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Alkaline protease production from alkalophilic *Bacillus* sp. isolated from natural habitats☆

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Abstract

Bacillus strains isolated under extreme alkaline conditions (Izmir, Turkey), were screened and identified for high alkaline protease activity. Strains with high protease yields were optimized with respect to inoculum concentration, temperature, agitation speed, initial medium pH and incubation time. Three *Bacillus* strains coded as I18, L18 and L21 showed high potential, for alkaline protease activity (160–222 U/ml) among 85 isolates. The specific growth rates were estimated from the growth curves as 0.49 h^{-1} for I18, as 0.6 and 0.7 h^{-1} for L18 and L21, respectively. The optimum temperatures were determined as 30 °C for strain I18 and 37 °C for the strains L18 and L21. Similarly, the optimum agitation speeds were 100 rpm for I18 and 180 rpm for L18 and L21. For all three strains, the optimum inoculation ratio and incubation time, were determined as 5% (v/v) and 96 h, respectively. The optimum initial media pH was found as pH 10 for strain L18 and L21. *Bacillus* sp. L21 with the highest specific protease activity (60 U/mg protein) and a broader pH range was chosen for further study. The biomass and product yield for this strain was determined as 0.023 g cell/g glucose and 0.021 U/g glucose, respectively. The crude enzyme of this strain was further characterized and was determined as a bleach stable, serine alkaline protease with an optimum temperature of 60 °C and a pH of 11, with a potential to be a candidate for the applications in the detergent industry.
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Keywords: Alkaline protease; *Bacillus* sp.; Microbial enzymes; Enzyme production

1. Introduction

Proteases constitute 60–65% of the global industrial enzyme market most of which are alkaline proteases [1]. Most of these find applications in the food industry, in the meat tenderization process, peptide synthesis, for infant formula preparations, baking and brewing. Furthermore, they are used in pharmaceuticals and medical diagnosis, in the detergent industry as additives, as well as in textile industry in the process of dehairing and leather processing [2]. Currently a large proportion of the commercially available alkaline proteases are derived from *Bacillus* strains [3,4]. The reason for this is their high pH and temperature stability. Alkaline proteases belong to the group of proteases, which have either a serine center or are of metallo-type, exhibiting a wide pH range of pH 6–13. Among these are the serine proteases with industrial importance [3]. Few examples reported in the literature, are the studies conducted by Rebecca et al. [5], who produced a high nutritional value fish hydrolysate using *B. subtilis* protease, and O'Meara and Munro [6], who used commercial alkaline protease in the upgrading of lean meat waste to edible products. Another example is the application of the alkaline protease by Tanimoto et al. [7] in the enzymatic modification of zein to produce a non-bitter peptide fraction with high Fischer ratio for patients with hepatic encephalopathy.

Given the wide application of this enzyme, it is reported that in year 2005 the global proteolytic enzyme demand will increase dramatically to 1.0–1.2 billon dollars [8]. Therefore, taking this demand into account and knowing the geographic richness and biodiversity of our local environment with less industrial pollution, it is assumed that there is potential for alkalophilic

☆ This work proposes the optimization of the fermentation conditions of three *Bacillus* sp. isolated from natural habitats producing alkaline proteases. After the optimization process one isolate, coded L21 exhibiting high alkaline protease activity is chosen as the strain for further study. It is highly important to discover enzymes with novel properties, from species living under extreme conditions which could have wide industrial applications. Therefore this study is the initial work performed on such species and an initial step for further optimization processes.

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Bacillus species living in these environments. Discovering such species, producing proteases with novel characteristics will be of great value to the enzyme industry for different applications. With this as main objective, local soil of Izmir region and by-products of a leather industry working under highly alkaline conditions were screened for the isolation of these strains. Total of 85 isolates were screened for high alkaline proteolytic activity according to the method described by Horikoshii [9] and only three of them showed such a potential. These strains were coded as I18, L18 and L21. In this study, the goal was to further optimize the fermentation conditions of these strains whose phenotypic and genotypic identifications were completed and choose the strain with highest alkaline proteolytic activity, which could be a potential candidate for industrial use [10].

2. Materials and methods

2.1. Organisms and culture conditions

Under the direction of the Department of Biology at Izmir Institute of Technology, *Bacillus* sp. L18 and L21 were isolated from the by-products of a leather factory. Similarly, *Bacillus* sp. I18 was isolated from the soil of the campus by the Biology Department of Ege University. The phenotypic and genotypic studies of all three strains were completed by the Department of Biology at Izmir Institute of Technology [10].

These microorganisms were cultivated in a solution containing 1% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% potassium dihydrogen phosphate, 0.02% magnesium sulfate and 1% sodium carbonate where sterile sodium carbonate was added aseptically after the sterilization process. This formulation is known as Horikoshii reference medium [9]. For the solid media preparation 1.5% of agar was added to this formulation. Cultures were regenerated every 2–3 weeks on a fresh plate from the frozen stock culture. All assays were performed using the cell-free supernatant of the fermentation broth. The cell densities were determined by reading the optical densities at 600 nm and performing viable cell counts on the total broth. Biomass was also determined by drying the pellet obtained after centrifugation (at 5000 rpm for 10 min) at 65 °C for 24 h until it reached equilibrium weight. The cell-free supernatant was used to determine proteolytic activity, protein content and carbohydrate content.

2.2. Assay for proteolytic activity

Alkaline protease activity was determined by applying a modified form of the method given by Takami et al. [11]. According to this procedure 0.25 ml of glycine:NaCl:NaOH (50 mM, pH 10.5) buffer was incubated with 2.5 ml of 0.6% casein (Merck) dissolved in the same buffer at 30 °C until equilibrium was achieved. An aliquot of 0.25 ml of the enzyme solution was added to this mixture and incubated for 20 min. The reaction was stopped by adding 2.5 ml TCA solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid). After 10 min the entire mixture was centrifuged at 5000 × *g* for 15 min. The supernatant in the amount of 0.5 ml was mixed with 2.5 ml of 0.5 M Na_2CO_3 and 0.5 ml of Folin-Ciocalteu's phenol solution and kept for 30 min at room temperature. The optical densities of the solutions were determined with respect to the sample blanks at 660 nm using Varian Cary Bio 100 spectrophotometer. For these studies, one alkaline protease unit was defined as the enzyme amount that could produce 1 μg of tyrosine in 1 min under the defined assay conditions.

2.3. Total protein content

The total protein contents of the samples were determined according to the method described by Lowry [12]; the protein standard was bovine serum albumin (Sigma).

2.4. Total carbohydrate content

Total carbohydrate content was determined according to the phenol–sulfuric acid method [13]. The analytical grade materials were obtained from Sigma (St. Louis, USA). All the presented results were obtained from the average of two parallels.

2.5. Characterization of the crude protease enzyme

The crude protease obtained from the *Bacillus* sp. L21, which showed the highest potential for proteolytic activity, was further subjected to preliminary characterization study. Therefore, the effect of pH and temperature on activity and stability with the effect of various oxidizing agent, metal ion and inhibitors were studied. The procedures are outlined in detail below.

2.5.1. Effect of pH on activity and stability of protease

The effect of pH on the proteolytic activity of crude alkaline protease from *Bacillus* sp. L21, was determined by assaying the enzyme activity at different pH values ranging from 4.0 to 13.0 using the following buffer systems: acetate (pH 4.0), phosphate (pH 7.0), Tris–HCl (pH 9.0), glycine–NaOH (pH 10.5–11) and KCl–NaOH (pH 13.0). The concentration of each buffer was 0.1 M. The relative activities were based on the ratio of the activity obtained at certain pH to the maximum activity obtained at that range and expressed as percentage. The pH stability of the enzyme was investigated in the pH range of 4.0–13.0. Therefore, 2 ml of the crude enzyme was mixed with 2 ml of the buffer solutions mentioned above and incubated at 30 °C for 2 h. Afterwards, aliquots of the mixtures were taken to measure the residual protease activity (%) with respect to the control, under standard assay conditions.

2.5.2. Effect of temperature on activity and stability of protease

The effect of temperature was determined by incubating the reaction mixture (pH 10.5) for 20 min at different temperatures ranging from 30 to 80 °C. The relative activities (as %) were expressed as the ratio of the proteolytic activity obtained at certain temperature, to the maximum activity at the given temperature range. In order to determine the thermo stability of the enzyme, experiments were conducted by measuring the residual activity after incubation at various temperatures ranging from 30 to 60 °C, for 30 and 60 min, in the presence and absence of 0.05 M Ca^{2+} ion.

2.5.3. Effect of oxidizing agent, metal ion and inhibitors activity of protease

The effects of Ca^{2+} ion as a metal ion, H_2O_2 as an oxidizing agent, EDTA and PMSF as inhibitors, on alkaline protease activity were investigated to further characterize the enzyme. The crude alkaline protease was pre-incubated with the above-mentioned chemicals for 1 h at 30 °C; afterwards the residual activity (%) was tested by standard proteolytic activity assay.

3. Result and discussion

3.1. Determination of the specific growth rates

The first sub-culture to be used as the inoculum for the actual fermentation was prepared by transferring a loop-full of culture of each strain from fresh Horikoshii agar plate, into 25 ml Horikoshii broth media and incubated at 37 °C and 180 rpm for 24 h. During this time period, samples were taken for viable cell count and optical density (data not shown), in order to estimate the specific growth rates. The inoculum concentration for the actual fermentation of each strain was based on the optical density at the end of the exponential phase, corresponding to 5% (v/v). The aim was to standardize the inoculum amount for each experiment based on the growth rate of the first subculture. The specific growth rates were calculated from the slope of the loga-

rithmic phase as 0.49, 0.60, and 0.70 h^{-1} for I18, L18 and L21, respectively. According to these, *Bacillus* sp. L21 was the strain with highest specific growth rate.

3.2. Effect of the incubation temperature

The effect of the incubation temperature on the proteolytic activity was determined by inoculating 5% inoculum of each culture into 50 ml of Horikoshii medium (in 250 ml of Erlenmeyer flask) and incubating at 30, 37, 45 and 55 °C at 180 rpm for 96 h. Based on the maximum protease activities (Table 1), strain I18 has temperature optima at 30 °C whereas strains L18 and L21 have at 37 °C (additional experiments performed using strain I18 revealed that the optimum temperature was not below 30 °C; data not shown). The optical density results presented on the same table indicated that, for strains I18 and L18, there was an inverse relationship between the enzyme synthesis (based on the specific protease activities) and cell growth for the given temperatures. Only at 45 °C, strain L18 with a very low optical density and protease activity opposed this statement, which could be due to slower cell growth at this temperature producing therefore less enzyme. According to these data, one could conclude that protease enzyme is a non-growth related product, which is common in many microbial enzyme fermentations. For strain L21 an optimum temperature of 37 °C promoted cell growth as well as enzyme synthesis compared to the other temperature ranges studied. One should also take into account that the enzyme synthesized at 45 °C and higher temperatures might face the risk of denaturation of the protein, as well as degradation due to the proteolytic activity of the protease produced. This could contribute to the lower activity results obtained in this study as well. Therefore, it is very difficult to make solid justi-

Table 1
Optical densities, total and specific enzyme activities of strains I18, L18 and L21 at different incubation temperatures, agitation speeds and inoculum ratios, after 96 h of incubation

Strains	Factors	Levels	Optical density	Protease activity (U/ml)	Specific protease activity (U/mg protein)
I18	Temperature (°C)	30	3.52	132.2	27.3
		37	4.56	128.0	14.9
		45	4.26	127.1	16.3
		55	No growth	No growth	No growth
	Agitation (rpm)	100	5.08	159.5	31.97
		180	4.56	128.0	14.87
		250	3.24	141.4	20.73
		325	4.12	134.6	15.42
	Inoculum (v/v, %)	1	6.74	51.9	6.9
		2.5	6.63	26.7	3.7
		5	3.52	132.2	27.3
		10	4.99	28.3	3.8
L18	Temperature (°C)	30	5.47	133.8	28.9
		37	4.97	222.1	49.0
		45	2.56	85.0	18.3
		55	No growth	No growth	No growth
	Agitation (rpm)	100	5.15	161.2	19.49
		180	4.97	222.1	49.02
		250	3.78	142.3	16.55
		325	5.09	135.3	13.61
	Inoculum (v/v, %)	1	3.58	61.3	7.4
		2.5	3.85	42.7	4.9
		5	4.97	222.1	49.0
		10	2.67	36.8	3.7
L21	Temperature (°C)	30	2.72	163.0	42.7
		37	5.32	216.3	60.1
		45	4.66	92.1	14.6
		55	No growth	No growth	No growth
	Agitation (rpm)	100	5.06	106.7	16.94
		180	5.32	216.3	60.09
		250	2.90	94.2	8.91
		325	4.35	186.0	30.24
	Inoculum (v/v, %)	1	5.47	161.6	21.8
		2.5	5.12	167.7	22.3
		5	5.32	216.3	60.0
		10	5.52	176.8	25.1

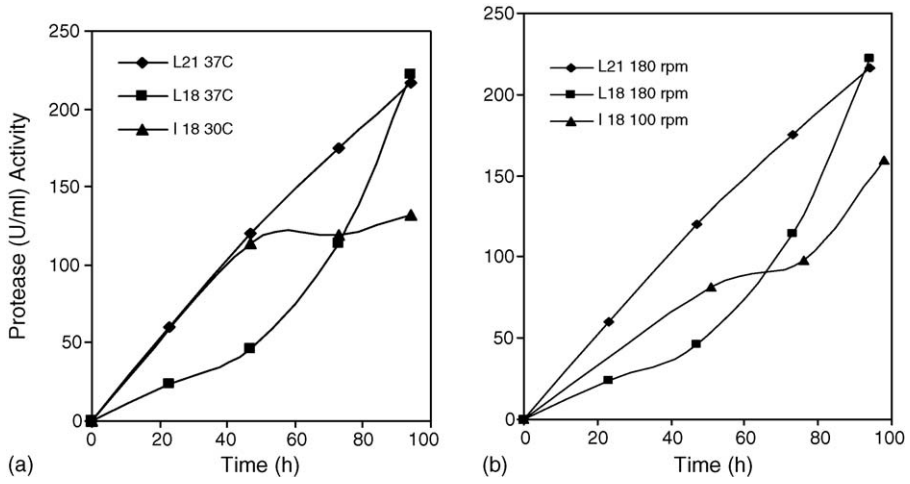

Fig. 1. Protease enzyme profiles of the strains I18, L18 and L21 at (a) optimum temperatures; (b) optimum agitation speeds.

fications according to the given data. From the enzyme profiles (Fig. 1a), it was observed that, L18 and L21 reached almost the same enzyme level; however, the rate of enzyme synthesis was much faster with strain L21 compared to the others. Considering either total or specific enzyme activities, in both cases strain I18 was the less performing strain. This difference among the strains could be attributed to the sources of isolation, where I18 was isolated from soil and L18 and L21 were isolated from leather by-products. No growth was detected for any of the strains at 55 °C.

A comparison of the literature on the characteristics of alkaline *Bacillus* strains producing alkaline proteases revealed that most of the alkaline *Bacillus* strains were of mesophilic type with temperature optima of 30–37 °C. With this regard, these strains were in agreement with the literature [1,2,11,14–16].

3.3. Effect of agitation speed

The effect of the agitation speed on the protease enzyme synthesis was determined, by inoculating 5% of each first subculture into 50 ml of Horikoshii media and incubating at 37 °C and 100, 180, 250 and 325 rpm under the same fermentation conditions. Based on the total and specific protease activities (Table 1), 100 rpm was determined to be the optimum agitation speed for strain I18 and 180 rpm for the others (L18 and L21). In the correlation of the agitation speed to the cell growth, it was observed that for all three strains, there was a trend where an increase in agitation speed up to 250 rpm resulted in low optical cell density, beyond which the cell densities started to increase with an increase in the agitation speed to 325 rpm. This could be explained besides the characteristics of the strain, with a better oxygen and nutrient transfer rate at this speed, promoting more cell growth. It was obvious from the given data, that all three strains did not require higher agitation speeds for enzyme synthesis, probably due to the less requirement for oxygen. This phenomena again brings up the issue of non-growth related product formation, observed in many microbial enzyme fermentations as discussed in Section 3.2. Based on the total protease activities, L18 was the dominant strain. However, based on specific activities this pictures changed, where L21 became the superior strain with 18.4% and 46.7% more specific protease activity than the strains I18 and L18, respectively. This was also supported with the enzyme profiles at optimum agitation speeds shown in Fig. 1b. In the reported literature, most of the agitations speeds used for this type of strains are between 180 and 220 rpm. Therefore, our results support these findings. [1,3,4,17].

3.4. Effect of inoculation ratio

To determine the effect of inoculation ratio, 50 ml of the Horikoshii media was inoculated, with 1%, 2.5%, 5% and 10% (v/v) of the first subculture of each strain. Strain I18 was incubated at 100 rpm and 30 °C; L18 and L21 were incubated at 180 rpm and 37 °C, since these were the predetermined optimum conditions. An increase in the inoculation ratio from 1% to 5% decreased the optical cell density of the strain I18, beyond where a raise was observed after 10% inoculation (Table 1). An explanation for this would be that, 1% or 2.5% inoculation ratio did not cause an overload of cells facing nutrient or oxygen limitation. However, after 5% this changed, where this concentration became probably a threshold changing the pathways of cell growth towards enzyme synthesis. A 10% inoculation however, was so high where the nutrients were consumed faster and overall resulted into less cell growth compared to 1% and 2.5%. But this seemed to be still within the pathways towards cell growth rather than enzyme synthesis, therefore resulted into lower enzyme activity. The effect of inoculation ratio on strain L21 seemed not to be significant on cell growth, because almost same optical cell densities were obtained at all inoculation levels used. An increase to 10% decreased the cell growth significantly for strain L21. It was obvious that, the inoculation ratio had different impacts on cell growth and enzyme synthesis depending on the characteristics of the strains. Based on these findings, the optimum inoculation ratio was determined as 5% (v/v) for all three strains considering protease activities. Strain L21 was

Table 2
Total protease activities of strains I18, L18 and L21 at different incubation times

Strain	Protease activity (U/ml)		
	73 h	96 h	125 h
I18	97.5	159.5	81.3
L18	114.0	222.1	243.7
L21	175.2	216.3	224.0

chosen as the best performing strain, based on the comparisons with respect to total and specific enzyme activities at this optimum ratio. Since, the specific enzyme activity gives an indirect indication on the purity of the enzyme, this value is desired to be as high as possible. Therefore, L21 was thought to produce less side by-products than the other two strains. An inoculation ratio of 2–5% was an optimum value for *Bacillus* type of strains reported in the literature, which supported our findings [14,18].

3.5. Effect of incubation time

In order to determine the optimum incubation time for maximum enzyme production, 50 ml of Horikoshii media inoculated with 5% (v/v) of the first subculture of each strain, was incubated at the predetermined optimum conditions (I18: 30 °C and 100 rpm; L18 and L21: 37 °C and 180 rpm) for 73, 96 and 125 h. According to the results taken at different time intervals (Table 2), it was determined that the optimum incubation time for all there strains was around 96 h even though, numerically from the data the highest activity was achieved for strain L18 and L21 at 125 h. This conclusion was based on the estimation of the activity gain of 3.3% for strain L21 and 8.8% for L18 versus a time extension of 29 h. In order to make right judgments and decisions, one has to estimate the cost, whether extending the fermentation time and therefore increasing the operating cost would be compensated by the extra gain that would be obtained through the extra enzyme yield. Our experience is that, shorter fermentation time would be much more profitable in industrial scale compared to the extra enzyme yield. The decrease observed in the protease activity of strain I18 at 125 h could be because of the hydrolysis of the enzyme by the protease itself. The literature reports a broad incubation time ranging from 24 to 120 h for *Bacillus* strains, which includes our range as well [14,19,20].

3.6. Effect of media pH

The effect of initial media pH was determined for strains L18 and L21, by adjusting the Horikoshii media to different initial pH using 6 N NaOH. Inoculation was performed with 5% (v/v) inoculum of each subculture and incubated at 37 °C and 180 rpm for 96 h. I18 was not taken in this set of experiment since, L18 and L21 strains were the promising strains for high protease activities and further study was performed on these two strains. The protease activity and optical density profiles (Fig. 2) at different pH values demonstrated that, L21 has a much broader pH range than L18. This suggested that strain L18 was a strict alkaline with a narrow pH range, whereas L21 might range to be

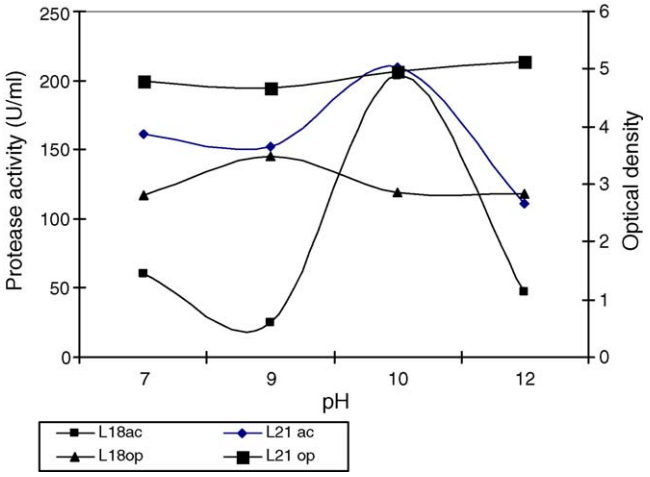

Fig. 2. Growth pH profiles of L18 and L21 at 37 °C and 180 rpm in standard Horikoshii media incubated for 96 h ("ac" stands for activity; "op" stands for optical density absorbance).

a neutral to alkaline. Strain L21 seemed to be much more stable against pH changes with respect to protease activity; however, same conclusion could not be drawn for the other strain. Cell growth seemed to be effected by pH changes similarly for both strains; even so L18 has overall a lower optical cell density than L21. With this regard L21 seemed to be a better candidate for further study.

3.7. Yield factors at optimum conditions

Biomass and product yield factors were estimated using the protease activities reported in Table 1 and carbon utilization profiles in Fig. 3. The predetermined optimum conditions as given in Section 3.5 were used with 96 h of incubation. The inoculation ratio for all three strains was 5% (v/v). The initial pH of the Horikoshii media was 10, which was already determined to be the optimum pH for L18 and L21. In the calculations of the yield factors maintenance effects and endogenous metabolism were neglected, the biomass was the dry cell weight of the culture. An inverse relationship between biomass and product yield was observed (Table 3). This relation was very much pronounced

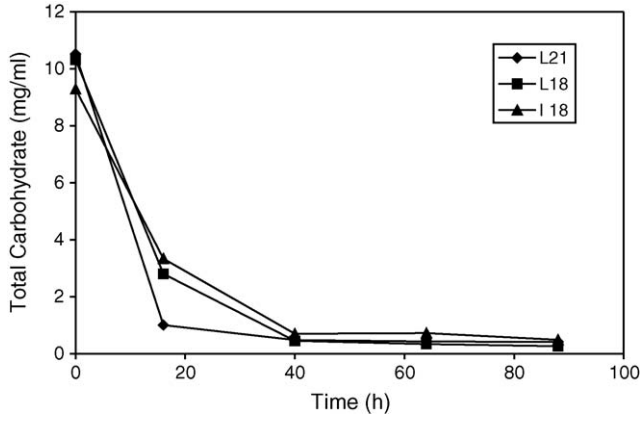

Fig. 3. Total carbohydrate profiles of I18, L18 and L21 at optimized fermentation conditions.

Table 3
Biomass and product yields of strains I18, L18 and L21 at optimized conditions after 96 h of incubation

Strain	Biomass ($Y_{x/S}$) (g dcell/g glucose)	Product yield ($Y_{P/S}$) (U protease/g glucose)
I18	0.017	0.014
L18	0.011	0.022
L21	0.023	0.021

for strain L18 compared to the other two. Therefore, one could conclude that the enzyme production was not growth related. However, for the other two organisms (I18 and L21) fermentation conditions favoring the growth seemed not to adversely effect the product formation. These results were confirmed with carbohydrate utilization profiles (Fig. 3), which showed that the carbohydrate was faster utilized in strain L21 compared to the others. This brought up the issue, whether controlled carbohydrate supply at the end of 40 h would increase product formation of this strain (L21) further or not. Therefore, this would be a task to be studied in future. Another drawback would be that faster carbohydrate consumption would increase raw material cost. Therefore, a balance between the cost of the raw material and the gain from the product yield has to be established. With this respect strain L18 seemed to be a more efficient strain with less carbohydrate consumption but still with higher product yield. Since, this strain resulted in lower specific activity with less pH stability, it was not considered for further study. Comparison among the strains demonstrated that, strain L21 was the strain with high biomass and product yield. The biomass and product yield for this strain was determined as 0.023 g cell/g glucose and 0.021 U/g glucose, respectively.

Table 4
Effect of various reagents on activity of alkaline protease

Agent	Residual activity (%)
Control	100.0
H_2O_2 (5%, v/v)	81.8
H_2O_2 (15%, v/v)	93.6
EDTA (0.01 M)	92.8
$CaCl_2$ (0.01 M)	89.7
PMSF (0.01 M)	1.5

3.8. Characterization of the crude enzyme obtained from L21

Since strain L21, demonstrated a higher potential for alkaline protease activity, it was world wide to further characterize this enzyme and determine its potential applications. Therefore, a preliminary study, as outlined in Section 2 on the characterization of this enzyme was carried out. According to this study (Fig. 4 and Table 4), it was determined, that this enzyme was bleach stable and belonged to the serine alkaline family with an optimum temperature of 60 °C and a pH of 11. This conclusion was based on the findings that this enzyme retained 93% of its activity after 1 h of incubation with EDTA and was completely inhibited by 0.01 M PMSF. As it is known EDTA is a specific inhibitor of metallo-type protease and PMSF is known to sulphonate the essential serine residue in the active site of the protease, resulting in a total loss of enzyme activity [21]. This inhibition profile suggested that the protease produced from *Bacillus* sp. L21 belongs to the family of serine proteases. Many of the *Bacillus*-derived alkaline proteases reported so far, belong to the class of serine proteases [22]. In addition, the stability of

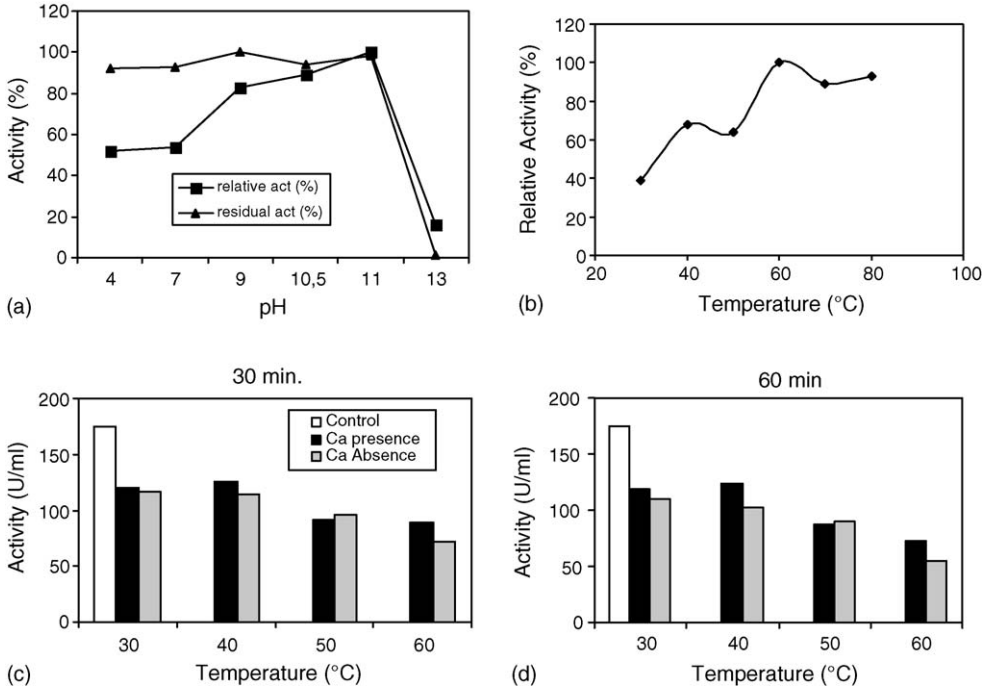

Fig. 4. (a) Effect of pH on activity and stability of *Bacillus* sp. L21; (b) effect of temperature on activity; (c) effect of temperature on the stability at 30 min incubation; (d) effect of temperature on the stability at 60 min incubation.

the enzyme in presence of EDTA is advantageous for its use as a detergent additive.

The crude protease retained 90% of its activity at 80 °C and was stable over a broad pH range from 4 to 11. It was also thermostable over a temperature range of 30–50 °C, retaining 30% of its activity at 60 °C after 1 h of incubation in the absence of Ca^{2+} ion. In the process of detergent formulation, where alkaline proteases are commonly added, chelating agents are included to overcome the problem of water hardness. In the presence of such chelating agents; however, the Ca^{2+} from the weak-binding site of the alkaline protease can easily be stripped-off thus greatly affecting the thermal stability of the detergent enzyme under application conditions. Therefore, enzymes such as the one introduced in this study, where the effect of Ca^{2+} for stability at low temperatures below 40 °C was not significant, could offer tremendous benefit for detergent application.

Another detail study on the growth medium (using various carbon, nitrogen and elements) optimization for this strain was carried out using response surface methodology. Soybean meal at 3 g/l, maltose 50 between the ranges of 30 and 40 g/l and tween 80 at 0.35 g/l produced an activity of 307 U/ml, which corresponded to 40% increase. [23].

4. Conclusion

In this paper three *Bacillus* strains I18, L18 and L21 isolated from local environment of Izmir Turkey region, with possible alkaline protease activity were compared and their fermentation conditions (temperature, agitation speed, inoculum concentration, incubation time and initial media pH) were optimized. Based on the results, strain I18 had optimum conditions as 30 °C, 100 rpm, and 5% inoculum with an incubation time of 96 h. Whereas these data were, 37 °C and 180 rpm, 5% inoculum and 96 h of incubation time for L18 and L2. In all experiment I18 strain was the less performing strain. L18 and L21 showed a closer performance with the main difference in their pH profiles. Taking all the results into account, L21 strain was the strain of choice for further optimization study and a potential candidate with alkaline proteolytic activity for future industrial applications. Based on the preliminary characterization of the crude enzyme, it was determined that, this enzyme was a bleach stable alkaline protease belonging to the serine alkaline family. The optimum pH and temperature were determined as 11 and 60 °C.

Overall, the highest protease activity (222 U/ml) achieved in this study was lower compared to the regular proteases used industrially, which are mainly in the purified form. One should also take into account that the present protease activity in this study is from a crude enzyme, obtained purely from a strain, which did not go through any strain improvement study in this regard. Furthermore, a characterization after purification and possible application study of this enzyme would be a task to do in future. With this respect this paper, is just an initial study conducted on three strains producing alkaline proteases with possible novel characteristics, carrying the potential to be candidate for industrial use. Considering the biodiversity of our environment it is highly important to discover new enzymes from isolates living under extreme conditions, which could have novel properties that could contribute to the current enzyme potential.

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References

[1] Banerjee CU, Sani RK, Azmi W, Soni R. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. Process Biochem 1999;35:213–9.
[2] Joo HS, Kumar CG, Park GC, Paik SR, Chang CS. Oxidant and SDS stable alkaline protease from *Bacillus clausii* I-52: production and some properties. J Appl Microbiol 2003;95:267–72.
[3] Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 2002;59:15–32.
[4] Joo HS, Kumar CG, Park GC, Kim KT, Paik SR, Chang CS. Optimization of the production of an extra cellular alkaline protease from *Bacillus horikoshii*. Process Biochem 2002;38:155–9.
[5] Rebecca BD, Pena-Vera MT, Diaz Castaneda M. Production of fish protein hydrolysates with bacterial proteases, yield and nutritional value. J Food Sci 1991;56:309–14.
[6] O'Meara GM, Munro PA. Selection of a proteolytic enzyme to solubilize lean beef tissue. Enzyme Microb Technol 1984;6:181–5.
[7] Tanimoto SY, Tanabe S, Watanabe M, Arai S. Enzymatic modification of zein to produce a non-bitter peptide fraction with a very Fisher ratio for patients with hepatic encephalopathy. Agric Biol Chem 1991;55:1119–23.
[8] Godfrey T, West S. Introduction to industrial enzymology. In: Industrial enzymology. 2nd ed. New York: Stocholm Press; 1996. p. 1–7.
[9] Horikoshii K. Extracellular enzymes. Isolation procedures for alkaline enzymes. In: Alkaliphiles. Tokyo: Kodensha Ltd.; 1999. p. 147.
[10] Akbalik G, Günes H, Yavuz E, Yasa I, Harsa S, Elmacı SZ, et al. Identification of extracellular enzyme producing alkalophilic bacilli from Izmir province by 16S-ITS rDNA RFLP. J Appl Microbiol 2004:766–73.
[11] Takami H, Akiba T, Horikaoshi K. Production of extremely thermostable alkaline protease from *Bacillus* sp. No. AH-101. Appl Microbiol Biotechnol 1989;30:120–4.
[12] Lowry OH, Rosebrough NJ, Farr AL, Randall JR. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.
[13] DuBois M. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28(3):350–6.
[14] Mabrouk SS, Hashem AM, El-Shayeb NMA, Ismail MS, Abdel-Fattah AF. Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC 21415. Bioresour Technol 1999;69:155–9.
[15] Çalık P, Özçelik ŞÖ, Çalık G, Özdamar TH. Enzyme ion exchanger interactions in serine alkaline protease production: theory, equilibria and kinetics. Biochem Eng J 2002;3623:1–12.
[16] Puri S, Khalil O, Gupta R. Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. Curr Microbiol 2002;44:286–90.
[17] Liu BL, Tzeng YM. Optimization of growth medium for the production of spores from *Bacillus thuringiensis* using surface response methodology. Bioprocess Eng 1998;18:413–8.
[18] Kanekar PP, Nilegaonkar SS, Sarnaik SS, Kelkar AS. Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. Bioresour Technol 2002;85:87–93.
[19] Beg QK, Gupta R. Purification and characterization of an oxidation stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. Enzyme Microb Technol 2003;32:294–304.

[20] Chu IM, Lee C, Li TS. Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. Enzyme Microb Technol 1992;14:755–61.

[21] Kumar CG. Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. Lett Appl Microbiol 2002;34:13–7.

[22] Gessesse A. The use of nug meal as a low-cost substrate for the production of alkaline protease by the alkaliphilic *Bacillus* sp. AR-009 and some properties of the enzyme. Bioresour Technol 1997;62:59–61.

[23] Tari C, Genckal H, Tokatli F. Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. Process Biochem, in press.