Production of Alkaline Lipase from *Kluyvera* KB4

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ABSTRACT

***Kluyvera** KB4 produced alkaline lipase in a medium containing oil as inducer. Optimum conditions for lipase production were determined in terms of composition of medium, pH, temperature and time of incubation. The highest lipase productivity was obtained from a medium with corn oil as carbon source and peptone as nitrogen source. Oils seem to be an essential carbon source, since in a medium containing sorbitol without addition of oil the lipase production was very low, approximately only 14%. However, higher concentration of oil (2%) suppressed the lipase production. Production of lipase was greatly influenced by pH of the medium and incubation temperature. It was optimally active at pH 7.5 and 37 °C in medium containing peptone and corn oil 1 %, respectively. Under optimum condition (pH 7.5, at 37 °C), the highest lipase activity was obtained after 36 h of incubation.*

Keywords: Alkaline lipase, *Kluyvera* sp., lipase production.

INTRODUCTION

Lipase (EC 3.1.1.3) hydrolyzes ester triglycerides to diglicerides, monoglycerides, fatty acids and glycerol. This enzyme is widely produced by molds (Brockerhoff and Jensen 1974, Ohnishi et al. 1994, Xia et al. 1996) and bacteria such as *Pseudomonas, Achromobacter, Staphyloccoccus,* etc. (Crueger and Crueger 1989, Lin et al. 1996). Alkaline lipase, which active in alkaline conditions, has been extracted from *Bacillus* sp., *Alcaligenes* spp., *Pseudomonas,* etc. (Lesuisse et al. 1993, Kokusho et al. 1982, Kojima et al. 1994, Lin et al. 1996). Industrial applications of alkaline lipase are for fat dispersal to enhance cleaning ability of detergents, for degreasing hides in leather industry and for substitute of pancreatic lipase in digestive medicine (Yamane 1987).

Genera of *Kluyvera* occurred in food, soil, and sewage (Krieg and Holt 1984). This genera is a rod, gram negative, motile, and glucose fermenting. The *Kluyvera* produces α-ketoglutaric acid during glucose fermentation. Production of lipase from this bacterium has not been reported yet.

In the previous paper, we reported the isolation of alkaline lipase producing bacteria from several raw hides. Isolate KB4 was found as the highest producer of the enzyme. In this paper we describe the identification of the bacterium and effects of medium compositions and fermentation conditions for production of alkaline lipase from bacterium KB4.

MATERIALS AND METHODS

Microorganisms

Bacterium strain of KB4 isolated from rawhide (goat skin) (Rumiyati and Indrati 1998) was used through out this study. The morphological and physiological characteristics of the bacterium were studied. It was identified by Api E20 test kit (BioMereux, German). These characteristics were then used for identification by the Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984).

Medium and cultivation.

Cells were grown on the Samad's medium (Samad et al. 1990) containing 0.50 % peptone, 0.05 % $MgSO_4$, 0.10 % KH_2PO_4, 0.10 % $NaNO_3$, 0.50 % sorbitol, 1.00% (v/v) palm oil. Unless otherwise noted, incubation was carried out at 37 °C for 48 h with reciprocal shaking. Effect of various nitrogen sources were tested

using the same medium containing 0.5 and 1.0 % of NH_4Cl, $(NH_4)_2SO_4$, corn steep liquor, malt extract, urea, or yeast extract in place of peptone. Effect of inducers were also tested using the same medium by replacing palm oil with corn oil, cotton seed oil, olive oil, peanut oil, sesame oil, soybean oil, or sunflower seed oil at concentration of 0.5, 1.0, 1.5, and 2.0 %,

Enzyme preparation

Culture medium was centrifuged at 20000 *g* for 15 min at 4 °C. The resulting supernatant was assigned as a crude extracelluler enzyme.

Enzyme assay

Enzyme activity was determined using the Razak et al. (1995) and Kohno et al. (1994) methods with slight modification. Assay mixtures composed of 4 ml olive oil emulsion which was preincubated at 37 °C for 5 min; 40 μl $CaCl_2$. $2H_2O$ 20 mM; and 2 ml of enzyme solution. This reaction mixture was incubated at 37 °C for 30 min in a shaker incubator, and the reaction was terminated with the addition of 6 ml ethanol. The resulting fatty acids were titrated using NaOH 10 mM up to pH 9.0 and phenolphthalein as an indicator. Olive oil emulsion was prepared by mixing polyvinyl alcohol 2 % in phosphate buffer pH 7.5 with olive oil (2 : 1 v/v).

One unit of enzyme activity (U) is defined as the amount of enzyme producing 1 μ mol equivalent of fatty acid per minute under these assay conditions, and was calculated by the method of Xia et al. (1996).

Analytical methods

Protein content was measured with the method of Lowry et al. (1951) which was modified by Peterson (1977) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Morphological and physiological characteristics of isolate KB4 are shown in Table 1. This KB4 was a rod, gram negative, motile, spore forming bacterium, and facultatively anaerobic. From these data it belongs to the family of *Enterobacteriaceae* (Krieg and Holt 1984). Furthermore, KB4 showed catalase positive, produced gas and acid from glucose, indole positive, citrate utilisation at 37 °C positive, VP test negative and lysine carboxylase positive. From these data together with data shown in Table 1, the bacterium KB4 was thought to be *Kluyvera* sp. (Krieg and Holt 1984).

Table 1. **Characteristics of isolate KB4.**

No.	Test	Result
1.	Shape	Rod
2.	Yellow pigment	-
3.	Gram	-
4.	Motility	+
5.	Spore formation	+
6.	Production of N_2 gas	+
7.	Catalase	+
8.	Oxidase	-
9.	β-Galactosidase	+
10.	Arginine dihydolase	+
11.	Lysine decarboxylase	+
12.	Ornithine decarboxylase	+
13.	Urease	+
14.	Tryptophane deaminase	-
15.	Gelatinase	-
16.	Citrate utilisation (at 37 °C)	+
17.	H_2S production	-
18.	Indole production	+
19.	VP test	-
20.	Growth on MacConkey Medium	+
21.	Growth at 37 °C	+
22.	Assimilation of :	
	- Glucose	+
	- Mannitol	+
	- Inositol	+
	- Sorbitol	+
	- Rhamnose	-
	- Sucrose	+
	- Melibiose	-
	- Amygdalin	+
	- Arabinose	+
23.	Fermentation of Glucose	+
24.	Lipase, corn oil	+

Production of lipase from *Kluyvera* KB4 was affected by the presence of nitrogen in the medium. All nitrogen sources used in the experiment could increase the production of the enzyme (Fig. 1). Among them malt extract was the poorest which slightly increased the activity, whereas peptone was the best which increased the activity 2.4 times higher compared to the control (medium with no addition of nitrogen). Figure 1 also shows that the bacterium was able to use both organic and inorganic nitrogen sources for production of lipase.

The concentration of nitrogen sources did not significantly affect the enzyme activity, while at the concentration of 1 % slightly decreased the activity, except for peptone. This indicated that nitrogen was necessary at low concentration (0.5 %), whereas at higher concentration it could suppress the production of lipase enzyme. Several reports indicated that peptone could also enhance microbial lipase production such as *Aspergillus oryzae* (Ohnishi et al. 1994), and *Rhizopus rhizopodiformis* (Samad et al. 1990). While lipase from *Ophiostoma piceae* and *Candida rugosa* were enhanced with ammonium sulphate and urea, respectively (Gao and Breuil 1995, Rao et al. 1993).

Effect of nitrogen sources on the enzyme specific activity was shown in Fig. 1. Both at concentration of 0.5 % and 1.0 %, organic nitrogen sources such as corn steep liquor, malt extract, yeast extract and peptone decreased the specific activity, while inorganic nitrogen sources such as ammonium sulphate, ammonium chloride, and urea increased the specific activity. These indicated that organic nitrogen induced the production of not only lipase but also other extracellular proteins or enzymes, whereas *Kluyvera* KB4 used inorganic nitrogen for production of lipase.

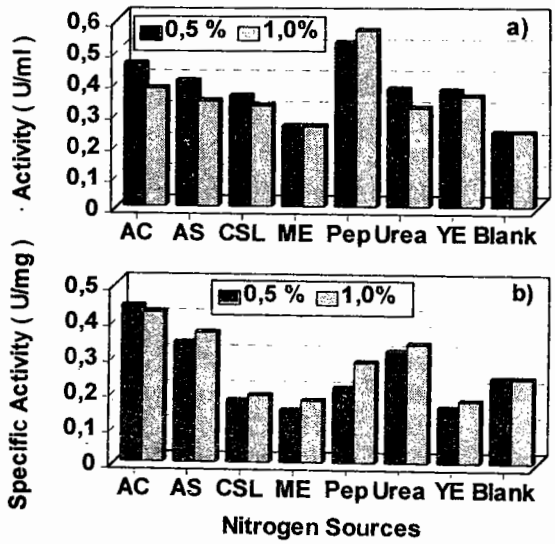

Figure 1. Effect of nitrogen sources on the activity (a) and specific activity (b) of lipase.
AC, ammonium chloride; AS, ammonium sulphate; CSL, corn steep liquor; ME, malt extract; Pep, peptone; YE, yeast extract.

Figure 2 shows effects of several oils as inducer o activity and specific activity of lipase. Generally, th activity and specific activity of lipase increased with th increasing of concentration of inducer reaching maximum at 1.5 %, after that they declined with th increase of concentration. Corn oil was found as the bes inducer, which increased both activity and specifi activity of lipase to the highest at concentration of 1.0% However, at concentration of 1.5 % the result was th same as that of 1.0 %. The production of lipase from thi bacterium was very low when no inducer was added t the medium (14 % from the highest activity). Differen inducers have been reported to enhance microbial lipas production such as oleic acid and soybean oil fo *Aspergillus oryzae* (Ohnishi et al. 1994), and olive oi and peanut oil for *Ophiostoma piceae* (Gao and Breui 1995).

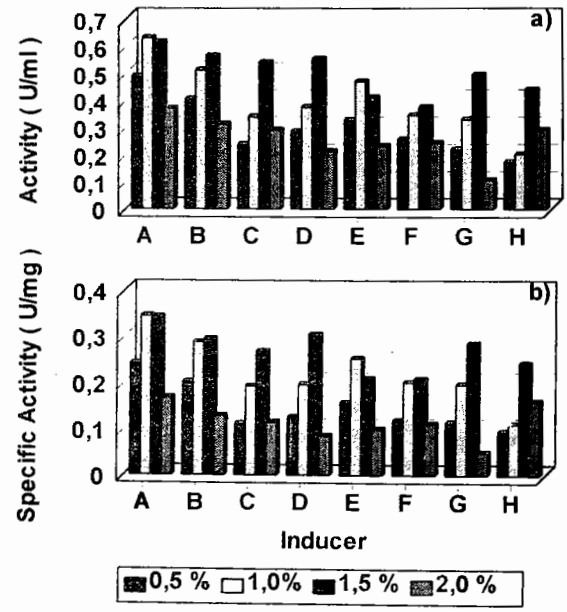

Figure 2. **Effect of inducer on activity (a) and specific activity (b) of lipase.**
A, Corn oil; B, Cotton seed oil; C, Olive oil; D, Palm oil; E, Peanut oil; F, Sesame oil; G, Soybean oil; H Sunflower seed oil.

Effect of medium pH on lipase production showr in Fig. 3. In this experiment the medium containec peptone and corn oil 1.0 %, respectively. As shown ir Fig. 3 production of lipase is affected by medium pH. I increases gradually with the increase of medium pH from 6.5 to 7.5, further increase of medium pH results ir

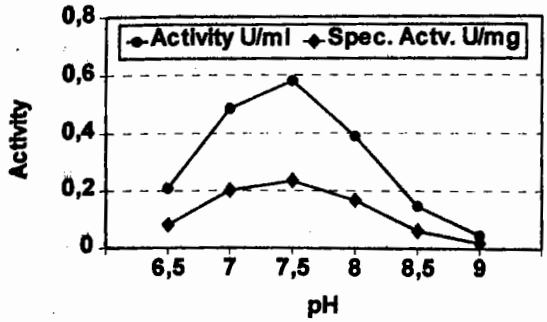

Figure 3. Effect of medium pH on the activity (●) and specific activity (♦) of lipase.

decrease of the enzyme production. At an initial pH of 9.0 the activity and specific activity of lipase were the lowest. These data indicate that even though the enzyme was stable in alkaline condition from pH 8.0 to 11.0 (Rumiyati and Indrati 1998) production of the enzyme was good in the neutral conditions, which was the best at pH 7.5. This might be correlated with the bacterium growth that decreased gradually when the medium pH increased (data not shown). In addition to this optimum pH, pH for production of the enzyme was the same as pH medium for isolation of the bacterium (pH 7.4) (Rumiyati and Indrati 1998). For other microbial lipases, optimal pH for the enzyme production was 7.0 for *Candida deformans, Humicola lanuginosa,* and *Calvatia gigantea,* and 8.8 for *Aspergillus flavipes* (Seitz 1974, Christakopoulos et al. 1992, Savitha and Ratledge 1992).

The temperature of incubation (Fig. 4) also affected production of the enzyme. It increased gradually when the temperature of incubation increased, reaching the maximum at 37 °C, after that the production of lipase decreased with the increase in incubation temperature. The temperature for the highest production of lipase was

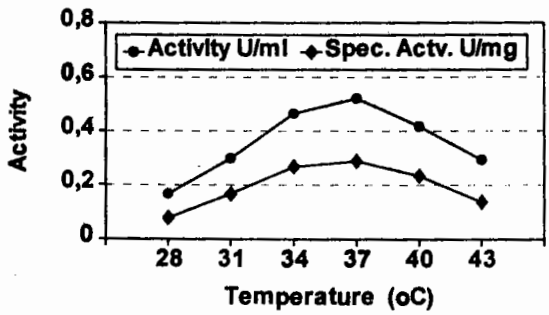

Figure 4. Effect of temperature of incubation on the activity (●) and specific activity (♦) of lipase.

the same as the temperature for isolation of *Kluyvera* KB4 (37 °C). The optimal temperature for lipase production from *Kluyvera* KB4 was the same as that of *Ophiostoma piceae* (Gao and Breuil 1995) and *Candida cylindracea* (Seitz 1974). Different optimal temperatures have been reported for other microbial lipase, they were 24–25 °C for *Aspergillus oryzae,* 30 °C for *Candida rugosa,* 40 °C for *Geotrichum candidum* and *Rhizopus delemar,* 45 °C for *Candida deformans* and *Humicola lanuginosa,* and 60 °C for *Staphylococcus aureus* 226 (Ohnishi et al. 1994, Seitz 1974, Rao et al. 1993, Lazar and Schroder 1992, Papaparaskevas et al. 1992).

Production of lipase during the growth of *Kluyvera* KB4 is shown in Fig. 5. The bacterium was grown at 37 °C on medium containing corn oil and peptone 1 %, repectively, which was adjusted to pH 7.5. The activity and specific activity of enzyme increased during incubation time and was maximum at 36 hours of incubation. Prolonged incubation resulted in a decrease of the an enzyme activities. This production of lipase was associated with the growth of bacterium. Different maximum time of incubations have been reported for other microbial lipase, which were: 22 h for *Pseudomonas aeruginosa,* 48 h for *Candida rugosa,* 72 h for *Ophiostoma piceace* and *Geotrichum candidum,* and 96h for *Aspergillus oryzae* (Chartrain et al. 1993, Rao et al. 1993, Gao and Breuil 1995, Hang and Woodams 1990, Ohnishi et al. 1994).

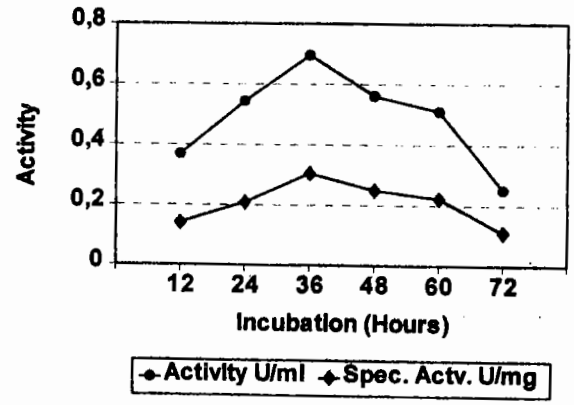

Figure 5. Effect of incubation time on the activity (●) and specific activity (♦) of lipase.

CONCLUSION

Bacterium KB4 was thought to be the genus *Kluyvera* sp. It produced a highest lipase on a medium

containing peptone 1.0 % and corn oil 1.0 % with an initial pH of 7.5, whereas the best incubation conditions were at 37 °C for 36 hours. It was found that these conditions for lipase production were the same as the conditions for the isolation of *Kluyvera* KB4. Oils were essential, since with no addition of oil to the medium, the enzyme production was only 14 %.

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REFERENCES

Brockerhoff, H., and Jensen, R.G. 1974. Lipolytic enzymes. Academic Press, New York.

Chartrain, M., Marcin, C., Katz, L., Salmon, P., Brix, T., Buckland, B., and Greasham, R. 1993. Enhancement of lipase production during fed-batch cultivation of *Pseudomonas aeruginosa* MB 5001. J. Ferment. Bioeng., 76:487–492.

Christakopoulos, P., Tzia, C., Kelos, D., and Macris, B.J. 1992. Production and characterisation of extracellular lipase from *Calvatia gigantea.* Appl. Microb. Biotech., 38 : 194–197.

Crueger, W., and Crueger, A. 1989. Biotechnology : A textbook of industrial microbiology. Sinauer Associates Inc., Sunderland, M.A.

Gao, Y. and Breuil, C. 1995. Extracellular lipase production by a sapwood-staining fungus, *Ophiostoma piceae.* World J. Microb. Biotech., 11:638-642.

Hang, Y.D. and Woodams, E.E. 1990. Lipase production by *Geotrichum candidum* from sauerkraut brine. World. J. Microb. Biotech., 6:418-421.

Kawase, T., Hashimoto, T., Fujii, T., and Minagawa, M. 1985. J. Jpn. Oil Chem. Soc. (Yukagaku), 34:22.

Kohno, M., Kugimiya, W., Hashimoto, Y., and Morita, Y. 1994. Purification, characterisation, and crystallisation of two types of lipase from *Rhizopus niveus.* Biosci. Biotech. Biochem., 58:1564-1568.

Kojima, Y., Yokoe, M., and Mase, T. 1994. Purification and characterisation of an alkaline lipase from *Pseudomonas fluorescens* Ak 102. Biosci. Biotech. Biochem 58:1564-1568.

Kokusho, Y., Machida, H., and Iwasaki, S. 1982. Production and properties of alkaline lipase from *Alcaligenes* sp. strain no. 679. Agric. Biol. Chem., 6:1743-1750.

Krieg, N.R., and Holt, J.G. 1984. Bergey's manual of Systematic bacteriology. Vol. I. Williams and Wilkins, Baltimore, U.S.A.

Lazar, G. and Schroder, F.R. 1992. Degradation lipids by fungi. In Microbial degradation of natural products. Winkelmann, G. (Ed.). pp. 267-291. Weinheim, VCH.

Lesuisse, E., Schanck, K., and Colson, C. 1993. Purification and preliminary characterisation of the extracellular lipase of *Bacillus substilis* 168, an extremely basic pH tolerant enzyme. Eur. J. Biochem., 216:155-160.

Lin, S.F., Chiou, C.M., Yeh, C.M., and Tsai, Y.C. 1996. Purification and partial characterisation of an alkaline lipase from *Pseudomonas pseudoalcaligenes* F-111. Appl. Environ. Microb, 62:1093-1095.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.L. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Ohnishi, K., Yoshida, Y., and Sekiguchi, J. 1994. Lipase production of *Aspergillus oryzae.* J. Ferment. Bioeng., 77:490-495.

Papaparaskevas, D., Christakopoulos, P., Kekos, D., and Macris, B.J. 1992. Optimising production of extracellular lipase from *Rhodotorula glutinis.* Bitechnol. Lett., 14:397-402.

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry et al. Which is more generally applicable. Anal. Biochem., 83:346-356.

Rao, P.V., Jayaraman, K., and Lakshmanan, C.M. 1993. Production of lipase by *Candida rugosa* in solid state fermentation. 1: Determination of significant process variables. Proc. Biochem., 28:385-389.

Razak, C.N.A., Salleh, A.B., Basri, M., and Yunus, W.M.Z. 1995. Screening, isolation and characterisation of lipolytic microbes. Indonesian Food Nutr. Prog., 2:9-11.

Rumiyati, V.S.P. and Indrati, R. 1998. Isolasi bakteri penghasil enzim lipase alkali dari kulit. Agritech. (Accepted).

Samad, M.Y.A., Salleh, A.B., Razak, C.N.A., Ampon, K., Yunus, W.M.Z.W., and Basri, M. 1990. A lipase from newly isolated thermophylic *Rhizopus rhizopodiformis.* World J. Microb. Biotech., 6:390-394.

Savitha, J., and Ratledge, C. 1992. An inducible, intracellular, alkalophilic lipase in *Aspergillus flavipes* grown on triacylglycerols. World J. Microb. Biotech., 8:129-131.

Seitz, E.W. 1974. Industrial application of microbial lipases: a review. J. Am. Oil Chem. Soc. 51:12-16.

Xia, J., Chen, X., and Nnanna, I.A. 1996. Activity and stability of *Penicillium cyclopium* lipase in surfactant and detergent solutions. J. Am. Oil Chem. Soc., 73:115-120.

Yamane, T. 1987. Enzyme technology for the lipid industry: An engineering overview. J. Am. Oil Chem. Soc., 64:1657-1662.