Biosynthesis of protease from Lactobacillus paracasei: Kinetic analysis of fermentation parameters

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Fifteen strains of Lactobacillus species, isolated from different samples of curd were screened for their ability to produce more extracellular protease. The proteolytic activities of these strains based on casein hydrolysis showed a variation of 1.26-5.80 U ml\(^{-1}\), with Lactobacillus IH\(_5\) showing the maximum activity and was identified as L. paracasei. Different cultural conditions for enhanced production of protease by L. paracasei were optimized. The optimal conditions for production of the enzyme were an incubation temperature of 35°C and a medium p\(\mathrm{H}\) of 6.0. The maximum proteolytic activity of L. paracasei (7.28 U ml\(^{-1}\)) was achieved after 48 h of cultivation. The kinetic parameters such as product yield (\(Y_{\text{p},\text{r}}\)), growth yield (\(Y_{\text{w},\text{r}}\)), specific product yield (qp) and specific growth yield (qs) coefficients also revealed that the values of experimental results were kinetically significant.

**Keywords:** Protease, Lactobacillus, Kinetics, Medium p\(\mathrm{H}\), Incubation temperature

Proteases are the enzymes that hydrolyze the peptide linkages of proteins to simpler proteins, peptides and amino acids. Unlike other enzymes, they are considered as mixture of enzymes\(^1\) and include proteinases, peptidases and amidases, which hydrolyze intact proteins, peptides or peptones and amino acids, respectively. They are generally divided into three broad categories:\(^2\) i) acidic proteases which show maximum activity between p\(\mathrm{H}\) 2.0-5.0; ii) neutral proteases, with a maximal activity at p\(\mathrm{H}\) 7.0 or around 7.0 and are inhibited by metal chelating agents\(^3\), and iii) alkaline proteases having maximum activity at p\(\mathrm{H}\) 9.0-11.0 and are unaffected by metal chelating agents and cleave a wide range of peptide bonds\(^4\). A wide range of microorganisms in their extracellular environment produces proteases. Extracellular proteases are important for the hydrolysis of protein substrates in the cell-free environment and enable the cell to absorb and utilize the hydrolytic products of the substrates\(^5\). They have also been exploited commercially for protein degradation in a number of industrial processes\(^6\).

Lactic acid bacteria (LAB) play an important role in dairy and meat fermentation processes and have a great influence on the quality and preservation of the end products. As they produce lactic acid, thus causing the lowering of p\(\mathrm{H}\) and the proteolysis of the substrate to release peptides and free amino acids, which subsequently affect the flavor and texture of the end products\(^7\). A large number of LAB species have been employed for the production of yogurt and different types of cheeses, therefore, the study of their proteolytic system has received much attention in the recent years. Their proteolytic system consists of cell envelope-associated proteinases (Prt P), specific peptide and amino acid transport systems and several cytoplasmic peptidases\(^8\).

A fairly large number of Lactobacillus species are known to produce various types of proteolytic enzymes\(^8\). The most commonly used species for the purpose include Lactobacillus bulgaricus\(^9\), L. rhamnosus\(^10\), L. casei\(^7,11\), L. paracasei\(^12\), L. helveticus and L. delbrueckii\(^13,14\), L. brevis, L. cellobiosus, L. fermentum and L. plantarum\(^15\). Many of these species are used as starters in cheese manufacturing; however, some are the non-starter usual floras of cheeses. The proteolytic enzymes of Lactobacillus species are highly specific and can be loosely grouped into a Pr/P\(_{\text{Ir}}\)-type\(^16\). However, some Lactobacillus proteases have relatively broad specificity, resulting in a large number of possible cleavage sites on the substrate\(^17\).

In the present investigation, the proteolytic activities of various Lactobacillus species isolated from the curd, optimization of the cultural conditions

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for maximum production of protease and kinetic analysis of the results have been studied.

**Materials and Methods**

**Organism, growth and maintenance**

A potent *Lactobacillus* strain was isolated from the curd using nutrient agar casein plates. The strain was isolated on the basis of clear zone of casein hydrolysis. The culture was grown and maintained on nutrient agar-lactose medium. The bacterial slopes were incubated at 37°C for 48 h and then stored at 4°C. However, the strain was transferred weekly on to the fresh slopes.

**Culture characterization**

All the selected isolates were morphologically and biochemically characterized. Cell morphology, Gram staining, biochemical tests, carbohydrate fermentation profile and the ability to produce lactic acid were studied to confirm the genus designation. The most potent strain was identified with the help of Bacterial Identification Kits (Oxoid, UK).

**Inoculum preparation**

The inoculum of *Lactobacillus* was prepared in 250 ml Erlenmeyer flasks containing 50 ml of nutrient broth-lactose medium. The flasks were sterilized in an autoclave at 121°C (15 lb/in² pressure) for 15 min. After cooling, the medium was aseptically inoculated with a loopful of bacteria from a 48 h old slope. The flasks after inoculation were incubated for 24 h on a rotary incubator shaker (Gallenkamp, UK) at 37°C and 200 rpm.

**Fermentation experiments**

The fermentation experiments for production of protease from *L. paracasei* were carried out in 250 ml shake flasks. The 50 ml of fermentation medium containing (mg/ml): Tryptone, 15; yeast extract, 5.0; meat extract, 5.0; MgSO₄·7H₂O, 0.2; MnSO₄·4H₂O, 0.05; Tween 80, 1 ml (pH 6.5) and 2% lactose and 0.5% glucose separately autoclaved, was added to each flask and sterilized in an autoclave. After cooling, the medium was inoculated with already prepared 1 ml of the inoculum containing 3-4 × 10⁸ CFU/ml. The flasks were then placed in a rotary type incubator shaker (Gallenkamp, UK) for the said interval of time. After incubation, fermented broth was centrifuged at 5,000 rpm (Sigma Laboratory Centrifuge, Model: 3K30, Germany) for 10 min and the supernatant was assayed for protease activity.

**Assay of protease**

The assay of protease was carried out as described previously¹⁸. Casein (1% solution in 0.1 M Phosphate buffer of pH 6.0) was incubated with 1 ml of enzyme sample at 30°C for 1 h. The reaction was arrested by addition of 5 ml of 5% trichloroacetic acid (TCA) solution. The mixture was centrifuged at 5000 rpm for 10 min and 1 ml of supernatant was mixed with 5 ml of alkaline reagent. To this mixture, 1 ml of 1 N NaOH was added to make the contents of the tube alkaline. After 10 min, 1 ml of Folin and Ciocalteau reagent (diluted with distilled water at ratio 1:1) was added to the test tubes and mixed. The blue colour produced was measured with UV spectrophotometer (CECIL, CE 7200, Cambridge, England) at 700 nm after 30 min.

One unit of protease activity was defined as the amount of enzyme required to produce an increase of 0.1 in optical density at 700 nm under the defined conditions.

**Analysis of residual sugar**

The amount of residual sugar in the fermented broth was measured by DNS method¹⁹.

**Kinetic and statistical analysis**

The experimental data were statistically analyzed by the method described previously²⁰. Duncan’s multiple range test was applied under one-way ANOVA. Significance was presented in the form of probability (p ≤ 0.05) values.

Kinetic parameters for batch fermentation experiments were determined according to the methods described previously²¹,²². The following kinetic parameters were studied: i) Maximum specific growth rate (µ) max per h – The value of (µ) max was calculated from plot of ln x vs. time of fermentation; ii) Product yield coefficient (Y p/x = U/mg: the amount of enzyme produced per amount of biomass): Its value was determined by the equation: Y p/x = dp/dx; iii) Growth yield coefficient (Y s,y = mg/mg: the amount of biomass produced per amount of sugars consumed): Its was determined by the equation: Y s,y = dx/ds; iv) Specific product yield coefficient (q p = U/mg/h: the amount of enzyme produced per amount of sugar consumed): The value of q p was determined by the equation: q p = Y p/x . (µ) max; and v) Specific growth yield coefficient (q s = mg/mg/h: amount of biomass produced per amount of sugars consumed per h): Its value of q s was determined by the equation: q s = Y s/s . (µ) max.
Results and Discussion

Screening of isolates

Fifteen different strains of *Lactobacillus*, which were capable of producing protease were screened for higher production of the enzyme (Table 1). Among all the strains screened, *Lactobacillus* IH₈ gave the maximum production of protease (5.80 U ml⁻¹) and thus was selected for subsequent investigations.

Culture characterization

Proteolytic isolates were morphologically and biochemically characterized. As shown in the Table 2, the strains were non-motile, anaerobic, Gram-positive and rod-shaped bacteria, showing negative catalase and benzidine tests. The strains were capable of fermenting glucose, fructose, lactose and mannose, but not galactose and produced lactic acid as the main end product. They were confirmed from Bergey’s Manual of Determinative Bacteriology that the isolates belonged to the genus *Lactobacillus*. The strain IH₈ was identified as *L. paracasei* with the help of Bacterial Identification Kits.

Effect of medium pH

The pH of the culture medium is one of the most important factors in most studies on microbial cultures. So, the effect of pH of medium on the production of protease by *L. paracasei* was investigated and the results are shown in Fig. 1a. The optimum pH of the fermentation medium for growth of *L. paracasei* (8.60 mg/ml) and subsequent production of protease (7.20 U ml⁻¹) was 6.0. So, it was inferred that the organism was an acidophile and grew well in an acidic medium. All other values of the medium pH (4.5-8.0) supported less growth of the organism (5.0-8.0 mg/ml) and showed decreased production of protease (4.42-6.82 U ml⁻¹). Earlier studies also reported the optimum pH for production of protease by *Lactobacilli* as 6.0. While, pH 6.5 was found to be the best medium pH for protease production from *L. rhamnosus*.

| Table 1—Screening of *Lactobacillus* species for the production of protease in shake flasks
| Values given are mean of three parallel replicates with a standard deviation of each. Incubation period, 48 h; incubation temperature: 35 ± 1°C; medium pH, 6.5 |
| Strain | Protease activity (U ml⁻¹) | Strain | Protease activity (U ml⁻¹) |
| IH₁ | 2.34 ± 0.015 | IH₈ | 5.80 ± 0.02 |
| IH₂ | 4.40 ± 0.030 | IH₉ | 4.00 ± 0.041 |
| IH₃ | 1.82 ± 0.005 | IH₁₀ | 2.24 ± 0.020 |
| IH₄ | 3.22 ± 0.02 | IH₁₁ | 1.26 ± 0.020 |
| IH₅ | 2.88 ± 0.015 | IH₁₂ | 3.68 ± 0.015 |
| IH₆ | 1.42 ± 0.030 | IH₁₃ | 2.94 ± 0.011 |
| IH₇ | 5.20 ± 0.030 | IH₁₄ | 2.72 ± 0.005 |
| IH₁₅ | 3.20 ± 0.041 |

Least significance difference (LSD) = 0.03349; Significance level = 0.05; Non-significant ranges = a-n; letters in superscript shows that the values differ significantly

Table 2—Morphological and biochemical characterization of the isolates

| Morphology | Straight rods |
| Motility | Non-motile |
| Gram’s staining | Positive |
| Growth in anaerobic conditions | Positive |
| Production of lactic acid as main end product | Positive |
| Catalase | Negative |
| Benzidine reaction | Negative |
| Carbohydrate fermentation profile | Capable of fermenting glucose, fructose, lactose and mannose, but not galactose |

Fig. 1—(a): Effect of medium pH on the production of protease by *Lactobacillus paracasei* [Each value is an average of three parallel replicates. Y error bars indicate the standard error of mean. Incubation period, 48 h; incubation temperature, 35 ± 1°C]; (b): Product and growth yield coefficients (Yₚₓ and Yₓₛ) for protease production by *L. paracasei* at different medium pH; and (c): Specific product and growth yield coefficients (qp and qx) for protease production by *L. paracasei* at different medium pH.
Kinetic analysis of results of revealed that the values of product yield coefficient ($Y_{p/x}$) showed a similar trend as the experimental values (Fig. 1b). However, the maximum value of growth yield coefficient ($Y_{x/s}$) was observed at pH 6.5, instead of 6.0, the optimal growth pH, so it can be inferred that the organism showed maximum growth at pH 6.5, in terms of cell mass production per unit substrate consumption. The values of specific product yield coefficient ($qp$) and specific growth yield coefficient ($qx$) also showed similar results (Fig. 1c). Present experiments also demonstrated that the organism was very sensitive to pH change towards alkaline values, because a slight increase in optimum pH resulted in much-reduced biomass and protease production.

**Effect of incubation temperature**

Effect of incubation temperature on the production of protease by *L. paracasei* was studied by varying the incubation temperature of fermentation flasks from 25-50°C. The maximum growth (8.40 mg/ml) and production of protease (7.10 U ml$^{-1}$) was achieved at an incubation temperature of 35°C (Fig. 2a). The strain was able to grow well even at 50°C (5.02 mg/ml, dry cell mass basis), but the optimum temperature for enzyme production was 35°C.

Kinetic analysis of results revealed the maximum value of product yield coefficient ($Y_{p/x}$) at incubation temperature of 35°C, which was in accordance with the experimental results (Fig. 2b). But the values of growth yield coefficient ($Y_{x/s}$) at incubation temperature of 35 and 40°C were almost same, indicating that the growth of *L. paracasei*, in terms of substrate consumption was same at both the temperatures. The specific product yield coefficient ($qp$) and specific growth yield coefficient ($qx$) also showed the similar values, as obtained during the experiments (Fig. 2c).

![Fig. 2](image1)

**Fig. 2**—(a): Effect of incubation temperature on production of protease by *L. paracasei* [Each value is an average of three parallel replicates. Y error bars indicate the standard error of mean. Incubation period, 48 h; medium pH, 5.5]; (b): Product and growth yield coefficients ($Y_{p/x}$ and $Y_{x/s}$) for protease production by *L. paracasei* at different incubation temperatures; and (c): Specific product and growth yield coefficients ($qp$ and $qx$) for protease production by *L. paracasei* at different incubation temperatures

![Fig. 3](image2)

**Fig. 3**—(a) Effect of incubation period on the production of protease by *L. paracasei* [Each value is an average of three parallel replicates. Y error bars indicate the standard error of mean. Medium pH: 5.5; incubation temperature: 35 ± 1°C]; (b): Product and growth yield coefficients ($Y_{p/x}$ and $Y_{x/s}$) for protease production by *L. paracasei* at different incubation periods; and (c): Specific product and growth yield coefficients ($qp$ and $qx$) for protease production by *L. paracasei* at different incubation periods
Effect of incubation time

Incubation time of a fermentation experiment has a direct relationship with the production of enzymes, so the effect of incubation time on production of protease and bacterial growth was investigated by incubating the experimental flasks for variable time intervals ranging from 12 to 72 h. The maximum production of protease (7.14 U ml\(^{-1}\)) and bacterial biomass (8.66 mg/ml) were achieved after 48 h of incubation (Fig. 3a), which corresponded to the logarithmic growth phase of the bacterium. The incubation period of more or less than 48 h did not show the promising results, as far as production of the enzyme was concerned. However, there was a steady decrease in the amount of protease produced by L. paracasei after 48 h of incubation. Earlier reports\(^ {26,27} \) also showed that Lactobacilli start producing proteases in the early exponential growth phase, but the maximum production usually occurred in the logarithmic growth phase.

Kinetic analysis of the parameter showed that the maximum amount of enzyme per unit biomass (\(Y_{ps} \)) was produced after 48 h of incubation. Also, the value of growth yield coefficient (\(Y_{sb} \)) was maximum at the start of fermentation, which declined gradually and reached minimum after 72 h (Fig. 3b). A rapid growth of organism was observed at the start of fermentation and then the growth profile became static, followed by the higher secretion of the enzyme. The values of specific product yield coefficient (\(q_p \)) and specific growth yield coefficient (\(q_s \)) were also maximum after 48 h of incubation (Fig. 3c).

Conclusion

Lactobacillus paracasei, a potent strain for the production of acid protease was isolated from curd. The strain may find use as a starter culture in the manufacture of cheese or the proteases produced by it can be directly used in cheese making.

References

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