J. Microbiol. Biotechnol. (2000), **10**(3), 349–354

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Isolation of High Yielding Alkaline Protease Mutants of *Vibrio metschnikovii* Strain RH530 and Detergency Properties of Enzyme

CHUNG, SO-SUN[1], YONG-UK SHIN[1], HEE-JIN KIM[1], GHEE-HONG JIN[2], HYUNE-MO RHO[3], AND HYUNE-HWAN LEE[1]*

[1]*Department of Bioscience and Biotechnology, College of Natural Sciences, Hankuk University of Foreign Studies, Yongin-Si, Kyunggi-Do 449-791, Korea*
[2]*R & D Center of Household Products and Personal Care, Cheiljedang Corp., Inchon 400-103, Korea*
[3]*Department of Molecular Biology, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea*

Received: January 22, 2000

Abstract A facultative alkalophilic gram-negative *Vibrio metschnikovii* strain RH530, isolated from the wastewater, produced several alkaline proteases (VAP) including six alkaline serine proteases and a metalloprotease. From this strain, high yielding VAP mutants were isolated by NTG treatment. The isolated mutant KS1 showed nine times more activity than the wild-type after optimization of the culture media. The production was regulated by catabolite repression when glucose was added to the medium. The effects of several organic nitrogen sources on the production of the VAP were investigated to avoid catabolite repression. The combination of 4% wheat gluten meal (WGM), 1.5% cotton seed flour (CSF), and 5% soybean meal (SBM) resulted in the best production when supplemented with 1% NaCl. The VAP showed a resistance to surfactants such as sodium-α-olefin sulfonate (AOS), polyoxy ethylene oxide (POE), and sodium dodecyl sulfate (SDS), yet not to linear alkylbenzene sulfonate (LAS). However, the activity of the VAP was restored completely when incubated with LAS in the presence of POE or Na_2SO_4. The VAP was stable in a liquid laundry detergent containing 6.6% SLES (sodium lauryl ether sulfate), 6.6% LAS, 19.8% POE, and stabilizing agents for more than two weeks at 40°C, but the stability was sharply decreased even after 1 day when incubated at 60°C. A washing performance test with the VAP exhibited it to be a good washing power by showing 51% and 60% activity at 25°C and 40°C, respectively, thereby indicating that the VAP also has a good detergency at a low temperature. All the results suggest that the VAP produced from the mutant strain KS1 has suitable properties for use in laundry detergents.

Key words: Alkaline proteases, mutagenesis, laundry detergent, enzyme stability, surfactant, washing performance, detergency

*Corresponding author
Phone: 82-335-330-4280; Fax: 82-335-333-1696;
E-mail: hyunelee@san.hufs.ac.kr

Because of the gradual developments over the past two decades, enzymes have now become an indispensable ingredient in laundry detergents [1, 3]. The most preferred enzyme is protease. For an enzyme to be used in laundry detergent, it must act and be stable at an alkaline pH. Furthermore, the enzyme must exhibit the activity in a detergent containing surfactants such as LAS (linear alkylbenzene sulfonate), AOS (sodium-α-olefin sulfonate), POE (polyoxy ethylene oxide), and SLES (sodium lauryl ether sulfate). Until now, it has been the general opinion that a detergent protease must have as wide a specificity as possible [1]. Many studies have been performed for the development of an alkaline protease for the use in laundry detergents from microorganisms [2, 4, 5, 6, 10, 12]. However, the properties and productivity of such an enzyme have been the major obstacles to commercialization.

To develop an alkaline protease suitable for laundry detergents produced using a microorganism, we isolated the facultative alkalophilic gram-negative *Vibrio metschinikovii* strain RH530 from wastewater [7, 9]. The strain was shown to produce six extracellular alkaline proteases (VAP) including major VapT, VapK, and a metalloprotease. Among these, VapT has already been cloned and characterized [8] and the other alkaline protease, VapK, was recently cloned and characterized by us (manuscript in preparation). The VAP produced by the *V. metschnikovii* strain RH530 exhibited properties suitable for an additive enzyme in laundry detergent by showing a maximal activity at pH 10.5 and at a temperature of 55°C, and resistance to several surfactants. However, the productivity of the VAP in *V. metschnikovii* strain RH530 was not high enough for industrial application.

This paper reports on the strain development and characterization of high yielding VAP mutants and the properties of the VAP under actual washing conditions for application to laundry detergents.

MATERIALS AND METHODS

Bacterial Strains and Chemicals

The *Vibrio metschnikovii* strain RH530 was isolated from wastewater as reported elsewhere [7, 9]. The N-methyl-N'-nitrosoguanidine (NTG) was purchased from Sigma Co. Surfactants (AOS, LAS, POE, and SLES) and the organic nitrogen sources (WGM: Wheat Gluten Meal; SBM: Soybean Meal) were obtained from Cheiljedang Corp. (Korea). The CSF (Cotton Seed Flour) was purchased from Pharmamedia (U.S.A.). All other chemicals were of reagent grade.

Media and Culture Conditions

The *V. metschnikovii* strain RH530 cells were routinely cultivated at 30°C on LSC medium which contained 1% tryptone, 0.5% yeast extract, 1% sodium chloride, and 100 mM sodium carbonate buffer (pH 10.5). When necessary, 0.1% LAS and 1% skim milk (Difco) were added to the LSC agar medium. For the production of VAP from the mutant KS1 strain, a combination of the indicated concentration of each nitrogen source prepared in 100 mM sodium carbonate buffer (pH 10.5) was used.

Mutagenesis and Isolation of Mutants

The *V. metschnikovii* strain RH530 cells were grown in the LSC medium until mid-logarithmic growth phase (about 1×10^8 cells/ml), harvested, suspended in a fresh LSC medium, and then treated with NTG (250 μg/ml) at 30°C for 30 min. The NTG treatment resulted in more than 99.9% death. The cells were washed twice with fresh LSC medium followed by plating on an agar medium containing 0.1-0.3% LAS and 1% skim milk. The colonies showing relatively large halos were selected and the enzyme activities of VAP were determined.

Enzyme Assay

The enzyme activity was determined by the method of Yanagida *et al.* [13] with modifications. Two-and-a-half milliliters of prewarmed 1% casein (Vitamine-free, Fluka) in 100 mM sodium carbonate buffer (pH 10.5) and 0.5 ml of the enzyme solution were mixed and the mixture was incubated at 30°C for 10 min. The reaction was terminated by the addition of a stopping mixture (0.22 M trichloroacetic acid, 0.22 M acetic acid, and 0.22 M sodium acetate), and then the mixture was centrifuged at 10,000 ×g at 4°C for 15 min. The absorbance at 280 nm was measured. To investigate the effect of a surfactant on the enzyme activity, the enzyme was incubated with an artificial substrate (N-Succinyl-Ala-Ala-Pro-Phe-p-Nitroalilid, Sigma Co.) in the presence of the indicated concentration of surfactant. One unit of protease activity (PU) was defined as the amount of the enzyme which catalyzed an increase of 0.1 absorbance per 10 min caused by the release of tyrosine. All the protease activities were expressed as the mean value of more than five independent experiments.

Stability of Vaps in the Liquid Laundry Detergent

To determine the stability of the VAP in a laundry detergent solution containing 6.6% SLES, 6.6% LAS, 19.8% POE, and stabilizing agents such as Na_2SO_4, the enzyme was mixed with the detergent solution and incubated at 40°C or 60°C. Thereafter, the enzyme activity was determined at time intervals for more than two weeks. To compare the stability, a commercially available alkaline protease (Novo Nordisk, Denmark) was used as the control.

Washing Performance Test

Washing trials were carried out as described by Umehara *et al.* [11] with modifications. A Terg-O-Tometer (US Testing Co.) was operated under typical Korean washing conditions as shown in Table 1. The protein-stained cotton fabric (AS10) was purchased from Wfk-Testgewbe GmEh (Germany). The cotton fabric was cut into 5×5 cm swatches. After washing, the fabric was ironed to avoid the effect of curvature on the reflection measured by the reflex meter (Kiwha Hitech. Co., Japan). The washing power (W) was determined as follows:

$$W(\%)=\frac{R_W-R_S}{R_O-R_S}\times 100$$

where, R_w is the reflex index after washing, R_O is the reflex index of the original, non-stained fabric, and R_S is the reflex index before washing. To compare the washing power, two control experiments were carried out in the same conditions except for the enzymes. Control 1 was performed without addition of alkaline protease as the negative control and control 2 was carried out with the same amount of commercially available alkaline protease as the positive control. (Novo Nordisk, Denmark).

Table 1. Washing conditions.

Articles	Conditions
Washing machine	Terg-O-Tometer
Rotation	300 rpm
Temperature	25°C, 40°C
Water hardness	50 ppm as $CaCO_3$
Washing time	10 min
Liquor volume	0.67 g liquid laundry detergent in 1 l of H_2O
Surfactant composition	6.6% SLES, 6.6% LAS, 19.8% POE, stabilizing agents
Enzyme used	1,000 PU
Clothes	Protein-stained AS-10 Cotton

Table 2. Isolation of mutants overproducing alkaline protease.

Strains	Growth[a] (OD_{600})	Protease activity (PU/ml)[b]	Relative activity (%)
RH530	4.7	230	100
L12-23	4.5	310	134.8
N4-8	6.4	690	300
KS1	5.9	892	387.8

[a]Cells were grown in LSC media.

[b]Mean value of more than three independent experiments.

RESULTS AND DISCUSSION

Isolation of High Yielding VAP Mutants

To isolate the high yielding VAP mutants through mutagenesis using NTG, all the colonies that appeared on the LSC agar medium containing 0.1% LAS were transferred to a medium containing 1% skim milk. Several colonies with a large clear halo were selected and inspected for the overproduction of VAP by the enzyme assay. As shown in Table 2, strain L12-23 showed about a 35% increase in protease activities, therefore, it was serially mutated with NTG. As a result, the mutant N4-8 showed more than a three-fold improvement in the production of VAP. Another mutant, KS1 derived from N4-8, showed even higher activities than N4-8. N4-8 and KS1 showed growth on a plate containing 0.3% LAS, in contrast to the limit of 0.1% wth L12-23. These results suggest that the resistance of the mutants to such a high concentration of LAS may promote the cells to secrete a large amount of VAP by destabilizing the membrane of the cells. However, no mutants were isolated that grow on a plate containing more than 0.3% LAS. This may be due to the fact that the biodegradability of the membrane by a high concentration of LAS kills the strains.

Effect of Glucose on the Production of VAP in Mutant Strain KS1

In order to identify the best conditions for the production of VAP in the mutant strain KS1, the effect of glucose was investigated. As shown in Table 3, the relative activity of the VAP was reduced to 37% by the addition of 2% glucose to the medium. Above 4%, the production of VAP was almost completely repressed. These facts suggest that the production of the enzyme was regulated by catabolite repression. Such catabolite repression in the production of alkaline protease has also been reported in *V. alginolyticus* [4]. To avoid catabolite repression, the effects of other carbon sources on the production of VAP were studied. All the carbon sources tested (including fructose, maltose, sucrose, and starch) repressed the production of VAP (data not shown). Therefore, it was necessary to grow the cells without supplying carbon sources, yet supplying growth factors such as amino acids directly from organic nitrogen sources.

Table 3. Effect of glucose concentration on the production of alkaline protease.

Glucose (%)	Growth[a] (OD_{600})	Protease activity (PU/ml)	Relative activity (%)
Control (0%)	6.62	1,148	100
1	9.3	432	37.6
2	9.6	430	37.5
3	10.3	286	24.9
4	10.2	32	2.8
5	9.8	20	1.7

[a]KS1 cells were cultured in LSC medium.

Effect of Organic Nitrogen Sources on the Production of VAP in Strain KS1

To study the effect of organic nitrogen sources on the production of VAP, SBM, WGM, and CSF were used. The production of VAP was affected differently by each organic nitrogen source. As shown in Table 4, variations in the concentration of each nitrogen source in a BPM media showed little effect on the production of VAP. However, combinations of various nitrogen sources resulted in a large increase in productivity (data not shown). Among the combinations, 4% WGM, 1.5% CSF, 3% SBM, and 1% NaCl, designated as PM medium, showed the best productivity (67% increase in productivity) compared to BPM (Table 5). However, an addition of each nitrogen source by more than the determined concentration was not accompanied by an increase in the productivity of VAP.

Table 4. Effect of different nitrogen sources on the production of alkaline protease.

Nitrogen sources[a] (%)		Protease activity (PU/ml)	Relative activity (%)
WGM	Control (2%)	1,656	100
	3	1,756	106.0
	4	1,876	113.2
	5	1,832	110.6
CSF	Control (0.5%)	1,656	100
	1.0	1,740	105.0
	1.5	1,804	108.9
	2.0	1,744	105.3
SBM	Control (2%)	1,656	100
	3	1,812	109.4
	4	1,752	105.7
	5	1,808	109.1

[a]KS1 cells were grown for 45 h in Basal Production Medium (BPM) containing 2% WGM, 0.5% CSF, 2% SBM, and 0.5% NaCl in a 50 mM sodium carbonate buffer (pH 10.5). To study the effect, the concentration of each nitrogen source in BPM media was changed as indicated.

Table 5. Comparison of alkaline proteases production.

Medium	Culture time (h)	Protease activity (PU/ml)	Relative activity[c] (%)
BPM[a]	20	752	
	45	1,524	
	67	1,322	
PM[b]	20	528	70.2
	45	2,172	142.5
	67	2,220	167.4

[a]Contains 2% WGM, 0.5% CSF, 2% SBM, and 0.5% NaCl.
[b]Contains 4% WGM, 1.5% CSF, 5% SBM, and 1.0% NaCl.
[c]Was determined by comparison of enzyme activity between BPM and PM at the corresponding time points. The relative activity (%) is an activity in PM at each time point/an activity in BPM at the corresponding point ×100.

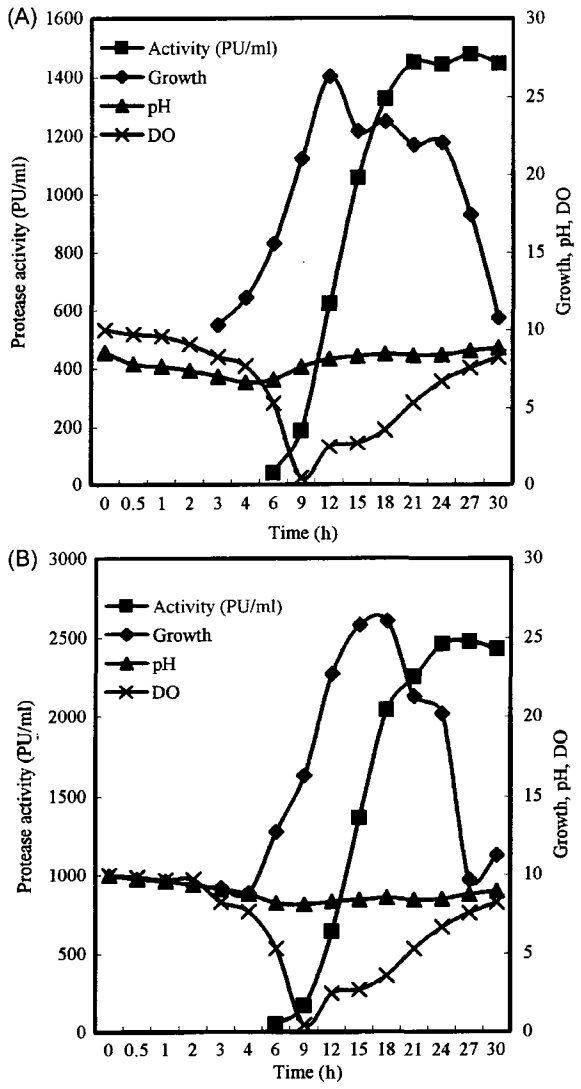

Fig. 1. Comparison of the fermentation time courses in the production of alkaline protease from N4-8 (A) and KS1 (B).
Cells were grown in a 5-l jar fermenter containing 2 l PM medium, which was pH-controlled, agitated at 500 rpm. Cell growth was measured at OD_{600}.

Comparison of the Fermentation Time Courses of VAP Production in Strains N4-8 and KS1

The production of VAP in N4-8 and KS1 was studied with a 5-l jar fermentor in PM media. Figure 1 shows the fermentation time course of the isolated N4-8 (A) and KS1 (B) mutants. The growth of N4-8 was more rapid than KS1, however, the productivity of N4-8 was lower than that of KS1. About a two-fold increase in protease activity was obtained in the KS1 mutant. The VAP produced in the medium was fairly stable and maintained constant activity after the stationary phase, however, the cells died abruptly thereafter. The remarkable stability of VAP in the culture media could be a good property for mass production by fermentation. However, further studies on the fermentation process are required.

Effects of Various Surfactants on the Activity of VAP

The effects of various surfactants on the activities of the VAP produced by mutant KS1 were investigated. The surfactants used were those generally used in commercial laundry detergents such as AOS, LAS, and POE. As shown in Fig. 2, the VAP showed resistance to AOS, POE and SDS. Furthermore, the relative activities were increased after incubation with AOS, POE, and SDS, compared to the control. These results can be explained in that the surfactants in the reaction mixture somehow change the conformation of enzymes, so that the substrates can easily attach the active sites of the enzymes. The LAS inhibited the activities of VAP strongly, yet the enzyme activities were restored completely when incubated with nonionic surfactant POE or Na_2SO_4, as shown in Fig. 3. However, the theoretical basis for the restoration of the enzyme activity of VAP should be further studied. The overall properties of the VAP are suitable for application to laundry detergent, because POE and Na_2SO_4, which restore

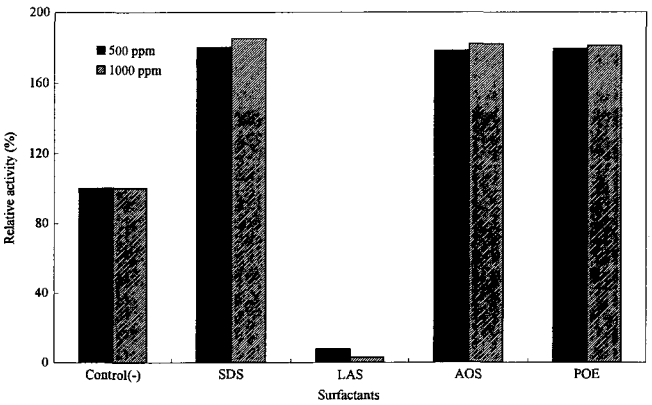

Fig. 2. Effect of various surfactants on the activity of alkaline proteases.
Indicated concentration of surfactant was added to the reaction mixture with an artificial substrate as described in Materials and Methods. Control shows the enzyme activities without the addition of each surfactant.

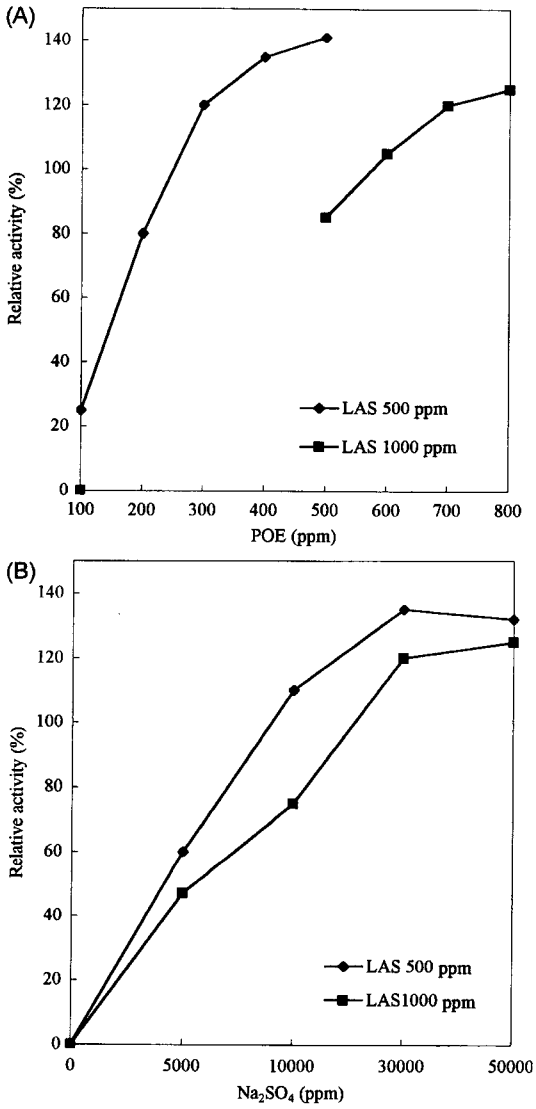

Fig. 3. Effect of POE (A) and Na_2SO_4 (B) on the activity of alkaline protease in the presence of LAS.
Enzymes were mixed with POE or Na_2SO_4 followed by addition of LAS.

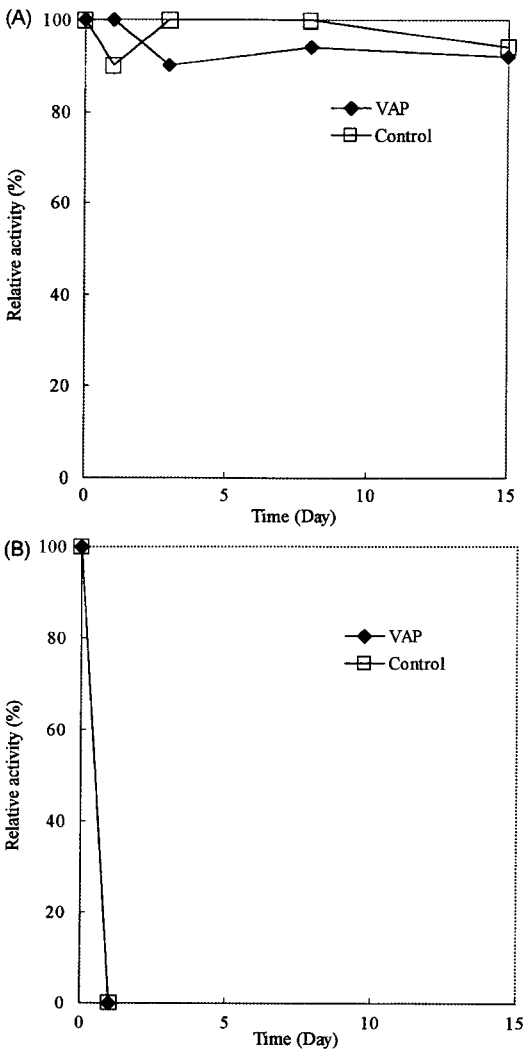

Fig. 4. Stability of alkaline protease in a laundry detergent solution at 40°C (A) and 60°C (B).
Control shows the activity of a commercially available enzyme at the same amount.

the VAP activity in the presence of LAS, are normal ingredients of commercial laundry detergent.

Stability of VAP in Liquid Laundry Detergent

For the VAP to be used in laundry detergent, the enzymes must be stable in a laundry detergent solution. To determine the stability, the VAP was added to a liquid laundry detergent, as described in Materials and Methods. The reaction mixtures were incubated at 40°C and 60°C for more than two weeks, by taking aliquots at time intervals. The activities of the proteases were analyzed. As shown in Fig. 4, the stability of the VAP was maintained for more than 2 weeks at 40°C, in contrast to a sharp decrease even after 1 day at 60°C. The same results were obtained in control experiments using commercially available alkaline protease. These results suggest that the detergency properties of the VAP were sufficient for use as an additive to laundry detergent, since a high stability was exhibited in a liquid laundry detergent.

Washing Performance Test of VAP

Figure 5 shows the results of the washing performance test of the VAP. The results demonstrate that the VAP had a good washing power at both low (25°C) and high temperatures (40°C) by showing 51% and 60% compared to 42% and 54% with commercially available alkaline protease, respectively. In particular, the VAP showed good washing power at low temperature. These properties suggest that the VAP can be applicable to laundry detergent, especially in the washing with cold water.

In conclusion, high yielding VAP mutants were isolated from *V. metschnikovii* strain RH530 by NTG treatment.

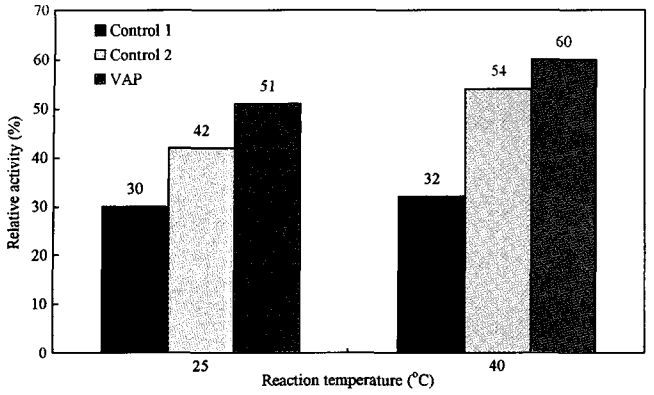

Fig. 5. Comparison of the washing power of alkaline protease.
Control 1 was the result of the washing performance obtained using the standard washing solution, as shown in Table 1, without addition of the alkaline protease and control 2 was with the addition of a commercially available detergent protease. Numbers represent the relative cleanness of stained clothes compared to unstained ones after washing.

The mutant strain KS1 produced five times more VAP than the wild-type strain when grown in an LSC medium. When the medium was optimized, a nine-times higher production of VAP was obtained. The VAP produced from the mutant KS1 exhibited good detergency as an additive enzyme in laundry detergent with optimal activities at pH 10.5 and at 60°C along with surfactant-resistance. Furthermore, the stability of VAP in a liquid laundry detergent and the results of the washing performance test showed potential usage in laundry detergent. VAP also showed a good washing power at low temperatures (below 20°C).

Acknowledgments

This work was supported by a grant from the Korean Ministry of Science and Technology through a G7 project.

REFFERENCES

1. Aaslyng, D., E. Gormsen, and H. Malmos. 1990. Mechanistic studies of proteases and lipase for the detergent industry, pp. 26-28. *In* Paper presented at the SCI conference, *Recent Advances in the Detergent Industry*, held at the University of Cambridge, U.K.
2. de Boer, A. S., F. Priest, and B. Diderichsen. 1994. On industrial use of *Bacillus licheniformis*; a review. *Appl. Microbiol. Biotechnol.* **40:** 595-598.
3. Dambmann, C., P. Holm, V. Jensen, and M. Nielsen. 1971. How enzyme got into detergents. *Dev. Indus. Microbiol.* **12:** 11-23.
4. Deane, S. M., F. T. Robb, and D. R. Woods. 1987. Production and activation of a SDS-resistant alkaline serine exoprotease of *V. alginolyticus*. *J. Gen. Microbiol.* **133:** 391-398.
5. Kim, Y. O., J. K. Lee, H. K. Kim, Y. S. Park, and T. K. Oh. 1996. Purification and characterization of an alkaline protease from *Bacillus licheniformis* NS70. *J. Microbiol. Biotechnol.* **6:** 1-6.
6. Kim, Y. O., J. K. Lee, K. Sunitha, H. K. Kwon, and T. K. Oh. 1999. Minor thermostable alkaline protease produced by *Thermoactinomyces* sp. E79. *J. Microbiol. Biotechnol.* **9:** 469-474.
7. Kwon, Y. T., J. O. Kim, S. Y. Moon, H. H. Lee, and H. M. Rho. 1994. Extracellular alkaline proteases from alkalophilic *Vibrio metschnikovii* strain RH530. *Biotechnol. Lett.* **16:** 427-434.
8. Kwon, Y. T., J. O. Kim, S. Y. Moon, Y. D. Yoo, and H. M. Rho. 1995. Cloning and characterization of the gene encoding an extracellular serine protease from *Vibrio metschnikovii* strain RH530. *Gene* **152:** 59-63.
9. Kwon, Y. T., S. Y. Moon, J. O. Kim, Y. H. Kho, and H. M. Rho. 1992. Characterization of extracellular proteases from alkalophilic *Vibrio* sp. Strain RH530. *Kor. J. Microbiol.* **30:** 501-506.
10. Mabrouk, S. S., A. M. Hashem, N. M. A. El-Shayeb, A. M. S. Ismail, and A. F. Abdel-Fattah. 1999. Optimization of alkaline protease productivity of *Bacillus licheniformis* ATCC21415. *Bioresource Technol.* **69:** 155-159.
11. Umehara, G., Y. Masago, T. Mukaiyama, and O. Okumura. 1990. Behavior of alkaline lipase on detergency. *J. Jpn. Oil Chem. Soc.* **39:** 321-326.
12. Xiaohang, M. and X. Guanchui. 1990. Isolation of a bacterial strain (BsO_8) producing alkaline protease and properties of the enzyme production. *Weishengwuxue Zazhi* **10:** 20-24.
13. Yanagida N., T. Uozumi, and T. Beppu. 1984. Specific excretion of *Serratia marcescens* protease through the outer membrane of *Escherichia coli*. *J. Bacteriol.* **166:** 937-944.