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# OPTIMIZATION OF THE CULTURE CONDITIONS FOR PROTEASE AND CELLULASE PRODUCTION BY TWO BACTERIAL STRAINS *BACILLUS AMYLOLIQUEFACIENS* (D4F1) AND *BACILLUS PUMILUS* (D4H3) ISOLATED FROM *LEMNA POLYRHIZA* FED FISH (*CIRRHINUS MRIGALA*) GUT.

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**ABSTRACT**
Cellulase and protease producing autochthonous bacteria were isolated from the gut regions of *Cirrhinus mrigala* (Hamilton) treated *with Lemna polyrhiza* meal. Two bacterial strains, *Bacillus amyloliquefaciens* D4F1 and Bacillus *pumilus* D4H3, isolated from the foregut and hindgut regions, respectively and were identified by both phenotypic characters and 16S rDNA sequence analysis. Cellulase and protease production was enhanced by optimizing the culture conditions. The effects of moisture, pH, temperature, fermentation period, inoculum size, different nitrogen sources, vitamins and surfactants on Cellulase and protease production by these two strains were evaluated. Cellulase and protease production by the two strains in an optimum pH range of 6.5-7.5. Minimum enzymes production was observed at 50-55$^{o}$C while maximum activity recorded at 40$^{o}$C. To standardize the fermentation period for cellulase and protease production, production rate was measured at 12-h intervals up to 120 h. Enzyme production increased at 96 h of fermentation in both strains. Best moisture content for this two isolates was 10-15% to produce both enzymes. Beef extract was the most promising nitrogen source for D4F1 and D4H3 for protease and D4F1 for cellulase production (except D4H3; tryptophan in cellulase production). In case of cellulase production, most promising carbon source was glucose for D4F1, fructose for D4H3 and for protease production, fructose and lactose were found to be ideal carbon source in case of D4F1 and D4H3, respectively.

**KEY WORDS:** Fermentation conditions, Fish gut bacteria, *Cirrhinus mrigala*, Gut enzymes, Optimization.

## INTRODUCTION

Gastrointestinal tract of a fish have the ability to adapt the changes in functional demands that take place during the life history (e.g., metamorphosis, anadromous or catadromous migrations and from day-to-day due to seasonal shifts in diet or environmental conditions) (Karila, 1998). They modulate the composition of digestive gut wall, exocrine pancreas and liver and allow fish rapidly and reversibly to alter the characteristics of the GI tract and other organ systems to adapt to changes in the contents of the GI tract, such as amounts and types of nutrients, pH, ionic composition, and to environmental conditions (Holst *et al*., 1996). These fluids contain a great range of compounds that may stimulate or inhibit the growth and composition of the intestinal microbiota. (Laubitz *et al*., 2003). The fish gut microbiota may help the host by producing a range of digestive enzymes like amylase, protease and lipase and also may help in degradation of cellulose by producing cellulase and other anti-nutrients like phytic acid, tannin etc. Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells. Enzymes are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries.

Microbial proteases account for approximately 60% of the total enzyme sales in the world (Banik and Prakash, 2004). Proteases are one of the most important groups of industrial enzymes with broad applications including meat tenderization, detergents, cheese-making, de-hairing, baking, waste management and silver recovery (Gupta *et al*., 2002). Of all proteases, alkaline proteases produced by Bacillus species are of significant importance in detergent industry due to their high thermal and pH stability.

Isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process of enzyme production for industrial uses (Parekh *et al*., 2002). In the aquaculture industry, proteases are the useful enzyme for the preparation of high quality functional feeds through bioconversion of low-cost feed materials because

  

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the worldwide sustainability of the aquaculture industry depends on the availability of low-cost, high quality feeds (Esakkiraj *et al*., 2007). Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Chu, 2007). Bacteria belonging to Bacillus sp. are by far the most important source of several commercial microbial enzymes (Ferrero *et al*., 1996; Kumar *et al*., 1999; Sookkheo *et al*., 2000; Singh *et al*., 2012; Gupta *et al*., 2002a; Beg and Gupta, 2003; Shafee *et al*., 2005; da Silva *et al*., 2007; Chu, 2007).

However, information regarding production of proteases by fish gut bacteria is scarce (Bairagi *et al*., 2002; Shafee *et al*., 2005; Esakkiraj *et al*., 2007; Mondal *et al*., 2008). Microbial enzymes have the advantage of large scale production by established fermentation techniques. To establish a successful fermentation process, it is necessary to make the environmental and nutritional conditions favorable for the microorganism for over production of the desired metabolite (Ray *et al*., 2007).

Cellulose is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bio resource produced in the biosphere (Jarvis, 2003; Zhang and Lynd, 2004). It is the most abundant biomass on Earth (Tomme *et al*., 1995). Cellulose is commonly degraded by an enzyme called cellulase. Therefore, it has become of considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources. Cellulase is the enzyme that hydrolyses the beta 1,4 glycosidic bonds in the polymer to release glucose units(Nishida *et al*., 2007). Cellulose containing wastes may be agricultural, urban, or industrial in origin; sewage sludge might also be considered a source of cellulose since its cellulosic content provides the carbon needed for methane production in the anaerobic digestion of sludge. Agricultural wastes include crop residue, animal excreta and crop-processing wastes, slashing generated in logging, sawdust formed in timber production, and wood products in forestry originated activities. This cellulose-degrading enzyme can be used, for example, in the formation of washing powders, extraction of fruit and vegetable juices, and starch processing (Camassola and Dillon, 2007). This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Bahkali, 1996; Magnelli and Forchiassin, 1999; Shin *et al*., 2000; Immanuel *et al*., 2006).

However, the application of bacteria in producing cellulase is not widely used. Enzyme production is closely controlled in microorganisms and for improving its productivity these controls can be ameliorated. Cellulase yields appear to depend upon a complex relationship involving a variety of factors like inoculums size, pH value, temperature, presence of inducers, medium additives, aeration, growth time, and so forth (Immanuel *et al*., 2006). Enormous amounts of agricultural, industrial, and municipal cellulosic wastes have been accumulating or used inefficiently due to the high cost of their utilization processes (Lee et al., 2008).

So, investigation is therefore required to establish the culture condition to scale up enzyme (protease and cellulase) production in an individual fermentation process for industrial uses. That's why the aim of the present study was to optimize the environmental and nutritional parameters for fermentation to enhance protease and cellulase production by the bacterial strains, Bacillus *Bacillus amyloliquefaciens* D4F1 and Bacillus *pumilus* D4H3, isolated from the foregut and hindgut regions, respectively, of mrigal (*Cirrhinus mrigala* (Hamilton) treated *with Lemna polyrhiza* meal.

## MATERIALS AND METHODS

### *Isolation of bacteria and Growth medium:*

Two bacterial strains, *Bacillus amyloliquefaciens* D4F1 and Bacillus *pumilus* D4H3, isolated from the foregut and hindgut regions, respectively, of mrigala (*Cirrhinus mrigala* (Hamilton) and were identified by both phenotypic characters and 16S rDNA sequence analysis. Both strains were cultured in 4 % tryptone soya broth for 24 h at 37 ± 2$^{o}$C to obtain an average viable count of 9.75 9 107 cells ml-1 culture broth. This was used as the inoculum for the production medium, as required. The culture medium used for protease production (Peptone–gelatin- medium) contained (g l-1): Beef extract, 3; Peptone, 5 and Gelatin, 4 (pH 7) and for cellulase (Carboxy-methyl-cellulose medium) contained (g l-1): Tryptone, 2; $KH_2PO_4$, 4; $Na_2HPO_4$, 4; $MgSO_4$, $7H_2O$, 0.2; $CaCl_2$, 0.001; $FeSO_4$, $7H_2O$, 0.004 and CMC, 10.

### *Enzyme assay:*

  

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Liquid specific production medium of 20 ml was inoculated with 0.5ml of inoculum obtained from the seed culture and incubated for 48-96 h at the same temperature. The contents of the culture flasks were centrifuged (9,000×$g$, 10 m, $4^0$C), and the cell-free supernatant was used for enzyme assay.

***Protease assay***

Liquid media (broth) were used for the quantitative assay of protease production from the two bacterial strains. Protease activity was measured following the caseinase assay method developed by Walter (1984). One protease unit (U) was expressed as microgram tyrosine liberated per mg protein per unit time.

***Cellulase assay***

The production of reducing sugars due to cellulolytic activity was measured at 540 nm according to the method of Denison and Koehn (1977), modified by Sadasivam and Manickam (1996). Carboxymethlycellulose (1.5%) was used as the substrate. The enzyme activity was expressed as the microgram glucose released per mg protein per unit time.

***Optimization of culture conditions:***

Optimization of culture conditions of the bacterial isolates, to be used in fermentation of plant ingredients were carried out at pH 7.0, 37 ± 2 °C, for 72 h, if not stated otherwise.

***Optimization of pH and temperature***

The most suitable pH of the fermentation medium was determined by adjusting the pH of the culture medium at different levels in the range of pH 5.5–9. In order to determine the effective temperature for enzyme production by the selected strains, incubation was carried out at 25, 30, 37, 40, 45, 50 and 55 °C.

***Optimization of incubation period***

Fermentation period is an important parameter for enzyme production by microorganisms. Some microorganisms produce the target metabolite maximally in their exponential growth phase, whereas some in their stationary growth phase. In this experiment, fermentation was carried out for 24, 48, 72, 96 and120 h and enzyme production was determined.

***Optimization of moisture content in fermentation process***

To determine the optimum moisture content in the fermentation process, the microorganisms were cultured in specific medium, which was prepared by moistening the substrate with a basal medium. The moisture content of the fermentation medium varied from 5 to 100 %.

***Effect of nitrogen sources***

For determination of the appropriate nitrogen source for enzyme production by the isolates, the fermentation medium was supplemented with four inorganic (ammonium nitrate, ammonium chloride, ammonium sulphate and potassium nitrate) and six organic (arginine, asparagine, tryptophan, tyrosine, gelatin and beef extract) nitrogen compounds at 0.2 % level, replacing the prescribed nitrogen source of the fermentation medium.

***Effect of carbon sources***

To detect the appropriate carbon source for enzyme production by the isolates, the fermentation medium was supplemented with five different carbohydrates namely, glucose, sucrose, lactose, fructose, maltose and starch at 1.0 % level, replacing the prescribed carbon source of the fermentation medium.

***Statistical Analysis***

The data were subjected to analysis of variance (ANOVA) by Origin 6.1 software. Duncan's multiple range test (Duncan, 1955) was employed to test differences among means. The significance of differences was tested at the significance level P = 0.05.

## RESULTS

  

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The fermentation conditions for the two isolates D4F1 (identified as *Bacillus amyloliquefaciens*) isolated from the foregut and D4H3 (identified as *Bacillus pumilus*) isolated from the hindgut of *C. mrigala* were optimized for scaling up production of the enzymes cellulase and protease. For successful fermentation it is necessary to optimize the nutritional and environmental conditions favorable for the microorganism for over production of the desired metabolite. Optimization of different parameters like moisture content of the fermentation medium, temperature, pH, incubation period, nitrogen and carbon sources were carried out. The bacterial strains were cultured in TSA broth at 37 °C for 24 h. This was used as inoculum for the production medium used in the optimization experiment. The incubation temperature used was 37 °C unless stated otherwise.

***Optimization of pH:***

The initial pH in the basic medium influenced the cellulase and protease production. The pH optima of the fermentation medium for highest cellulase and protease production were 7.0 for both the strains, D4F1 and D4H3. Maximum (26.75±1.02 U) cellulase production by the strain D4F1 was recorded at pH 7, whereas the strain D4H3 recorded highest (20.20±0.87 U) cellulase production at pH 7.0 (Fig. 1). Reduction of the initial pH to 6.5 significantly lowered the production of cellulase by the bacterial strains. Increase in pH to 7.5 also significantly reduced the cellulase production by the strains. In case of protease, the strain D4F1 resulted highest (0.65±0.04 U) protease production at pH 7.0, whereas the strain D4H3 showed the highest (0.11±0.02 U) protease production at pH 7.0. As in the case of cellulase, reduction of the initial pH to 6.5 significantly lowered the production of protease by the bacterial strains and an increase in pH to 7.5 significantly reduced the protease production by the strains.

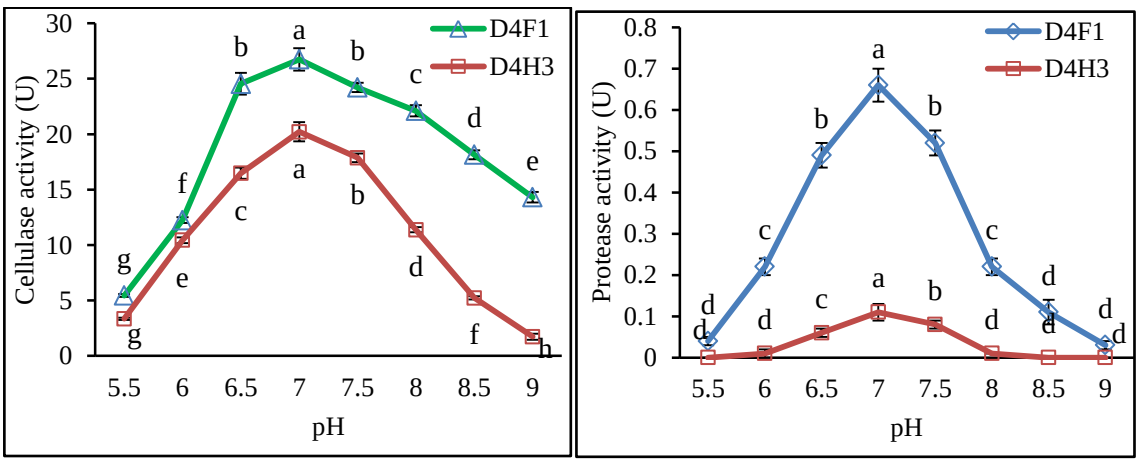


**Figure 1.** Effect of different pH on cellulase and protease production by the bacterial isolates. Error bars show deviation among three replicates; Means with different letters are significantly different ($P < 0.05$)

***Optimization of temperature:***

The initial temperature in the basic medium influenced the cellulase and protease production. The temperature optima of the fermentation medium for highest cellulase and protease production was 40 °C for both the strains, D4F1 and D4H3. Maximum (28.52±0.65 U) cellulase activity was showed by the strain D4F1 at temperature 40 °C, whereas the strain D4H3 showed the highest (22.24±0.43 U) cellulase activity at temperature 40 °C. Reduction of the initial temperature to 37 °C significantly lowered the production of cellulase by the bacterial strains (Fig. 2). Increase in temperature to 45 °C also significantly reduced the cellulase production by the strains. In case of protease, the strain D4F1 showed the highest protease production which was 0.99±0.04 U at temperature 40 °C, whereas the strain D4H3 showed the maximum (0.24±0.05 U) protease production at temperature 40 °C. Reduction of the initial temperature to 37 °C significantly lowered the production of protease by the bacterial strains and increase in temperature to 45 °C also significantly reduced the protease production by the strains.

  

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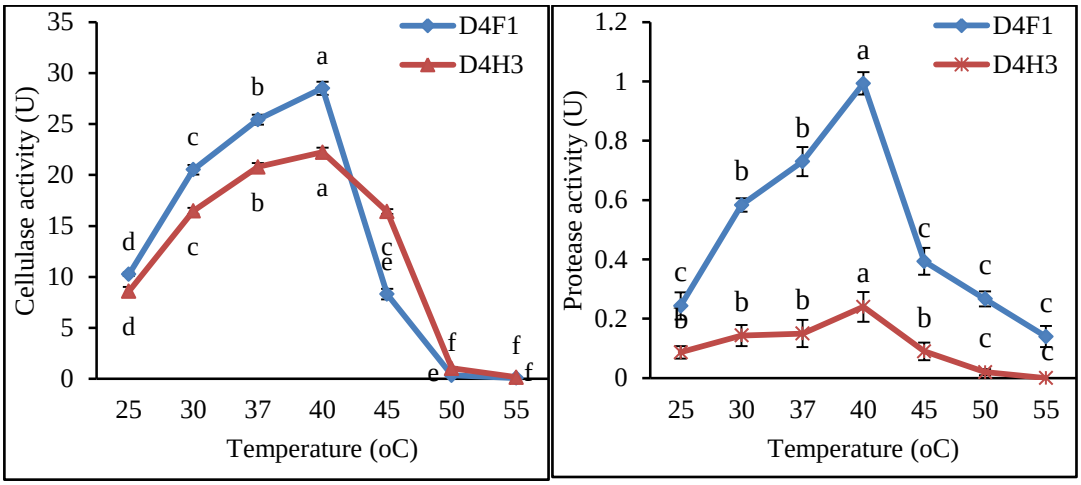


**Figure 2.** Effect of different temperature on cellulase and protease production by the bacterial isolates. Error bars show deviation among three replicates; Means with different letters are significantly different ($P < 0.05$)

***Optimization of incubation period:***

Optimization of incubation period is crucial for enhancing production of enzymes because microorganisms specifically produce different metabolites at different phases of growth. The cellulase and protease activities varied at different incubation period during shake flask culture of the two strains. The highest cellulase activity was detected after incubation for 96 h for both the strains D4F1 and D4H3. The highest protease production was detected at incubation period of 96 h for both the strains D4F1 and D4H3 and thereafter the protease production declined (Figure 3).

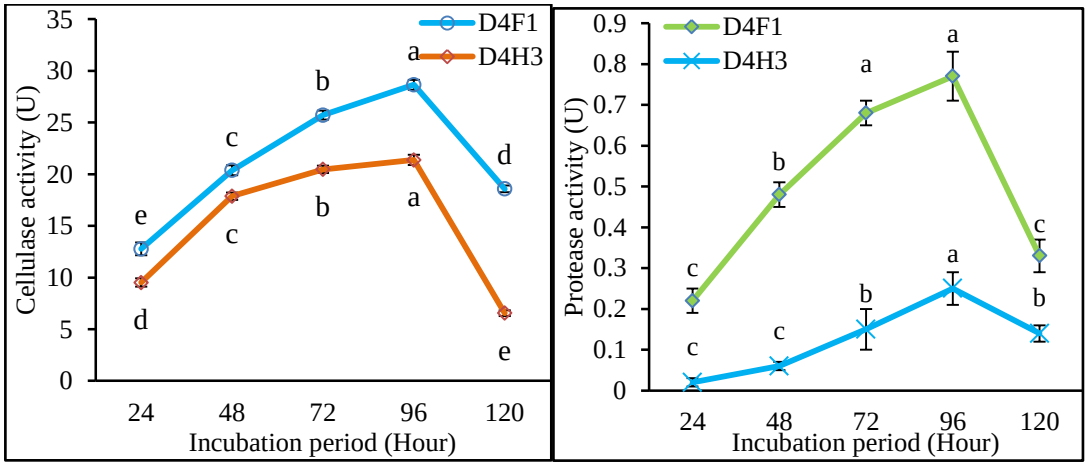


**Figure 3.** Effect of different incubation period (24-120) on cellulase and protease production by the bacterial isolates. Error bars show deviation among three replicates; Means with different letters are significantly different ($P < 0.05$)

***Optimization of moisture content:***

Moisture content is an important parameter for enzyme production under solid state fermentation conditions using particular substrates. It is crucial to provide optimized water level that controls the water activity of the fermenting substrate for achieving maximum metabolite production. The optimum moisture content detected for cellulase production was 10% for both the strains D4F1 and D3H4, whereas in case of protease production, the optimum moisture content in the production medium was10% and 15% for the strains, D4F1 and D3H4, respectively (Figure 4).

  

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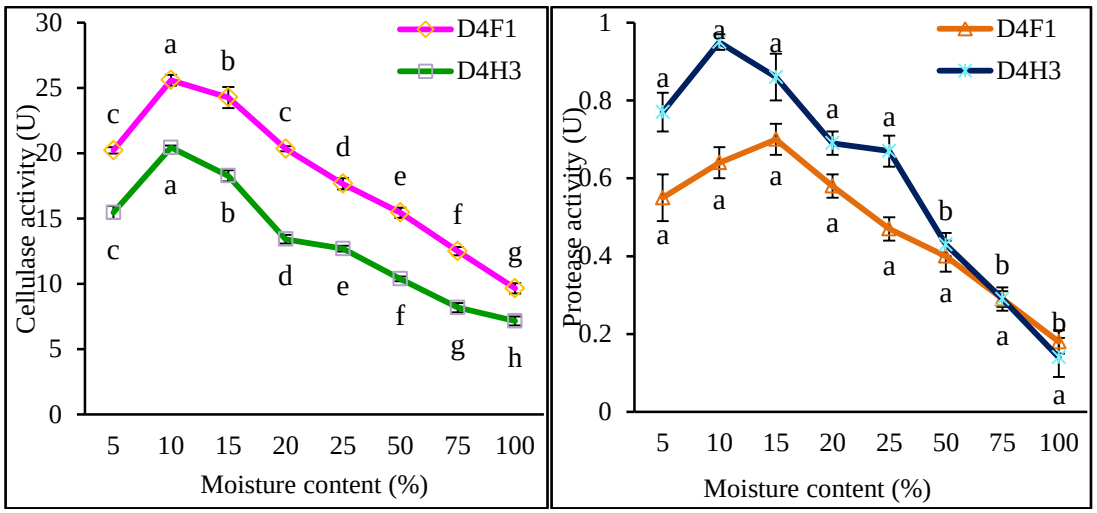


**Figure 4.** Effect of different moisture content (5-100) on cellulase and protease production by the bacterial isolates. Error bars show deviation among three replicates; Means with different letters are significantly different ($P < 0.05$).

***Optimization of nitrogen source:***

Nitrogen sources, including organic and inorganic, play an important role in the synthesis of enzymes. Inorganic nitrogen sources can be used quickly by the bacteria, while organic nitrogen sources can supply many cell growth factors and amino acids, which are needed for cell metabolism and enzyme synthesis. Therefore, both organic and inorganic nitrogen sources were used in the fermentation process (Fig. 5 and Fig. 6). The fermentation medium was supplemented with four inorganic (ammonium nitrate, ammonium chloride, ammonium sulphate and potassium nitrate) and six organic (arginine, L-asparagine, tryptophan, tyrosine, beef extract and gelatin) nitrogen compounds at 0.2 % level, replacing the prescribed nitrogen source. Beef extract was detected as best nitrogen source for D4F1, whereas tryptophan was found to be the best nitrogen source for D4H3. In case of protease, beef extract was detected as the best carbon source for both the strains D4F1and D4H3.

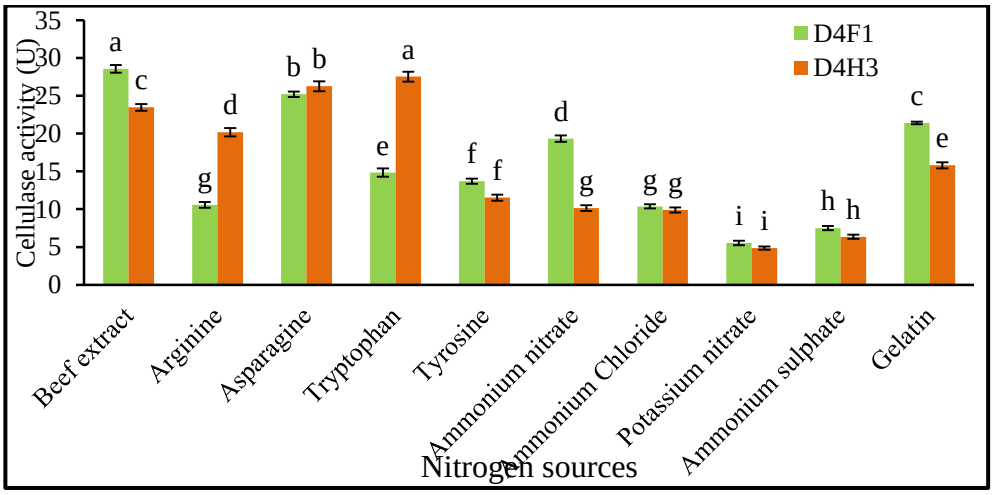


**Figure 5.** Effect of different nitrogen source on cellulase production by the bacterial isolates. Error bars show deviation among three replicates; Means with different letters are significantly different ($P < 0.05$).

  

Trends in Fisheries Research
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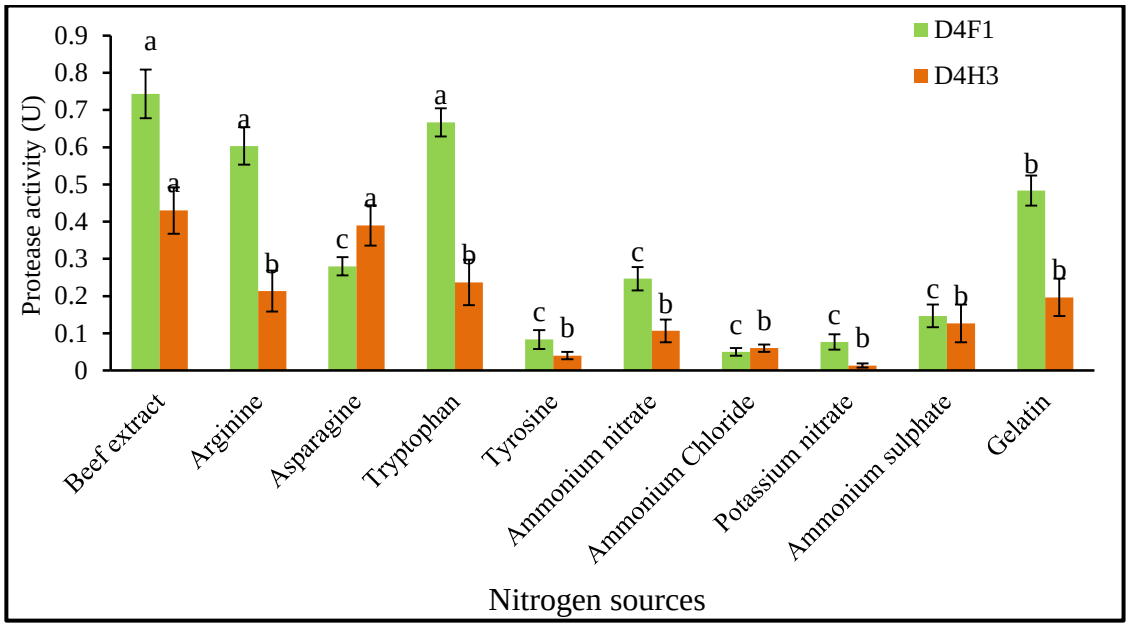


**Figure 6.** Effect of different nitrogen source on protease production by the bacterial isolates. Error bars show deviation among three replicates; Means with different letters are significantly different ($P < 0.05$).

***Optimization of carbon source:***

Carbon is a major component of cells and hence the influence of different carbon sources on cellulase and protease production were studied (Fig. 7). Six different carbohydrates, namely, glucose, sucrose, maltose, starch, lactose and fructose were used at 1.0 % level, replacing the prescribed carbon source of the fermentation medium. Glucose was detected as the best carbon source for cellulase production by D4F1, whereas the strain D4H3 maximally utilized fructose as the carbon source. But in case of protease, fructose was detected as the best carbon source for protease production by D4F1, whereas the strain D4H3 maximally utilized lactose as the carbon source.

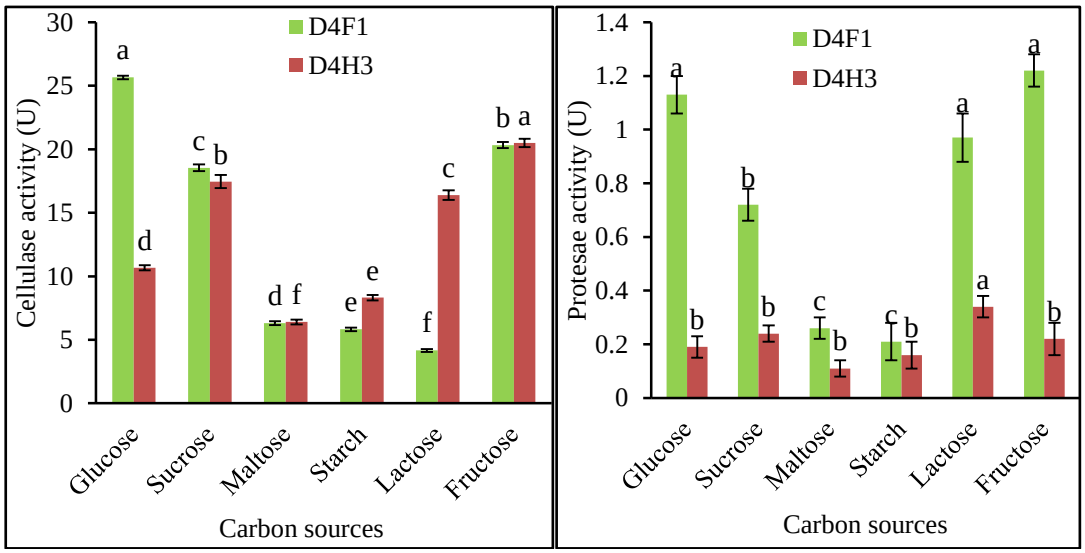


**Figure 7.** Effect of different nitrogen source on cellulase and protease production by the bacterial isolates. Error bars show deviation among three replicates; Means with different letters are significantly different ($P < 0.05$)

**DISCUSSION**

  

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Optimization is defined as need of environmental and nutritional parameters for growth and production (Winkler, 1991). Medium optimization is the first and foremost step to enhance the production in both laboratory experiments as well as in fermentation industries. Each and every element should be in proper amount for satisfying the metabolic process which will supply sufficient energy for maximum biomass production (Ray *et al*., 2012b). Nutrient requirement of the microorganism in aerobic condition can be calculated according to the equation:
Carbon and energy source + nitrogen source + oxygen+ other requirements → Biomass + products + CO2+ H2O + heat
Fermentation process of microorganism that is used to grow cells, it is necessary to monitor and control parameters starting from the selection of optimum carbon and nitrogen sources, moisture content, pH, temperature, incubation period etc. Changes in one of these parameters can have a dramatic effect on the yield of cells and the stability of protein product. The high rate of metabolism supports the critical period of metabolite production. Consequently, adequate and timely supply of carbon and nitrogen can be key factors affecting peak productivity levels and their duration. The meaning of optimization in this context needs careful consideration of the environmental and nutritional parameters for growth and production (Ray *et al*., 2007).

In the present study, highest cellulase production was exhibited by this two selected bacterial strains at 10% moisture content which is similar to the findings of Ray *et al*. (2007). They reported maximum cellulase production by two bacterial strains *Bacillus subtilis* and *B. circulans* at 10% moisture content. But in case of protease, the strain D4H3 showed highest activity at 10% moisture content, whereas D4F1 showed highest activity at 15% moisture content.
Most microorganisms grow optimally within a wide pH range. Ray *et al*. (2007) also reported higher cellulase production by *Bacillus subtilis* CY5 and *B. circulans* TP3, isolated from the GI tracts of common carp, *Cyprinus carpio*, and Mozambique tilapia, *Oreochromis mossambicus*, respectively in an optimum pH range of 7.0 to 7.5. Immanuel *et al*. (2006), however, reported that the cellulolytic enzyme, endogluconase from *Cellulomonas*, *Bacillus*, and *Micrococcus* spp., isolated from estuarine coir netting effluents hydrolyzes substrate in the pH range of 4.0 to 9.0, with maximum activity at pH 7.0.

The enzyme maintained its stability over a wide pH range (6.0 to 8.0), but had maximum activity at pH 7.0. Some earlier studies reported that pH 7.0 appears to play a decisive role in cellulose digestion for maximum production by *Clostridium thermocellum* and *Cellulomonas* sp. (Garcia-Martinez *et al*., 1980; Prasetsan and Doelle, 1987). Rajoka (2004), however, reported pH 7.3 as optimum for production of β-cellobiohydrolase (CBH) in *Cellulomona sflavigens*. It has been noted that the important characteristic of most microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production (Kurmar and Tagaki, 1999). Most microorganisms grow optimally within a wide pH range. The enzyme maintained its stability over a wide pH range (6.0 to 8.0), but had maximum activity at pH 7.0. In the present study, maximum cellulase activity was also recorded at pH 7.0 by the two strains D4F1 and D4H3. Ray *et al*. (2012b), however, reported maximum protease activity at pH between 6.0 and 6.5 by two strains, *Bacillus licheniformis* BF2 and *Bacillus subtilis* BH4 isolated from the foregut and hindgut regions, respectively, of bata, *Labeo bata*. Increasing the pH beyond the optimum level may interfere with the amino acid composition of the enzyme thereby decreasing the enzyme activity (Esakkiraj *et al*., 2007). Enzyme production by bacterial isolates is largely dependent on pH of the medium. Geethanjali and Subash (2011) reported that protease production from *Bacillus subtilis* isolated from the midgut of *Labeo rohita* was highest at pH 9.0. A similar result was reported regarding protease production by *Bacillus proteolyticus* CFR 3001 isolated from fish waste (Bhaskar *et al*., 2007). In the present study, maximum protease activity was recorded at pH 7.0 by two strains, D4F1 and D4H3.

Like pH, temperature is one of the most important parameters essential for successful fermentation reaction. Microorganisms grow slowly at a temperature below or above the normal growth temperature because of a reduced rate of cellular production (Ray *et al*., 2007). If the growth temperature is too high but not lethal, there may be a premature induction of target protein expression. In the present study, for cellulase production by the two selected bacterial strains 40$^{o}$C was found to be most effective. Production started to decline after further increase in temperature. Immanuel *et al*. (2006) also recorded maximum endoglucanase activity in *Cellulomonas*, *Bacillus*, and *Micrococcus* sp. at 40$^{o}$C at neutral pH. Further increase in pH and temperature reduced the enzyme activity considerably. Ray *et al*. (2007) observed 40$^{o}$C as most effective for cellulase production by *B. subtilis* CY5 and *B. circulans* TP3. Production started to decline after further increase in temperature. Rajoka *et al*. (1998) and Rajoka (2004) however, observed suppressed exoglucanase production at temperatures higher than 30$^{o}$C in *Cellulomonas biazotea* and *C. flavigens*, respectively. At lower temperature, substrate transport across the cells is suppressed and lower product yields are attained. At higher temperature, the maintenance energy requirement for cellular

  

growth is high due to thermal denaturation of enzymes of the metabolic pathway (Aiba *et al*., 1973) resulting in maximum production. In the present study, optimum temperature for protease production by two strains, D4F1 and D4H3 (identified as *Bacillus amyloliquefaciens* and *Bacillus pumilus*, respectively) was 40$^{o}$C. This finding also match with (Sonia *et al*., 2013) highest cellulase activity all the four bacteria *Pseudomonas fluorescens*, *Bacillus subtilus*, *E. coli*, and *Serratia marscens* at 40$^{o}$C.

Production of enzyme started to decline after further increase in temperature. Contrary to the present observation, Hoshino *et al*. (1997) reported maximum protease activityin *Pseudomonas* sp. isolated from fish intestine at low temperature of 15$^{o}$C, whereas at 40$^{o}$C, the activity was minimum. Sankaralingam *et al*. (2012) also recorded the highest protease production by *Shigella* sp. at 40$^{o}$C. Ray *et al*. (2012b) reported 40$^{o}$C as most effective for protease production by two fish GI tract bacterial strains, *B. licheniformis* BF2 and *B. subtilis* BH4. Esakkiraj *et al*. (2007) however, observed maximum protease activity in a fish gut isolate, *B. cereus* at 60$^{o}$C. da Silva *et al*. (2007) reported 70$^{o}$C as the optimum temperature for protease production by thermophilic *Bacillus* sp. strain SMIA-2 isolated from soil. At lower temperature, substrate transport across the cells is suppressed and lower product yields are attained, whereas at higher temperature, the maintenance energy requirement for cellular growth is high due to thermal denaturation of enzymes of the metabolic pathway resulting in maximum production (Aiba *et al*., 1973). The present study indicates that protease from this two bacterial strains is thermophilic in nature which corroborates the thermophilic protease production by *B. cereus* (Kim *et al*., 2001; Esakkiraj *et al*., 2007) and *B. clausii* (Kumar *et al*., 2004).

Since fermentation duration is crucial, it is also important to find out the optimum period for enzyme production. Some organisms are reported to produce maximally in the log phase of growth, whereas some at their stationary phase (Ray *et al*., 2007). Singh (2012) optimized the culture condition for cellulase production from *Bacillus sphaericus* JS1 and recorded maximum cellulase production when the incubation time was 48 h. In the present investigation, however, maximum cellulase and protease production by the bacterial strains was obtained at 96 h fermentation. Krishna (1999) reported optimal production of cellulase at 72 h of incubation in *Bacillus subtilis* CBTK 106. Ray *et al*. (2007), however, reported maximum cellulase production by two fish GI tract bacterial strains, *Bacillus subtilis* CY5 and *B. circulans* TP3 after 96 h fermentation. Shafee *et al*. (2005) recorded maximum protease activity in *B. cereus* at 170-rpm agitation speed after 48 h of incubation.

Most industrially used microorganisms can utilize inorganic or organic nitrogen sources. Inorganic nitrogen may be supplied as ammonia gas, ammonium salts or nitrates and as amino acids, protein or urea. It was found that the growth was faster with the supply of organic nitrogen, and a few microorganisms also were found to have absolute requirement for amino acids. However, amino acids are more commonly added as complex organic nitrogen sources which are non-homogenous, cheaper and readily available (Ray *et al*., 2007). In the present study, complex nitrogen compound, beef extract at 0.2% level proved to be the best for D4F1 and amino acid tryptophan at the same concentration for D4H3for cellulase production. In the present study, inorganic nitrogen sources and other amino acids namely, aspergine and tyrosine were the poor nitrogen sources for cellulase synthesis. On the contrary, Spiridonov and Wilson (1998) found that $NH_4$ compounds are the most favourable nitrogen sources for protease and cellulase synthesis.

Production of extracellular proteases has been shown to be sensitive to repression by different carbohydrate and nitrogen sources (Levisohn and Aronson, 1967; Haulon *et al*., 1982). Effects of a specific nitrogen supplement on protease production differ from organism to organism although complex nitrogen sources are usually used for alkaline protease production (Kurmar and Tagaki, 1999). In the present study, two different sources of nitrogen were used. They were six organic nitrogen (beef extract, arginine, asparagine, tryptone, tyrosine and gelatin), and four inorganic nitrogen (ammonium chloride, ammonium nitrate, ammonium sulfate and potassium nitrate) sources. Among the nitrogen sources tested, beef extract resulted in the highest activity of protease in the two bacterial strains D4F1 and D4H3. The results of the present study are in agreement with those of Yang and Lee (2001) and Shafee *et al*. (2005), which reported increased production of protease by *Streptomyces rimosus* and *B. cereus* strain 146, respectively in the presence of beef extract. Ray *et al*. (2012b) also reported that beef extract was the most effective nitrogen source in alkaline protease production by *B. licheniformis* BF2 and *B. subtilis* BH4. Organic nitrogen sources have also been reported to be the best for protease production by *B. stearothermophilus* F1 (Razak *et al*., 1995), *B. mojavensis* (Beg *et al*., 2003) and *B. subtilis* (Geethanjali and Subash, 2011).

  

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Carbohydrates or their derivatives induce most of the cellulolytic enzymes. Thus, the carbon sources play an important role in enzyme production (Kubicek and Penttilä, 1998). It is generally accepted that cellobiose (Fritscher *et al*., 1990), cellobiono-d-1,5-lactone (Kubicek *et al*., 1988), lactose (Morikawa *et al*., 1995; Bailey and Tähtiharju, 2003) and sophorose (Mach *et al*., 1995) enhance the production of endo and exoglucanase as well as b-glucosidase. Nochure *et al*. (1993) reported fructose as the best inducer for avicellase in *Clostridium thermocellum*. Bagga *et al*. (1989) identified lactose as the best inducer of endoglucanase and cellobiohydrolase. Trehalose was found to be the best inducer of cellulases in a *Clostridium* sp. (Thirumale *et al*., 2001). However, the highest cellulase yields were obtained on cellulose containing carbonsources (Bhat and Bhat, 1997; Kubicek and Penttilä, 1998). Ahmed *et al*. (2003) reported that cellulase production increased significantly when xylan or CMC was used as a carbon source, whereas glucose had inhibitory effect on cellulase production. Maximum production of β-cellobiohydrolase and FPase was evident using fructose among the monomeric saccharides and cellobiose among the disaccharides (Hanif *et al*., 2004). Juhász *et al*. (2005) reported that high cellulolytic activity could be reached using steam pretreated corn as carbon source. In the present study, glucose was found to be the most suitable source of carbon for the cellulase production in D4F1 and fructose induced maximum cellulase production in D4H3.

In the microbial culture medium, the type of carbon sources is considered as an important nutrient factor for protease production (Esakkiraj *et al*., 2009). In the present study, fructose was found to be the most suitable source of carbon for the protease production in D4F1 and lactose induced maximum protease production in D4H3.

## CONCLUSION

On the basis of the above experimental findings, it is concluded that different physical parameters like temperature, pH, moisture content, incubation period, carbon and nitrogen sources etc. have a crucial role for cellulose and protease production by the two strains, D4F1 and D4H3. Maximum cellulase and protease production by strains, D4F1 and D4H3 was recorded at 40˚C, pH 7, incubation period 96 h, moisture content 10% (except D4H3; 15% in case of protease production). Beef extract was the most promising nitrogen source for D4F1 and D4H3 for protease and D4F1 for cellulase production (except D4H3; tryptophan in cellulase production). In case of cellulase production, most promising carbon source was glucose for D4F1, fructose for D4H3 and for protease production, fructose and lactose were found to be ideal carbon source in case of D4F1 and D4H3, respectively. This present investigation will be helpful to standardize the requirements for optimum production of cellulase and protease by enzyme-producing fish gut bacteria and might help to better fish feed formulation, pharmaceutical and food industries. Further investigations are, however, required to make use of the full potential of these organisms for cellulase and protease production by employing genetic, biochemical, and microbial engineering techniques.

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