

# EFFECT OF CULTURE PARAMETERS ON PROTEASE AND CELLULASE PRODUCTION BY TWO BACTERIAL STRAINS, *Corynebacterium alkanolyticum* ATH3 AND *Bacillus licheniformis* CBH7 ISOLATED FROM FISH GUT

GOUTAM BANERJEE<sup>1,2</sup>, ANKITA NANDI<sup>1</sup> and ARUN KUMAR RAY<sup>1,\*</sup>

<sup>1</sup>Department of Zoology, Visva-Bharati University, Santiniketan 731235, India

<sup>2</sup>Department of Biochemistry, University of Calcutta, Kolkata 700019, India

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## ABSTRACT

Microbial protease and cellulase are in high demand by different industries due to their minimal cost and availability. This study was aimed to maximize the production of protease and cellulase using two bacteria, *Corynebacterium alkanolyticum* ATH3 and *Bacillus licheniformis* CBH7, isolated from fish gut. This study demonstrated the effect of different culture parameters in protease and cellulase production using two different bacterial strains. Results of this study clearly indicated the importance of different parameters such as moisture content, pH, incubation temperature, incubation period, inoculum size, carbon sources and nitrogen sources in enzyme production. The most critical parameters affecting the enzymes production were pH, temperature, carbon and nitrogen sources. Further investigations are required to enhance the enzymes production using genetic engineering.

**Keywords:** Bacterial strains, culture parameters, product maximization, protease and cellulase

## INTRODUCTION

Protease catalyzes the hydrolysis of protein and cellulose forming amino acids, while cellulase catalyzes glucose (Ray *et al.* 2012a). Protease and cellulase are biotechnologically very important and mainly isolated from different living organisms such as plants, bacteria and fungi (Saddler 1993; Gupta *et al.* 2002; Banerjee *et al.* 2015b). Gut bacteria are proven to produce various substances such as vitamin K, riboflavin, and various enzymes (e.g. protease, amylase, cellulase, lipase, phytase) (Ray *et al.* 2012a). Proteases isolated from microbial source are preferred over the enzymes isolated from plant and animal sources because they possess all the characteristics required for their biotechnological application (Rao *et al.* 1998; Beg & Gupta 2003). Proteases constitute 50 – 65% of the global industrial enzymes market (Banerjee *et al.* 2015b). Proteases from microbial sources are widely used in different industries such as food, textile and

beverages (Pastor *et al.* 2001). Proteases are useful for preparing high quality functional feeds through bioconversion of low-cost feed materials (Esakkiraj *et al.* 2007). Among several bacterial strains, genus *Bacillus* is considered to be the most important commercial enzyme producers (Ray *et al.* 2012a; Banerjee & Ray 2016).

Cellulose, a polymer of glucose, is the primary structural component of most plant cell walls. Although this polysaccharide is the most common carbohydrate on earth, relatively few animals are able to utilize this resource efficiently (Goodenough & Goodenough 1993; He *et al.* 2015). Utilization of cellulose as a nutrient source requires enzymes that cleave beta-1, 4 glycosidic bonds between constituent sugars. The enzyme referred to as cellulases, are required to split beta-1, 4 glycosidic bonds in the polymer to release glucose units (Barr *et al.* 1996). Although a large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of extracellular enzymes capable of completely hydrolyzing crystalline cellulose *in vitro*. Fungi are

\* Corresponding author: aray51@yahoo.com

the main cellulase-producing microorganisms, though a few bacteria and actinomycetes are also recently reported to be good producer of cellulase (Ray *et al.* 2012a; Lugani *et al.* 2015; Banerjee & Ray 2016).

Microbial enzymes have the advantage of large scale production using established fermentation techniques. A successful fermentation process requires favorable environmental and nutritional conditions for the microorganisms (Ray *et al.* 2007). Information on the optimum conditions for protease and cellulase production using fish gut bacteria is limited (Esakkiraj *et al.* 2007; Ray *et al.* 2007, 2012b). This study was aimed to optimize the environmental and nutritional parameters to enhance protease and cellulase production using two bacterial strains, *Corynebacterium alkanolyticum* ATH3 and *Bacillus licheniformis* CBH7, isolated from the gut of climbing perch, *Anabas testudineus* and walking catfish, *Clarias batrachus*, respectively.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Medium

Two bacterial strains *Corynebacterium alkanolyticum* ATH3 (GenBank accession no. JX656749) and *Bacillus licheniformis* CBH7 (GenBank accession no. HQ005269) were isolated from the gut of two species of Indian air-breathing fish, i.e. climbing perch, *Anabas testudineus* and walking catfish, *Clarias batrachus*, respectively (Banerjee & Ray 2013; Banerjee *et al.* 2015a). Both isolates were cultured in Tryptone Soya Agar broth for 24 hours at 30 °C to increase the viable count. The bacterial culture medium was prepared according to Ray *et al.* (2012b).

### Enzyme Assay

Extracellular protease activity was assayed following the method of Walter (1984). The amount of tyrosine released in the assay medium was measured at 273 nm. Cellulase activity was estimated according to the method of Denison and Kohen (1977). The production of reducing sugar (glucose) from CMC substrate through cellulolytic activity was measured at 540 nm. One unit of specific activity is defined as amount of product released per milligram protein per milliliter enzyme per minute. Protein

concentration was determined according to Lowry *et al.* (1951) using bovine serum albumin as a standard.

### Effect of Different Physical Parameters on Enzyme Production

#### *Moisture content maximization*

In order to determine the optimum moisture content, bacteria were cultured in protease (peptone-gelatin) and cellulase media (carboxymethylcellulose), which were prepared by moistening the media with basal salt (elaborate basal salt) solution. Moisture content of the fermentation medium ranged from 5 to 75%.

#### *Effect of pH of the medium*

Optimization of suitable pH for enzymes production was determined by culturing the bacterial strains in Tryptone Soya Agar broth at different pH ranging from 5.0 - 10.0.

#### *Incubation temperature maximization*

Optimum temperature of incubation for protease and cellulase production was determined by culturing the bacterial strains at increasing temperature ranging from 20 to 50 °C with 10 °C intervals.

#### *Incubation period maximization*

Incubation period is one of the most important parameter in fermentation process. To determine the optimum incubation period, enzymes activity was measured up to 120 hours at an interval of 24 hours.

#### *Effect of inoculum size*

For this purpose, fermentation media were seeded with 1%, 2%, 3%, 4%, 5% and 6% seed culture in Tryptone Soya Agar broth.

#### *Effect of different carbon sources*

In order to optimize the suitable carbon source for enzyme production, fermentation media were prepared with different carbon sources such as glucose, maltose, sucrose, lactose, arabinose, fructose and starch. These alternative carbon sources at 0.2% level were used to replace the original carbon source in the media.

#### *Effect of different nitrogen sources*

To optimize the appropriate nitrogen source, the fermentation media were supplemented with both organic (arginine, L-asparagine, tryptone, beef extract, tyrosine, gelatin) and inorganic

(ammonium chloride, ammonium sulphate, sodium nitrate and potassium nitrate) nitrogen sources at 0.2% level replacing the original nitrogen source in the media.

### Statistical Analysis

The analysis of variance for different values was carried out in Microsoft Excel 2007. One way ANOVA followed by Duncan's Multiple Range Test (Duncan 1955) was conducted at significance level of  $p < 0.05$ .

## RESULTS AND DISCUSSION

Protease and cellulase production using the two bacterial strains at different moisture content (5 - 75%) are tabulated in Table 1.

Protease production using the two bacterial strains was the highest at 10% moisture content ( $3.79 \pm 0.155$  U in *Corynebacterium alkanolyticum* ATH3 and  $2.16 \pm 0.132$  U in *Bacillus licheniformis* CBH7). Maximum cellulase activity was recorded at 15% moisture content ( $8.34 \pm 0.144$  U in *C. alkanolyticum* ATH3 and  $6.17 \pm 0.115$  U in *B. licheniformis* CBH7). Medium maximization was the first and foremost step to enhance enzymes production in laboratory experiments and in fermentation industries. Each and every element should be in a proper amount to fulfill the

metabolic process requirements to supply sufficient energy for maximum biomass production (Ray *et al.* 2012b). Cellulase production reached the maximum level using two bacterial strains *Bacillus subtilis* and *Bacillus circulans* at 10% moisture content (Ray *et al.* 2007). Protease production reached the optimum level using *Bacillus cereus* AT at 120% moisture content (Vijayaraghavan *et al.* 2014). Moisture content was a crucial factor for producing bacterial extracellular enzyme, because water was required for growth, metabolic activities and biochemical process. Thus, optimum moisture content was required during fermentation process.

pH of the culture medium was another important factor controlling enzyme production and activity. In the present study, protease production exhibited by *C. alkanolyticum* ATH3 was maximum at pH 7.5 ( $3.81 \pm 0.096$  U), whereas *B. licheniformis* CBH7 showed the highest production at pH 8.0 ( $2.22 \pm 0.204$  U (Table 2).

On the other hand, cellulase production exhibited by these two bacterial strains was recorded to be the highest at pH 7.5 ( $8.41 \pm 0.83$  U in *C. alkanolyticum* ATH3 and  $6.27 \pm 0.216$  U in *B. licheniformis* CBH7, Table 2) and thereafter declined rapidly. Cellulase production using *Bacillus subtilis* CY5 isolated from the gastrointestinal (GI) tracts of common carp, *Cyprinus carpio* and using *B. circulans* TP3 isolated from the GI tracts of tilapia, *Oreochromis*

Table 1 Effect of moisture content on enzyme production

| Moisture content (%) | Specific activity of protease (U <sup>1</sup> ) |                    | Specific activity of cellulase (U <sup>2</sup> ) |                    |
|----------------------|---|--------------------|--|--------------------|
|                      | ATH3  | CBH7               | ATH3   | CBH7               |
| 5                    | $2.79 \pm 0.110^b$                              | $1.24 \pm 0.045^c$ | $6.91 \pm 0.183^c$                               | $4.18 \pm 0.183^d$ |
| 10                   | $3.79 \pm 0.155^a$                              | $2.16 \pm 0.132^a$ | $7.56 \pm 0.266^b$                               | $5.13 \pm 0.098^b$ |
| 15                   | $2.63 \pm 0.098^c$                              | $1.71 \pm 0.055^b$ | $8.34 \pm 0.144^a$                               | $6.17 \pm 0.115^a$ |
| 20                   | $1.22 \pm 0.045^d$                              | $0.63 \pm 0.08^d$  | $4.25 \pm 0.158^d$                               | $4.69 \pm 0.238^c$ |
| 25                   | $0.71 \pm 0.051^e$                              | $0.58 \pm 0.043^d$ | $2.45 \pm 0.062^e$                               | $4.31 \pm 0.091^d$ |
| 30                   | $0.42 \pm 0.06^f$                               | $0.49 \pm 0.034^e$ | $2.29 \pm 0.034^e$                               | $3.45 \pm 0.111^f$ |
| 50                   | $0.33 \pm 0.036^f$                              | $0.66 \pm 0.036^d$ | $2.04 \pm 0.055^f$                               | $3.88 \pm 0.135^e$ |
| 75                   | $0.29 \pm 0.018^g$                              | $0.42 \pm 0.062^e$ | $1.38 \pm 0.045^g$                               | $2.77 \pm 0.078^g$ |

Note: Data are presented as mean  $\pm$  SEM. n = 3 replications.

Numbers followed by the same letter did not differ significantly at  $p < 0.05$ .

U<sup>1</sup> =  $\mu$ g of tyrosine liberated/mg protein/minute.

U<sup>2</sup> =  $\mu$ g of glucose liberated/mg protein/minute.

Table 2 Effect of pH on protease and cellulase production

| pH  | Specific activity of protease (U <sup>1</sup> ) |                          | Specific activity of cellulase (U <sup>2</sup> ) |                          |
|-----|---|--------------------------|--|--------------------------|
|     | ATH3  | CBH7                     | ATH3   | CBH7                     |
| 5.0 | 0.49± 0.055 <sup>f</sup>                        | 0.28± 0.052 <sup>f</sup> | 2.09± 0.075 <sup>f</sup>                         | 2.39± 0.249 <sup>e</sup> |
| 5.5 | 0.58± 0.043 <sup>f</sup>                        | 0.71± 0.07 <sup>e</sup>  | 3.82 ± 0.17 <sup>e</sup>                         | 2.95± 0.301 <sup>d</sup> |
| 6.0 | 1.74± 0.13 <sup>d</sup>                         | 1.13± 0.052 <sup>d</sup> | 4.90± 0.262 <sup>d</sup>                         | 3.48± 0.153 <sup>d</sup> |
| 6.5 | 2.78± 0.193 <sup>c</sup>                        | 1.56± 0.085 <sup>c</sup> | 5.77± 0.145 <sup>c</sup>                         | 4.16± 0.202 <sup>c</sup> |
| 7.0 | 3.45± 0.16 <sup>b</sup>                         | 1.73± 0.088 <sup>b</sup> | 7.55± 0.124 <sup>b</sup>                         | 5.20± 0.215 <sup>b</sup> |
| 7.5 | 3.81± 0.096 <sup>a</sup>                        | 1.94± 0.134 <sup>b</sup> | 8.41 ± 0.83 <sup>a</sup>                         | 6.27± 0.216 <sup>a</sup> |
| 8.0 | 1.61± 0.052 <sup>d</sup>                        | 2.22± 0.204 <sup>a</sup> | 4.13± 0.410 <sup>e</sup>                         | 6.09± 0.705 <sup>a</sup> |
| 8.5 | 0.86± 0.021 <sup>e</sup>                        | 1.77± 0.160 <sup>b</sup> | 2.68± 0.461 <sup>f</sup>                         | 5.10± 0.306 <sup>b</sup> |
| 9.0 | 0.31± 0.01 <sup>f</sup>                         | 0.95± 0.052 <sup>d</sup> | 1.43± 0.104 <sup>g</sup>                         | 4.49± 0.514 <sup>c</sup> |

Note: Data are presented as mean±SEM. n = 3 replications.

Numbers followed by the same letter did not differ significantly at  $p < 0.05$ .

U<sup>1</sup> = µg of tyrosine liberated/mg protein/minute.

U<sup>2</sup> = µg of glucose liberated/mg protein/minute.

*mossambicus* was higher in optimum pH range of 7.0 to 7.5 (Ray *et al.* 2007). Protease activity was maximum at pH range of 6.0 to 6.5 using two bacterial strains, *Bacillus licheniformis* BF2 isolated from the foregut of bata fish (*Labeo bata*) and *Bacillus subtilis* BH4 isolated from the hindgut of bata fish (*Labeo bata*) (Ray *et al.* 2012b). Increasing pH beyond optimum level could interfere with amino acid composition of the enzyme leading to the significant decrease of the enzyme activity (Esakkiraj *et al.* 2007). Optimum protease production using *B. subtilis* and the highest protease activity were reached at pH 10.0 (Pant *et al.* 2015). Sethi *et al.* (2013) reported optimum pH

required for producing cellulase using several soil bacterial species. In a detailed investigation, Fagade and Bamigboye (2012) also optimized culture media parameters for cellulase production. The results of their study showed that pH 7.0 was optimum for bacterial strains *Pseudomonas putida*, *Bacillus subtilis* and *Bacillus licheniformis* I, however, pH 5.5 was recorded to be the best for cellulase production using bacterial isolate *Bacillus licheniformis* II.

Incubation period is an important parameter for enzyme production by the microorganisms. Some microorganisms produce maximally in their exponential growth phase, while other

Table 3 Effect of fermentation period on enzymes production

| Fermentation period (hours) | Specific activity of protease (U <sup>1</sup> ) |                          | Specific activity of cellulase (U <sup>2</sup> ) |                          |
|-----------------------------|---|--------------------------|--|--------------------------|
|                             | ATH3  | CBH7                     | ATH3   | CBH7                     |
| 24                          | 1.98± 0.121 <sup>d</sup>                        | 0.36± 0.062 <sup>d</sup> | 3.87± 0.326 <sup>d</sup>                         | 2.14± 0.141 <sup>e</sup> |
| 48                          | 2.72± 0.141 <sup>b</sup>                        | 0.88± 0.141 <sup>c</sup> | 5.23± 0.248 <sup>c</sup>                         | 2.99± 0.283 <sup>d</sup> |
| 72                          | 3.06± 0.206 <sup>b</sup>                        | 1.57± 0.262 <sup>b</sup> | 8.53± 0.655 <sup>a</sup>                         | 4.58± 0.643 <sup>c</sup> |
| 96                          | 3.95± 0.206 <sup>a</sup>                        | 2.32± 0.113 <sup>a</sup> | 8.07± 0.370 <sup>a</sup>                         | 6.42± 0.471 <sup>a</sup> |
| 120                         | 2.32± 0.225 <sup>c</sup>                        | 1.61± 0.347 <sup>b</sup> | 6.29± 0.266 <sup>b</sup>                         | 5.47± 0.481 <sup>b</sup> |

Note: Data are presented as mean±SEM. n = 3 replications.

Numbers followed by the same letter did not differ significantly at  $p < 0.05$ .

U<sup>1</sup> = µg of tyrosine liberated/mg protein/minute.

U<sup>2</sup> = µg of glucose liberated/mg protein/minute.

microorganisms exhibited maximum production in their stationary growth phase. Bacterial strains, *C. alkanolyticum* ATH3 and *B. licheniformis* CBH7 exhibited the highest protease activity ( $3.95 \pm 0.206$  U and  $2.32 \pm 0.113$  U, respectively) at 96 hours fermentation period (Table 3).

Cellulase production exhibited by *C. alkanolyticum* ATH3 was maximum at 72 hours of incubation period ( $8.53 \pm 0.655$  U). *B. licheniformis* CBH7 exhibited maximum cellulase production at 96 hours ( $6.42 \pm 0.471$  U) (Table 3). Singh and Kaur (2012) optimized the culture condition for cellulase production using *Bacillus sphaericus* JS1 and recorded maximum cellulase production at 48 hours of incubation period. In the year 2013, Suganthi *et al.* (2013) also optimized the culture condition of *B. licheniformis* and stated that the maximum protease production occurred at 24 hours of incubation period. The optimization of incubation period is crucial for obtaining maximum product and thus, several investigations have been conducted in this direction (Shafee *et al.* 2005; Ray *et al.* 2007; Sethi *et al.* 2013). Bacterial growth physiology (lag, log, stationary and decline phase) in liquid medium depends on incubation period. In general, maximum enzymes are produced during the log phase and then decreased rapidly due to interference of secondary metabolites.

Inoculum size is a crucial factor in fermentation process, as the duration of bacterial lag phase is highly dependent on inoculum quantity. The culture used to inoculate the fermentation medium must be in a healthy

condition. In this present experiment, a wide range of inoculum size (1 to 5%) was used to get the maximum yield. Protease production by *C. alkanolyticum* ATH3 and *B. licheniformis* CBH7 was the highest when the inoculum size was 2% ( $4.04 \pm 0.132$  U) and 3% ( $2.39 \pm 0.243$  U), respectively (Table 4).

Cellulase activities exhibited by *C. alkanolyticum* ATH3 ( $8.58 \pm 0.340$  U) and *B. licheniformis* CBH7 ( $6.51 \pm 0.473$  U) were found to be the highest at 3% inoculum size (Table 4). Inoculum quantity normally used is between 3% and 10% of the medium volume (Lincoln 1960; Meyrath & Suchanek 1972; Hunt & Stieber 1986). Lincoln (1960) stressed that bacterial inocula should be transferred in the logarithmic growth phase when the cells were still metabolically active. Shafee *et al.* (2005), however, reported that 4% is the optimum inoculum size for protease production in *B. cereus* strain 146. Similarly, Goyal *et al.* (2014) obtained the highest cellulase activity produced by *Bacillus* sp. at 1% inoculum size using rice straw as substrate. In recent years, the effects of inoculum sizes on protease and cellulase production have been investigated and published by several researchers (Suganthi *et al.* 2013; Agarwal *et al.* 2014; Lugani *et al.* 2015).

Temperature plays a critical role in maintaining normal body physiology in all living organisms, including bacteria. Maximum protease yield by *B. licheniformis* CBH7 was observed when incubation temperature was 40 °C ( $2.47 \pm 0.461$  U), while *C. alkanolyticum* ATH3 exhibited the highest protease production at 45 °C ( $4.18 \pm 0.57$  U) (Table 5).

Table 4 Effect of inoculum size on enzymes production

| Inoculum size (%) | Specific activity of protease (U <sup>1</sup> ) |                    | Specific activity of cellulase (U <sup>2</sup> ) |                    |
|-------------------|---|--------------------|--|--------------------|
|                   | ATH3  | CBH7               | ATH3   | CBH7               |
| 1                 | $2.98 \pm 0.208^b$                              | $0.82 \pm 0.06^c$  | $6.23 \pm 0.317^c$                               | $3.58 \pm 0.26^c$  |
| 2                 | $4.04 \pm 0.132^a$                              | $1.43 \pm 0.2^b$   | $7.16 \pm 0.416^b$                               | $5.63 \pm 0.70^b$  |
| 3                 | $3.10 \pm 0.384^b$                              | $2.39 \pm 0.243^a$ | $8.58 \pm 0.340^a$                               | $6.51 \pm 0.473^a$ |
| 4                 | $0.87 \pm 0.079^c$                              | $0.98 \pm 0.124^c$ | $3.23 \pm 0.245^d$                               | $3.42 \pm 0.205^c$ |
| 5                 | $0.21 \pm 0.069^d$                              | $0.27 \pm 0.036^d$ | $2.01 \pm 0.13^e$                                | $2.22 \pm 0.246^d$ |

Note: Data are presented as mean  $\pm$  SEM. n = 3 replications.

Numbers followed by the same letter did not differ significantly at  $p < 0.05$ .

U<sup>1</sup> =  $\mu$ g of tyrosine liberated/mg protein/minute.

U<sup>2</sup> =  $\mu$ g of glucose liberated/mg protein/minute.

Table 5 Effect of different temperature on enzymes activity

| Different temperature (°C) | Specific activity of protease (U <sup>1</sup> ) |                          | Specific activity of cellulase (U <sup>2</sup> ) |                          |
|----------------------------|---|--------------------------|--|--------------------------|
|                            | ATH3  | CBH7                     | ATH3   | CBH7                     |
| 20                         | 0.73± 0.081 <sup>c</sup>                        | 0.46± 0.043 <sup>c</sup> | 2.87± 0.182 <sup>d</sup>                         | 2.72± 0.25 <sup>d</sup>  |
| 30                         | 1.45± 0.105 <sup>d</sup>                        | 0.79± 0.19 <sup>c</sup>  | 5.13± 0.228 <sup>c</sup>                         | 3.79± 0.255 <sup>c</sup> |
| 35                         | 2.23± 0.246 <sup>c</sup>                        | 1.51± 0.173 <sup>b</sup> | 7.18± 0.55 <sup>b</sup>                          | 4.92± 0.56 <sup>b</sup>  |
| 40                         | 3.19± 0.42 <sup>b</sup>                         | 2.47± 0.158 <sup>a</sup> | 8.66± 0.762 <sup>a</sup>                         | 6.63± 0.34 <sup>a</sup>  |
| 45                         | 4.18± 0.42 <sup>a</sup>                         | 0.88± 0.131 <sup>c</sup> | 5.03± 0.232 <sup>c</sup>                         | 4.42± 0.482 <sup>b</sup> |
| 50                         | 1.99± 0.19 <sup>c</sup>                         | 0.45± 0.072 <sup>c</sup> | 1.89± 0.122 <sup>c</sup>                         | 2.34± 0.381 <sup>d</sup> |

Note: Data are presented as mean±SEM. n = 3 replications.

Numbers followed by the same letter did not differ significantly at  $p < 0.05$ .

U<sup>1</sup> = µg of tyrosine liberated/mg protein/minute.

U<sup>2</sup> = µg of glucose liberated/mg protein/minute.

On the other hand, the highest cellulase activity exhibited by these strains was observed at 40 °C (8.66±0.762 U for *C. alkanolyticum* ATH3 and 6.63±0.34 U for *B. licheniformis* CBH7) (Table 5). In this direction, Ray *et al.* (2007) observed and stated that 40 °C is the most effective temperature for cellulase production using *B. subtilis* CY5 and *B. circulans* TP3. Transport of enzymes to extracellular medium is lower at low temperature compared to that at high temperature (Rajoka 2004). However, at higher temperature, the high maintenance of energy requirement for cellular growth is needed due to thermal denaturation of enzymes of the metabolic pathway (Aiba *et al.* 1973), resulting in maximum production. Recently, Pant *et al.* (2015) reported 44 °C as the most effective temperature for protease production using *Bacillus subtilis*. On the other hand, Sethi *et al.* (2013) optimized the cellulase production using different soil bacterial strains and reported that 40 °C as the optimum temperature. Rasul *et al.* (2015) also isolated different bacterial strains from soil and sugar industries and optimized their cellulase production. Microorganisms grow slowly at a temperature below or above the normal growth temperature due to a reduced rate of cellular production (Ray *et al.* 2007). Therefore, it is important to maintain ambient temperature to obtain maximum production.

Effect of various carbon sources on protease and cellulase production were presented in Figure 1a and 1b, respectively.

Among seven carbon sources, fructose and sucrose were detected to be the most suitable carbon sources for protease production using *C. alkanolyticum* ATH3 (4.25±0.52 U) and *B. licheniformis* CBH7 (2.53±0.28 U). On the other hand, glucose was recorded to be more effective for cellulase production using *C. alkanolyticum* ATH3 (8.71±0.78 U) and *B. licheniformis* CBH7 (6.66±0.61 U). It is well established that carbohydrates induce most of the cellulolytic enzymes in bacteria and fungi (Morikawa *et al.* 1995). Rajoka and Malik (1997) used cellulose, cellulosic residues, xylan, cellobiose and CMC as carbon sources for the enhanced production of cellulases using *Cellulomonas* strain. Juhász *et al.* (2005) reported that high cellulolytic activity could be enhanced using steam-pretreated corn stover as carbon source. Carbon sources are very important for bacterial protease and cellulase production (Sethi *et al.* 2013; Suganthi *et al.* 2013; Pant *et al.* 2015).

Nitrogen sources are the building block of amino acids and considered to be the most important parameter in enzyme production. Among various inorganic and organic nitrogen sources, tryptone was detected to be the most important nitrogen source in both protease (4.33±0.388 U and 2.64±0.17 U) and cellulase (8.77±0.66 U and 6.72±0.38 U) production using *C. alkanolyticum* ATH3 and *B. licheniformis* CBH7 (Fig. 2a & 2b).

Recently, Lugani *et al.* (2015) tested the effect of different nitrogen sources (peptone, yeast

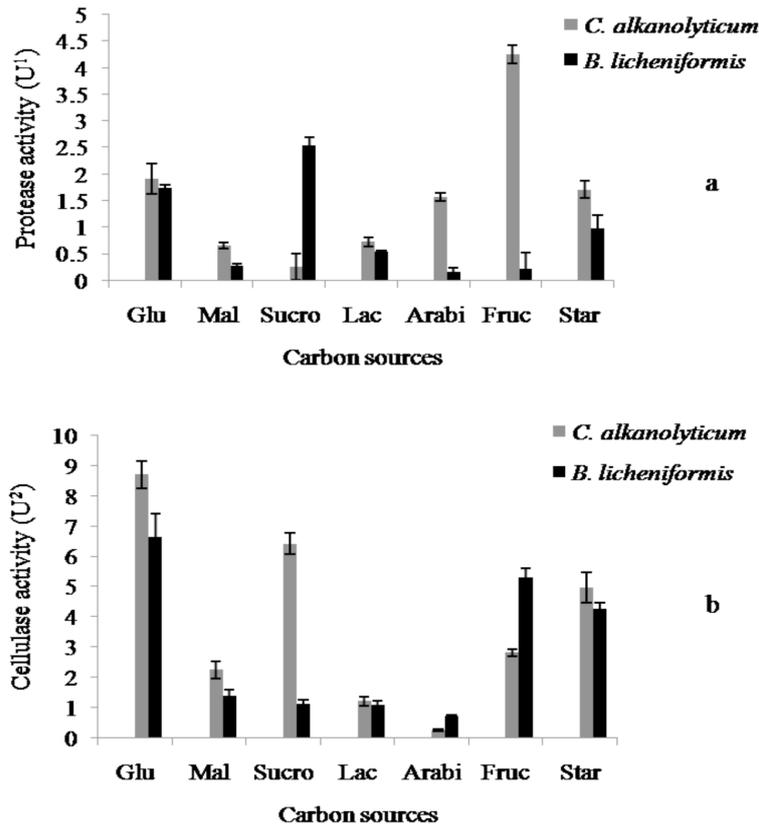


Figure 1 Effect of different carbon sources on enzyme production (Note: Glu = glucose; Mal = maltose; Sucro = sucrose; Lac = lactose; Arabi = arabinose; Fruc = fructose; Star = starch. Data are presented as mean±SEM. a = protease activity; b = cellulase activity. U<sup>1</sup> = µg of tyrosine liberated/mg protein/minute; U<sup>2</sup> = µg of glucose liberated/mg protein/minute)

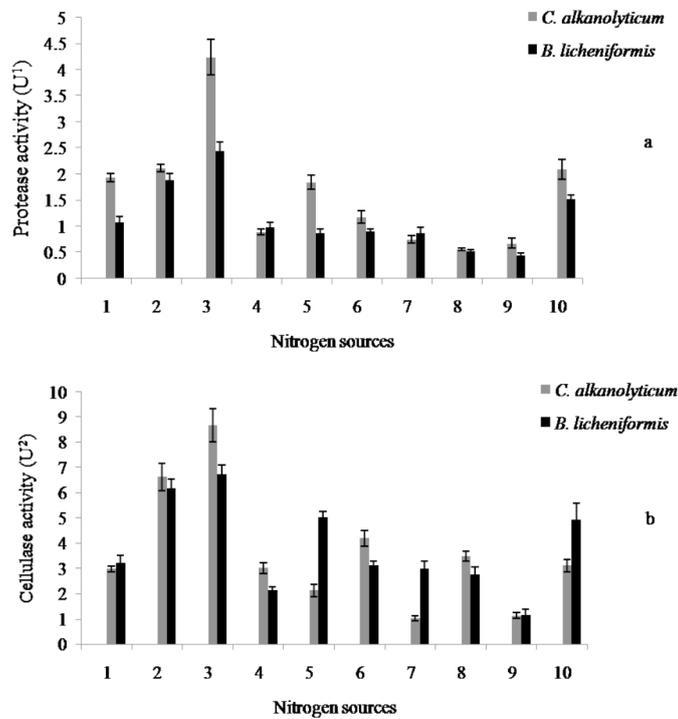


Figure 2 Effect of nitrogen sources on protease and cellulase production (Note: 1 = arginine; 2 = beef; 3 = tryptone; 4 = aspergine; 5 = tyrosine; 6 = NH<sub>4</sub>Cl; 7 = NaNO<sub>3</sub>; 8 = (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 9 = KNO<sub>3</sub>; 10 = gelatin. a = protease activity; b = cellulase activity. Data are presented as mean±SEM. U<sup>1</sup> = µg of tyrosine liberated/mg protein/minute; U<sup>2</sup> = µg of glucose liberated/mg protein/minute)

extract, ammonium sulphate, ammonium chloride and ammonium nitrate) on cellulase production from bacterial source. Results of their investigation reported peptone as the effective nitrogen source in culture medium of *Bacillus* sp. Y3. In this direction, Ray *et al.* (2007) stated that organic nitrogen sources exert better result compared to inorganic nitrogen sources in cellulase production using *Bacillus subtilis* CY5 and *B. circulans* TP3. In recent years, the effects of nitrogen sources on bacterial protease production have also been reported by several researchers (Ray *et al.* 2012b; Suganthi *et al.* 2013; Nisha & Divakaran 2014; Pant *et al.* 2015). In the present study, inorganic nitrogen sources and other amino acids such as L-asparagine and tyrosine were the poor nitrogen sources for cellulase production. On the contrary, Spiridonov and Wilson (1998) found that NH<sub>4</sub> compounds are the most favorable nitrogen sources for protein and cellulase synthesis.

Bacterial flora existing in digestive tract represents a very important and diversified enzymatic potential (Ray *et al.* 2012a; Banerjee *et al.* 2013; Banerjee & Ray 2016). Extracellular enzymes production using bacterial flora not only helps in digestion, but also has great application in different industries (Saha *et al.* 2006; Roy *et al.* 2009; Ringø *et al.* 2012; Banerjee & Ray 2016). Among several enzymes, microbial protease and cellulase have high demand in different biotechnological industries such as food, leather, beverage and detergent (Banerjee *et al.* 2015b). Production of these different types of extracellular enzymes largely depends on medium compositions and different physical factors (Ray *et al.* 2012b). Medium optimization is the most important step to enhance the production in laboratory experiments and in fermentation industries. Until now, reports on optimization of enzymes produced by gut bacteria are scanty, and thus, extensive research should be conducted to fulfill the demand.

## CONCLUSIONS

This study emphasized on production maximization of bacterial protease and cellulase due to their important contribution in several biotechnological industries. Bacterial strains *Corynebacterium alkanolyticum* ATH3 and *Bacillus*

*licheniformis* CBH7 as active producer of extracellular protease and cellulase, might be useful in different industries like bakery, dairy, meat, like textile, food, laundry, paper, biofuel and brewing sectors.

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