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Research Article

MICROBIAL LIPASES: PRODUCTION OF EXTRACELLULAR LIPASE ENZYME BY ALCALIGENES VISCOSUS (DOGE-1) STRAIN

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ABSTRACT : Industrially important extracellular lipase enzyme production was explored by utilizing microbial strain isolated from dairy effluents. Alcaligenes viscosus DOGE-1 strain isolated from dairy waste waters proved to produce extracellular lipase. Various growth factors were attempted to maximize the lipase production by this strain. Growth factors like NH_4PO_4, Peptone, Urea coupled with peptone, KH_2PO_4, Olive oil and pH were found to be favored the maximum lipase production. This microbial strain was found to have a high lipolytic activity.

Keywords: Extracellular lipase enzyme, A.viscosus DOGE -1 strain, growth factors

INTRODUCTION

Lipolytic enzymes are currently attracting significant attention because of their biotechnological potential. Most of the lipases used in industry are microbial enzymes, of both fungal and bacterial origin (Arpigny and Jaeger, 1999) Lipases are an important group of enzymes both physiologically and commercially as the use of lipases for a variety of biotechnological applications is rapidly and steadily increasing (Jaeger and Eggert, 2002). In general, lipases have promising applications in organic chemical processing, detergent formulation, synthesis of biosurfactants, the agrochemical industry, paper manufacture, nutrition, cosmetics and pharmaceutical processing (Sharma et al., 2002). Many companies market digestive enzymes prepared from plant and fungal lipases. Doctor's Holistic Market manufactures Chiro-Zyme, the digestive plant enzymes formula containing lipase from *Aspergills niger* and *Rhizopus oryzae*. Novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agrochemicals, and flavour compounds. Some of the industrially important chemicals manufactured from fats and oils by chemical processes could be produced by lipases with greater rapidity and better specificity under mild conditions. The chemo-, regio- and enantiospecific behavior of these enzymes has caused tremendous interest among scientists and industrialists (Saxena et al., 2003). Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. Microbial lipases are widely diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial applications. The commercial use of lipases is a billion-dollar business that comprises a wide variety of different applications (Jaeger et al., 1999). Lipases are valued biocatalysts because they act under mild conditions, are highly stable in organic solvents, show broad substrate specificity, and usually show high regio- and/or stereo selectivity in catalysis. Lipases are part of the family of hydrolases that act on carboxylic ester bonds. The physiologic role of lipases is to hydrolyse triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. In addition to their natural function of hydrolyzing carboxylic ester bonds, lipases can catalyse esterification, interesterification, and transesterification reactions in nonaqueous media.

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This versatility makes lipases the enzymes of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries (Houde et al., 2004). An important feature often overlooked is the high lipolytic activity of Alcaligenes species. Hence this study was carried out to explore the lipolytic activity of Alcalignes viscosus (DOGE-1) strain isolated from dairy industry waste waters.

MATERIALS AND METHODS

Isolation of Alcaligenes viscosus (DOGE-1) strain was done by enrichment on oil-grease from dairy effluents as sole carbon source. The nutrient media consists of nutrient agar, carbon source (1% w/v oli-grease), mineral media and trace elements stock solution (1% v/v). Mineral media has the following composition (gl^{-1}): NH_4NO_3 (4.0), KH_2PO_4 (0.53), Na_2HPO_4 (2.0), K2SO4 (0.1), MgSO4. 7 H_2O (0.10). Trace element stock solution has the following composition (gl-1): $CaCl_2$. 2 H_2O (1.5), NaCl (0.01), KI (0.83), $CaCl_2$. 6 H_2O (0.04). CuSO4. $5H_20$ (0.0125), $MnSO_4$. $4H_2O$(0.22), $ZnCl_2$ (0.28).

Pre-sterilized nutrient media was poured in 2 sets of petri dishes and allowed to solidify. The nutrient agar media was supplemented with cheese whey to allow luxuriant growth of this strain. A 5 ml of waste water sample was used for isolating of this microbe using loop dilution method and petri plates were incubated for 24 hrs at 37^0C. After incubation period, pure cultures were obtained by repeated streaking plate method. Stock cultures were prepared on agar slants containing 1% whey and isolates were identified using biochemical methods.

Physical & Bio-chemical characteristics of Alcaligenes viscosus (DOGE-1) strain :

A.viscosus (DOGE-1) strain is a gram-negative, aerobic, non-motile, asporogenous, rod-shaped bacteria isolated from dairy effluents. On nutrient agar it has produced dirty white colonies with no acid or gas production. It showed positive result to oxidase test and showed negative result in litmus milk and starch hydrolysis tests. Highly lipolytic in nature.

Lipolytic activity of A.visocsus (DOGE-1) strain:

In order to confirm the lipolytic activity of this strain, test was conducted in the following way. 0.5% of 10 ml propionate was taken in 20 ml test tube and loopful of culture mass was added into the solution. Development of intensively red colored solution confirmed lipolytic activity of this microbe.

Lipase enzyme assay:

Lipase enzyme assay was performed as described below:

The lipase activity was assayed calorimetrically by a method developed by Kwon et al. (1986). Culture filtrate (1 mL) was shaken with 2.5 mL of olive oil (70% oleate residues) emulsion (1:1 v/v) and 20 µL of 0.02 M CaCl2 in a water bath shaker at an agitation rate of 200 rpm. The emulsion was prepared by mixing together an equal volume of olive oil and 50 mM phosphate buffer with a magnetic stirrer for 10 minutes. The reaction mixture was shaken for 30 min at 70 °C. The enzyme reaction in the emulsion system was stopped by adding 6N HCL (1 mL) and isooctane (5 mL), followed by mixing using a vortex mixer for 30 s. The upper isooctane layer (4 mL) containing the fatty acid was transferred to a test tube for analysis. Copper reagent (1 mL) was added and again mixed with a vortex mixer for 30 s. The absorbance of the upper layer was read at 716 nm. Lipase activity was measured by measuring the amount of free fatty acid released from the standard curves of free fatty acids. One unit of lipase activity was defined as the amount of enzyme releasing 1 µmole of fatty acid per minute.

Effect of pH on Lipase Activity and Stability:

Enzymatic activity was measured at various pH values (pH 4–12) for 30 min at 70 °C. The substrate (olive oil) was prepared in 50 mM of various buffers; acetate buffer (pH 4.0–6.0), potassium phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 8.0–9.0), glycine-NaOH buffer (pH 9.0–11.0), and Na2HPO3/NaOH buffer (pH 11.0–12.0). The effect of pH on lipase stability was determined by incubating aliquots of lipase in buffers of different pH values for 30 min at 70 °C. Residual activity was assayed using method described by Kwon et al. (1986).

Effect of Temperature on Lipase Activity and Stability:

The optimum temperature for lipase activity was determined over the range of 0–100°C. The substrate was equilibrated at the required temperature for 5 min before the addition of enzyme. The effect of temperature on lipase stability was determined by incubating aliquots of lipase in Glycine-NaOH pH 9.0 at 0°C to 100°C. The residual activity was measured at the optimum temperature (70°C).

Effect of Metal Ions on Lipase Activity:

The effect of metal ions on lipase activity was studied according to the method described by Fairolniza Mohd Shariff et al. (2011). The activity of the purified L2 lipase was studied following incubation with 1 mM and 10 mM concentrations of various metal chlorides (Na+, K+, Mg2+, Ca2+, Fe2+, Mn2+, Zn2+, and Cu2+) in 50 mM Glycine-NaOH buffer pH 9.0 at 65 °C for 30 min. The residual activity was determined at 70 °C using olive oil emulsion and expressed as a percentage of activity without the metal chlorides. Effect of parameters like inorganic source, organic source, additives, carbon source, phosphates on lipase production by A.viscosus was studied using different concentrations and lipase was estimated using assay as described above.

RESULTS AND DISCUSSIONS

Table 1.1 shows the effect of inorganic source on lipase production. It was observed that $NH_4(H_2PO_4)$ acted as an effective inorganic source as was indicated by the high values of 5.2 Uml^{-1} at 36 hrs and dropped to 1.80 Uml^{-1} at 72 hrs. Next to $NH_4(H_2PO_4)$, urea was found to be an effective inorganic source as 1.6 Uml^{-1} of lipase was recorded at 12 hrs and it was raised to a maximum of 7.4 Uml^{-1} at 36 hrs. Least amount of lipase production was registered with $NaNO_3$ as was evident by low values.

Influence of additives on lipase production was shown in Table 1.2. It was noted that peptone and yeast were found to be the excellent additives as was evident by high enzyme production at a rate of 8.2 Uml^{-1} and 7.6 Uml^{-1} followed by Casein which induced at a rate of 3.6 Uml^{-1} . Least amount of lipase (0.7 Uml^{-1}) was produced when corn steep liquor was used as an additive. Soya bean meal and meat extract could induce lipase production more or less equally.

Table 1.1 Effect of Inorganic source on lipase production

S.No	Source	Concentration* (% w/v)	Lipase Activity (U/ml) at				
			12 hrs	24 hrs	36 hrs	48 hrs	72 hrs
1	NH_4Cl	1.5	0.6±0.09	1.7±0.04	2.4±0.03	1.3±0.06	0.07±0.01
2	$NH_4(H_2PO_4)$	1.5	2.7±0.01	3.6±0.07	5.2±0.11	2.6±0.01	1.8±0.06
3	NH_4NO_3	1.5	1.3±0.03	2.2±0.02	3.2±0.08	2.4±0.08	1.2±0.04
4	$NH_4(SO_4)_2$	1.5	0.9±0.06	2.1±0.01	4.3±0.04	3.1±0.03	1.3±0.07
5	$NaNO_3$	1.5	0.2±0.01	0.9±0.08	2.1±0.06	1.1±0.05	0.6±0.09
6	Urea	1.5	1.6±0.10	2.8±0.05	4.7±0.09	3.1±0.08	1.6±0.02

*Each concentration in triplicate

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Table 1.2 Effect of additives on lipase production

S.No	Source	Concentration* (% w/v)	Lipase Activity (U/ml) at				
			12 hrs	24 hrs	36 hrs	48 hrs	72 hrs
1	Beef extract	2.5	2.8±0.08	3.4±0.04	5.7±0.03	3.3±0.02	2.1±0.08
2	Casein	2.5	3.6±0.07	5.1±0.14	6.4±0.11	4.2±0.14	2.7±0.16
3	Corn steep liquor	2.5	0.7±0.02	1.8±0.08	2.4±0.05	1.1±0.07	0.6±0.03
4	Meat extract	2.5	2.3±0.14	4.1±0.07	6.7±0.20	3.6±0.03	1.8±0.01
5	Peptone	2.5	8.2±0.03	10.7±0.19	12.3±0.17	8.4±0.10	6.8±0.03
6	Soybean meal	2.5	2.1±0.13	3.3±0.04	5.4±0.16	3.0±0.03	1.6±0.09
7	Yeast extract	2.5	7.6±0.01	8.4±0.03	10.2±0.12	6.2±0.25	4.7±0.04

*Each concentration in triplicate

Effect of urea and organic nitrogen on lipase production (Table 1.3) showed that with increasing concentration of urea coupled with organic nitrogen, resulted in increasing lipase production. With a low concentration of (0.5%) urea and yeast extract, 5.4 Uml^{-1} of enzyme was produced and it was increased to 8.3 Uml^{-1} at higher concentration (1.5%). Peptone coupled with urea also showed same trend. But it was interesting to note that at any given concentration, peptone coupled with urea resulted in higher lipase production than yeast coupled with urea. The high lipase production with peptone and yeast (organic nitrogen source) can be attributed to presence of free amino acids in them which serve as readily available nitrogen source.

Table 1.3 Effect of Urea & Organic nitrogen on lipase production

S.No	Source	Concentration* (% w/v)	Lipase Activity (U/ml) at				
			12 hrs	24 hrs	36 hrs	48 hrs	72 hrs
1	Urea	0.5	3.7±0.08	5.6±0.03	10.8±0.15	5.8±0.04	2.7±0.07
2	Urea + Yeast	0.5	5.4±0.11	10.2±0.11	13.7±0.14	11.6±0.17	4.8±0.11
3	Urea + Yeast	1.0	6.3±0.11	11.7±0.10	13.4±0.18	10.2±0.22	5.1±0.12
4	Urea + Yeast	1.5	8.3±0.09	12.1±0.22	16.7±0.16	11.8±0.26	6.6±0.14
5	Urea + Peptone	0.5	4.7±0.07	9.3±0.11	11.8±0.21	7.5±0.15	3.2±0.12
6	Urea + Peptone	1.0	5.4±0.06	10.7±0.29	12.2±0.21	10.6±0.10	4.3±0.16
7	Urea + Peptone	1.5	10.3±0.023	14.2±0.18	19.5±0.19	13.4±0.26	9.8±0.10

*Each concentration in triplicate

Table 1.4 shows the effect of different carbon sources on lipase production. It was clearly indicated that oil-grease from dairy effluents acted as an efficient carbon source for lipase production as was evident by 13.6 Uml^{-1} of enzyme when compared to low enzyme production (3.1 Uml^{-1}) using fructose as a sole carbon source. Sucrose and lactose also acted as good carbon source as was evident by an enzyme production of 11.38 Uml^{-1} and 10.50 Uml^{-1} respectively. Studies with basal media showed poor enzyme production (0.4 Uml^{-1}) due to absence of any carbon source. Studies on effect of phosphate on lipase production (Table 1.5) showed that a 2% KH_2PO_4 at 36 hrs induced maximum lipase production. At this concentration of KH_2PO_4 till 36 hrs an increasing trend in lipase production was observed and after that at 48 and 72 hrs, there was a decreased trend. Here 2% KH_2PO_4 concentration was found to be the optimum and low lipase production at high concentrations (2.5%) may be due to the inhibitory effect of phosphate on the physiology of the tested microbe

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Table 1.4 Effect of Carbon source on lipase production

S.No	Source	Concentration* (% w/v)	Lipase Activity (U/ml) at				
			12 hrs	24 hrs	36 hrs	48 hrs	72 hrs
1	Basal Medium	0.5	0.4±0.03	0.9±0.01	1.4±0.03	0.7±0.02	0.4±0.05
2	Oil-grease*	0.5	13.6±0.18	18.4±0.16	22.7±0.12	16.1±0.18	12.4±0.16
3	Fructose	0.5	3.1±0.01	10.2±0.13	114.5±0.22	5.4±0.06	3.4±0.02
4	Galactose	0.5	4.2±0.06	9.7±0.16	15.3±0.18	10.4±0.25	3.9±0.08
5	Glucose	0.5	5.8±0.21	10.4±0.20	12.1±0.14	9.8±0.21	4.7±0.15
6	Lactose	0.5	10.5±0.26	12.7±0.11	16.2±0.11	10.6±0.11	8.6±0.15
7	Starch	0.5	3.6±0.12	8.7±0.14	11.8±0.16	9.4±0.12	4.1 ±0.17
8	Sucrose	0.5	11.3±0.23	14.8±0.17	20.7±0.19	13.6±0.18	9.3±0.11

*Each concentration in triplicate *Oil-grease extracted from dairy wastes

Table 1.6 shows effect of various salts on lipase production. Highest amount of lipase was recorded with $ZnSO_4$. At a concentration of 0.025%, a 36.6 Uml^{-1} of lipase was produced and it was decreased to 22.3 Uml^{-1} at a concentration of 0.20%. Least amount of lipase production was associated with KCl salt. $FeSO_4$ (0.02%) also could induce appreciable amounts of lipase production (29.6 Uml^{-1}). This study showed higher concentration of salts inhibited lipase production which was in agreement with the studies by Yamane et al. (1990). This may be due to accumulation of high salts around enzyme protein during lyophilization.

Table 1.5 Effect of Phosphate source on lipase production

S.No	Source	Concentration* (% w/v)	Lipase Activity (U/ml) at				
			12 hrs	24 hrs	36 hrs	48 hrs	72 hrs
1	Basal Medium	0.5	5.2±0.16	8.4±0.19	11.3±0.11	7.8±0.10	5.1±0.06
2	KH_2PO_4	0.05	13.2±0.28	18.6±0.15	20.3±0.28	15.1±0.17	10.8±0.10
3	KH_2PO_4	0.10	8.9±0.02	9.2±0.11	11.8±0.17	9.6±0.15	7.4±0.18
4	KH_2PO_4	0.50	13.1±0.23	16.7±0.19	18.4±0.16	12.8±0.12	11.3±0.24
5	KH_2PO_4	1.00	10.3±0.18	12.6±0.15	14.8±0.14	11.7±0.16	9.2±0.16
6	KH_2PO_4	1.50	9.6±0.11	10.2±0.17	13.4±0.12	10.3±0.19	8.1±0.11
7	KH_2PO_4	2.00	15.5±0.14	20.2±0.12	22.1±0.11	16.4±0.13	11.7±0.14
8	KH_2PO_4	2.50	8.7±0.06	9.2±0.14	11.4±0.13	9.2±0.13	6.9±0.12

*Each concentration in triplicate

Table 1.6 Effect of metal ions on lipase production

S.No	Salt	Concentration (% w/v)	Lipase Activity (Uml^{-1})
1	Control		33.0±0.23
2	KCl	0.025	18.6±0.24
		0.20	17.2±0.18
3	$MgCl_2$	0.025	20.8±0.13
		0.20	18.2±0.11
4	$CaCl_2$	0.025	23.7±0.16
		0.20	17.4±0.14
5	$ZnCl_2$	0.025	30.6±0.24
		0.20	22.3±0.16
6	$FeCl_2$	0.025	29.6±0.19
		0.20	19.1±0.24

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Effect of p^H on lipase production was shown in Table 1.7. It was observed that below and above an optimum p^H lipase production was greatly hampered. The high enzyme activity at pH 9 to 10 may be a result of the Ala for the first Gly-residue in the consensus sequence Gly-X-Ser-X-Gly

Effect of inducers on lipase production was showed in Table 1.8 and these studies indicated that olive oil was the suitable inducer as was evident by the high amounts of lipase production (28.4 $Uml^{-1)}$ at 72 hrs. Least amount of lipase was produced using ground nut oil as an inducer. Sunflower oil and coconut oil showed more or less equal amounts of lipase production.

Table 1.7 Effect of P^H on lipase production

S.No	P^H	Lipase Activity (U/ml) at				
		12 hrs	24 hrs	36 hrs	48 hrs	72 hrs
1	7.2	30.4±0.18	34.2±0.29	36.8±0.28	24.6±0.17	20.3±0.12
2	2.8	1.2±0.03	2.4±0.02	3.2±0.10	1.8±0.06	0.8±0.03
3	4.8	3.6±0.11	6.7±0.10	9.2±0.16	2.7±0.12	1.9±0.02
4	6.8	9.8±0.14	12.6±0.22	16.3±0.13	13.2±0.12	7.6±0.18
5	9.8	28.4±0.25	32.6±0.14	38.7±0.19	24.9±0.14	22.5±0.19
6	10.8	3.4±0.01	5.8±0.11	8.1±0.01	3.9±0.16	2.6±0.03
7	12.8	1.9±0.08	2.7±0.07	3.6±0.04	2.5±0.07	1.3±0.07

Table 1.8 Effect of Inducers on lipase production

S.No	Source	Concentration* (% w/v)	Lipase Activity (U/ml) at				
			12 hrs	24 hrs	36 hrs	48 hrs	72 hrs
1	Control	0.5	1.8±0.06	2.2±0.01	2.8±0.01	1.4±0.06	0.9±0.08
2	Castor oil	0.5	3.8±0.22	4.6±0.11	8.9±0.13	6.4±0.22	3.21± 0.16
3	Coconut oil	0.5	5.7±0.16	9.3±0.12	137.±0.16	8.6±0.16	4.9±0.11
4	Gingerly oil	0.5	10.8±0.23	17.4±0.25	23.8±0.19	16.4±0.21	9.7±0.09
5	Groundnut oil	0.5	2.6±0.19	3.8±0.18	5.9±0.16	3.7±0.07	1.8±0.02
6	Olive oil	0.5	14.9±0.31	18.7±0.11	28.4±0.19	18.6±0.16	12.7± 0.24
7	Paraffin oil	0.5	9.1±0.17	11.2±0.07	13.6±0.23	10.8±0.12	8.4±0.11
8	Sunflower oil	0.5	5.9±0.15	8.3±0.04	11.6±0.19	7.7±0.10	4.9±0.16

*Each concentration in triplicate; Control without inducers

CONCLUSIONS

he above study with tested strain showed its high lipolytic activity which is new contribution in the field of enzymology. The study also showed optimum conditions for the maximum production of lipase at industrial scale. Lipases are an extremely versatile group of bacterial extracellular enzymes that are capable of performing a variety of important reactions, thereby presenting a fascinating field for future research.

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