in Table (4). Among the tested compounds K^{+}, Ca^{+2} and Mg^{+2} were activated the enzyme protein, whereas Hg^{+2}, Cu^{+2} and Sn^{+2} inactivate it. The effect of Ca^{+2} may be due to the fact that it promotes the

alignment of the enzyme on the substrate molecule and neutralizes the fatty acids liberated from the substrate. Inhibition by Hg^{+2} may be due to their binding to an active thiol group of the enzyme. Similar observations were reported by Chopra *et al.* (1982); Gobbetti *et al.* (1996 a & b); Gao *et al.* (2000) and Rabie (2002).

Table (4): Effect of metal ions on lipase activity.

Metal ion	Relative activity (%)
None	100
K^{+}	133
Li^{+}	110
Ca^{+2}	157
Co^{+2}	88
Cu^{+2}	47
Hg^{+2}	27
Mg^{+2}	148
Mn^{+2}	119
Sn^{+2}	43
Fe^{+2}	80
EDTA	91

40 units of enzyme solution was assayed after mixing with 1 ml 0.002 M metal ion (chloride) solution at pH and temperature optima.
EDTA= ethylendiaminotetra acitic acid.

5- Effect of bile salts on enzyme activity:

Effect of some bile salts on enzyme activity is shown in Table (5). The enzyme was inhibited remarkably in the presence of these bile salts, and their effect was pronounced by increasing their concentrations. Bile salts can stimulate pancreatic lipases, but inhibited most of microbial lipases especially at higher concentration (Gao *et al.*, 2000).

Table (5): Effect of bile salts on lipase activity.

Bile salts	Final concentration	Relative activity (%)
Control	---	100
Sodium cholate	0.25	77
	0.50	49
Sodium deoxycholate	0.25	57
	0.50	38
Sodium taurocholate	0.25	72
	0.50	64

Bile salts concentrations= 0.25 or 0.50 % w/v.

6- Substrate specificity:

The data in Table (6) shows the hydrolyzing ability of this enzyme on a wide variety of lipid substrates. The enzyme hydrolyzed various lipids although the extent different each other. This may be due to its affinity and specificity of the enzyme – substrate complex. In general, the enzyme showed preference of vegetable oils to animal fats, and triglycerides. Several studies on lactic acid bacteria reported low lipase activity on triglycerides containing long chain fatty acids (Khalid and Marth, 1990). The lipase from *Lactobacillus plantarum* 2739 was most active on tributyrin but also moderately active on tripalmitin. Thus, the high activity on esters of butyric

acid and the large specificity indicated that *Lactobacillus plantarum* lipase may contribute to the flavor of ripening cheese. Similar observations were reported by Gobbetti *et al.* (1996b); Gao *et al.* (2000) and Rabie (2002).

Table (6): Substrate specificity of lipase.

Substrate	Relative rate of hydrolysis (%)
Olive oil	100
Corn oil	105
Cotton seed oil	95
Palm oil	110
Coconut oil	115
Soy bean oil	98
Sunflower oil	92
Wheat germ oil	89
Butter oil	97
Tallow fat	77
Triolein	75
Tristearin	87
Tripalmitin	86

Enzymatic oils hydrolysis:

The lipolysis of important oils (olive oil, coconut oil, butter oil and palm oil) in the field industrial and pharmaceutically processes with *Lactobacillus plantarum* lipase was carried out at the optimum temperature (35 °C), 5, 10, 15 and 20 units of enzyme were used for each lipolysis at 1, 2, 4 and 8 hours. The degree of hydrolysis (DH) was varied between different oils (Table, 7). The results also showed that higher degree of hydrolysis was observed with all oils, but with different extent. DH% reached 66.0, 68.5, 62.4 and 58.9 for olive oil, coconut oil, palm oil and butter oil, respectively after 8 hours of hydrolysis with 20 units of enzyme. In food processing, hydrolysis of butter oil and coconut oil can lead to the release of short – chain and medium chain saturated fatty acids and the development of hydrolytic rancidity and ketonic rancidity. Thus, these enzyme preparations are commercially available in food processing especially cheese ripening. Gao *et al.* (2000) and Rabie (2002) reported similar observations.

Table (7): Degree of hydrolysis (%) of some oils.

Enzyme conc. (Units)	Reaction time (hr.)	Degree of hydrolysis (%)			
		Olive oil	Coconut oil	Palm oil	Butter oil
5	1	6.6	7.5	5.6	4.5
	2	10.8	10.9	10.5	8.5
	4	18.6	19.5	15.5	13.6
	8	29.9	23.7	21.5	14.8
10	1	9.5	18.9	12.3	9.2
	2	18.4	24.6	21.8	17.8
	4	32.0	31.3	31.6	28.6
	8	45.6	43.6	33.9	36.8
15	1	20.2	23.0	18.9	14.7
	2	27.0	35.5	38.4	27.0
	4	45.4	46.0	45.8	38.5
	8	54.4	57.2	50.6	49.6
20	1	29.5	33.6	27.0	22.4
	2	44.9	46.8	47.5	36.9
	4	55.5	50.9	57.9	45.0
	8	66.0	68.6	62.4	58.9

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الحث على تكوين إنزيم الليباز المنتج من اللاكتوباسيلس بلانتريم ودراسة خواصه واستخدامه في تحليل بعض الزيوت والدهون.

وسام الدين إسماعيل على صابر.

قسم الميكروبيولوجي- معهد بحوث الأراضي والمياه والبيئة – مركز البحوث الزراعية – الجيزة

في الآونة الأخيرة اهتم العالم أجمع بعلوم البيوتكنولوجيا ومنها إنتاج الإنزيمات ودراسة خواصها واستخداماتها لأهميتها الكبرى في مجال الصناعات الغذائية والدوائية والصيدلانية وصناعة المنظفات وغيرها ومن أهم هذه الإنزيمات جنبا إلى جنب مع إنزيمات البروتيز والبكتيز والأميليز هي إنزيمات الليباز ولما كانت هناك دراسات قليلة على إنتاج هذا الإنزيم الهام من بكتيريا حمض اللاكتيك التي تعتبر من أهم المجاميع الميكروبية استخداما في الصناعات الغذائية وبخاصة صناعة المنتجات اللبنية ، فقد اتجهت الدراسة في هذا البحث لدراسة العوامل المحثة على إنتاج كميات كبيرة من إنزيم الليباز من بكتيريا اللاكتوباسيلس بلانتريم ودراسة خواص هذا الإنزيم المنتج واستخدامه في تحليل بعض الزيوت والدهون وقد أوضحت الدراسة النتائج التالية:-

١- وصلت أعلى إنتاجية من الإنزيم في اليوم الثالث من التحضين وكان الجلوكوز والببتون وزيت الزيتون هم أحسن مصدر كربون ونيتروجين ومصدر للليبيدات حثاً على الإنتاج العالى للإنزيم .
٢- كانت درجة pH ٦ و٣٠ و ٣٥ م° هى أفضل الظروف البيئية لإنتاج الإنزيم .
٣- كانت درجة pH 7.5 ، ٣٠ ، ٣٥ م° هى أنسب درجات الـ pH والحرارة لنشاط الإنزيم.
٤- أظهر الإنزيم ثبات عالي وشبه كامل تجاه درجات الـ pH وبخاصة في المدى من٥,٥ – ٩,٥ ، كما أظهر ثبات كامل تجاه درجات الحرارة حتى ٧٥م° ثم تناقص نشاط الإنزيم لحدوث تلفيات بدرجات الحرارة العالية لبروتين الإنزيم وهذا يعنى أن الإنزيم من النوع القلوي المتحمل لدرجات الحرارة العالية.
٥- كانت لبعض المعادن مثل البوتاسيوم والكالسيوم والمغنسيوم تأثير حثي عالي لنشاط الإنزيم في حين كان للزئبقيك والكوبلت تأثير مثبط للإنزيم.
٦- إضافة أملاح الصفراء لمخلوط التفاعل الإنزيمي أدى إلى تثبيط الإنزيم وبخاصة عند استخدام تركيزات عالية منها.
٧- نجح الإنزيم في تحليل كل مواد التفاعل المستخدمة ولكن بدرجات متفاوتة ويدل ذلك على مدى دقة التخصص بين الإنزيم ومادة التفاعل.
٨- استطاع الإنزيم تحليل كل من زيت الزيتون وزيت جوز الهند وزيت النخيل والسمن البلدي بدرجه عالية من التحلل مما يعنى أنه نجح في تحليل الليبيدات المحتوية على أحماض دهنية قصيرة ومتوسطه السلسلة وهذا يعنى أن مستحضرات هذا الإنزيم هامة من الناحية التجارية وبخاصة في الصناعات الغذائية وعلى وجه الخصوص في تسوية الجبن.