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OPTIMIZATION OF FERMENTATION CONDITIONS FOR CELLULASE PRODUCTION BY *BACILLUS SUBTILIS* CY5 AND *BACILLUS CIRCULANS* TP3 ISOLATED FROM FISH GUT

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Background. Microbial and fungal cellulases are known to hydrolyse cellulose, which is ingested as plant material by herbivorous/omnivorous fishes. Microbial enzymes have enormous advantage of being produced in large quantities by established fermentation techniques. The present investigation aims to optimize the environmental and nutritional parameters for fermentation to enhance cellulase production by two bacterial strains isolated from fish gastrointestinal tracts.

Materials and methods. Two bacterial strains, *Bacillus subtilis* CY5 and *Bacillus circulans* TP3, isolated from the gastrointestinal tracts of common carp, *Cyprinus carpio* L., and Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852), respectively were identified as potent cellulase producers. Both strains were cultured in tryptone soya broth for 24 h at 32 ± 2ºC, when average viable count of $9.75 \cdot 10^7$ cells · mL^{-1} culture broth was obtained. This was used as the inoculum for the production medium. The fermentation medium was seeded with 1.0%, 2.0%, 3.0%, 4.0%, and 5.0% inoculum (tryptone soya broth) and incubated in static culture at 40ºC to standardize the inoculum size for fermentation. The effect of different production parameters, such as fermentation condition, moisture, pH, temperature, inoculum size, and nitrogen sources on cellulase production by the isolated bacterial strains were studied.

Results. Cellulase yield was highest (26 U in *B. subtilis* and 20.2 U in *B. circulans*) in solid-state fermentation (SSF). Enzyme production in both the isolates increased in an optimum pH range of 7.0 to 7.5. Minimum cellulase production was observed at 45ºC, while maximum production was obtained at 40ºC. To standardize the fermentation period for cellulase production, production rate was measured at 12-h intervals up to 120 h. Enzyme production increased for 96 h of fermentation in both strains, and decreased thereafter. The enzyme production increased with increased inoculum size up to 3.0 percentage points. Asparagine as the nitrogen source was most effective in *B. subtilis* CY5, while beef extract proved useful in optimizing enzyme production by *B. circulans* TP3.

Conclusion. The results of this study will help to standardize the requirements for optimum production of cellulase by cellulase-producing fish gut bacteria and might contribute towards better fish feed formulation incorporating plant ingredients, especially in the larval stages when the enzyme system is not efficient.

Keywords: fermentation, cellulase production, optimization, fish gut bacteria

INTRODUCTION

Complete cellulose hydrolysis to glucose demands the action of exoglucanases (also called cellobiohydrolyses), endoglucanases and β-glucosidases. Exoglucanases (1,4-β-D-glucan cellobio-hydrolase, EC 3.2.1.91) are usually active on crystalline cellulose and are lacking from incomplete cellulase systems. Endogluconases (1,4-β-D--glucan-4-glucanohydrolase, EC 3.2.1.4) are more active against the amorphous regions of cellulose and they can also hydrolyse substituted celluloses, such as carboxymethylcellulose (CMC) and hydroxyethyl-cellulose (HEC). Cellobiohydrolases cleave disaccharide (cellobiose) units either from non-reducing or reducing ends, whereas endoglucanases hydrolyse the cellulose chain internally. β-glucosidases (EC 3.2.1.21) are needed to cleave cellobiose and other soluble oligosaccharides to glucose (Béguin 1990). Cellulase is used extensively in plant protoplast isolation, plant virus studies, metabolic investigations and genetic modification experiments. Apart from these applications, cellulases are increasingly being used as

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additives in pig and cattle feeds (Dovorak 2000, Siciliano-Jones 2001).

Cellulases are easily obtained from microbial and fungal sources, but vertebrates lack the ability to produce endogenous cellulases, and hence are reliant upon gastrointestinal microorganisms for cell wall degradation of ingested plants. The beneficial effects of microorganisms in the digestive processes of terrestrial animals are well established (Combe et al. 1976, McBee 1977, Savage 1977 a, b, Goldin 1986, Moriarty 1990). Some investigations have also suggested that microorganisms have a beneficial effect in fish digestive processes e.g., microbial breakdown of chitin (Minami et al. 1972, Goodrich and Morita 1977, Danulat and Kausch 1984, Kono et al. 1987), p-nitrophenyl-β-N-acetylgalactosamine and collagen (MacDonald et al.1986), cellulose (Stickney and Shumway 1974, Trust et al. 1979, Saha and Ray 1998, Bairagi et al. 2002 a, b, 2004, Saha et al. 2006), and the vitamin B_{12} producing ability of the bacteria (Sugita et al. 1991). However, the specific cellulolytic activity shown by the bacterial species is found to depend on the source (Saxena et al. 1993).

Microbial enzymes have the enormous advantage of being able to be produced in large quantities by established fermentation techniques. Enzyme production is closely controlled in microorganisms and therefore, to improve its productivity these controls can be exploited and modified. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size (carbon source and cellulose quality), pH value, temperature, presence of inducers, medium additives, aeration, growth time, etc. (Immanuel et al. 2006). Therefore, attention has been focused on studying the cellulolytic activity and cellulase enzyme production by several microorganisms in various products as well as in various environments. To establish a successful fermentation process it is necessary to make the environmental and nutritional conditions favourable for the microorganism for over-production of the desired metabolite. An elaborate investigation is therefore, required to establish the optimum conditions to scale up enzyme production in an individual fermentation process. Although there are reports on the influence of various fermentation variables on cellulase production by different bacteria and fungi isolated from various natural environments (Garcia-Martinez et al. 1980, Stewart and Parry 1981, Coral et al. 2002, Rajoka 2004, Immanuel et al. 2006), information on the optimum fermentation conditions for cellulase production by fish gut bacteria is lacking. In the present investigation, the environmental and nutritional parameters for fermentation were optimized to enhance cellulase production by the bacterial strains *B. subtilis* CY5 and *B. circulans* TP3, isolated from the gastrointestinal tract of common carp, *Cyprinus carpio* L., and Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852), respectively.

MATERIALS AND METHODS

Microorganisms and growth medium. The two bacterial strains *B. subtilis* CY5 and *B. circulans* TP3, isolated from the gastrointestinal tract of common carp, *C. carpio* and Mozambique tilapia, *O. mossambicus*, respectively (Bairagi et al. 2002 a), were identified as potent cellulase producers. Both strains were cultured in 4-% tryptone soya broth for 24 h at 37 ± 2ºC when an average viable count of $9.75 \cdot 10^7$ cells · mL^{-1} culture broth was obtained. This was used as the inoculum for the production medium, as required.

Medium composition. Carboxymethylcellulose-agar (CMC-agar) medium (g · L^{-1}): Carboxymethylcellulose, 10; KH_2PO_4, 4; Na_2HPO_4, 4; $MgSO_47H_2O$, 0.2; $CaCl_2$, 0.001; $FeSO_47H_2O$, 0.004; Tryptone, 2; Agar, 15; pH 7.0.

Enzyme assay. Liquid media were used for the quantitative assay of cellulase production from the two bacterial strains. Cellulase activity was measured according to the method of Denison and Koehn (1977). The production of reducing sugar (glucose) from CMC substrate through cellulolytic activity was measured at 540 nm by the dinitrosalicylic acid method using glucose as the standard. One cellulase unit (U) was defined as the amount of enzyme per millilitre culture filtrate that released 1 microgram glucose per minute.

Fermentation conditions. Fermentation was carried out at pH 7.0, 37 ± 2ºC, for 72 h, if not stated otherwise.

Optimization of moisture content in fermentation process. To determine the optimum moisture content in the fermentation process, the microorganisms were cultured in carboxymethylcellulose (CMC) medium, which was prepared by moistening CMC with a basal salt solution. The moisture content of the fermentation medium varied from 5%–100%.

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 5.5–8.5 using buffers. In order to determine the effective temperature for cellulase production by the selected strains, fermentation was carried out at 5ºC intervals in the range of 25 to 45ºC.

Optimization of period for cellulase fermentation. Fermentation period is an important parameter for enzyme production by microorganisms. In this experiment fermentation was carried out up to 120 h, and production rate measured at 12-h intervals.

Optimization of inoculum size for fermentation process. The inoculum volume was optimized for maximal enzyme production. The fermentation medium was seeded with 1.0%, 2.0%, 3.0%, 4.0%, and 5.0% seed culture (tryptone soya broth) and incubated in still culture at 37ºC.

Effect of nitrogen sources on cellulase production. To detect the appropriate nitrogen source for cellulase production by the isolates, the fermentation medium was supplemented with five inorganic (ammonium nitrate, ammonium chloride, ammonium sulphate, potassium nitrate and sodium nitrate) and five organic (arginine, L-asparagine, tryptophane, tyrosine and beef extract) nitrogen compounds at 0.2-% level, replacing the prescribed nitrogen source of the fermentation medium.

Statistical analysis. The data were subjected to analysis of

variance (ANOVA) using 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

Cellulase yield was highest (26 U in *B. subtilis* CY5 and 20.2 U in *B. circulans* TP3) when the moisture content in the fermentation medium was 10%. However, the production of cellulase by *B. circulans* TP3 at 10% moisture content was not significantly different from that at 15% and 20% (Fig. 1). Thus, cellulase yield increased under solid-state fermentation (SSF) conditions rather than under submerged fermentation (SmF), when the moisture content was 100%. Enzyme production in both the isolates increased with pH up to pH 7.5 although the value was not significantly different ($P < 0.05$) from those with pH 7 and 8 (Fig. 2). Production was much less up to pH 6.5, and declined again beyond pH 8. The effect of temperature on cellulase production by the bacterial isolates is depicted in Fig. 3. Minimum cellulase yield was observed when fermented at 45ºC, while maximum yield was at 40ºC. Cellulase production gradually increased for up to 96 h of fermentation in both strains and decreased thereafter (Fig. 4). Enzyme production increased gradually up to 3% inoculum size, but decreased thereafter. The enzyme production by both the strains in 3% inoculum size was however, not significantly different ($P < 0.05$) from that in 2% inoculum size (Fig. 5). The results of the effect of various nitrogen sources on cellulase production revealed that L-asparagine was most effective for *B. subtilis* CY5, while beef extract produced optimum results for *B. circulans* TP3 (Fig. 6). All the inorganic nitrogen sources and arginine and tyrosine among the organic sources tested were found to be poor nitrogen sources for cellulase production by both the strains.

DISCUSSION

Regardless of the fermentation process that is used to grow cells, it is necessary to monitor and control parameters starting from the selection of optimum carbon and nitrogen sources and including inoculum volume, 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.

Medium formulation is the foremost step for designing successful laboratory experiments for yield enhancement. The medium constituents must satisfy the elemental requirement for cell biomass and metabolite production; hence there must be adequate energy supply for biosynthesis and cell maintenance. The first step to consider is an equation based on the stoichiometry for growth and product formation. Thus, for an aerobic fermentation the reaction is as follows:

$$\text{Carbon and energy source + nitrogen source + oxygen + + other requirements} \longrightarrow \text{biomass + products + } CO_2 + H_2O + \text{heat}$$

This equation should be expressed in quantitative terms for economical designing of the medium to control the unspent nutrients. Thus, it is possible to calculate the minimal nutrient quantities that are needed to produce a sufficient amount of biomass. Substrate selection of a for enzyme production in a solid state fermentation (SSF) process depends upon several factors, mainly related with substrate cost and availability and thus may involve screening several agro-industrial residues. In the course of this study, macrophyte leaf meals such as *Lemna polyrhiza* and *Leucaena leucocephala* were considered as substrates for fermentation. In a SSF process, the solid substrate not only supplies nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. The substrate that provides all the required nutrients to the microorganisms growing in it should be considered as the ideal substrate. In the present experiment, CMC sodium salt (high viscosity) was used as the standard carbon source for optimizing cellulase production.

The requirement of water for growth and metabolic activities of microorganisms and the consequent potential of the water activity of the medium in controlling fermentation processes are well established (Hahn-Hägerdal 1986). SSF is distinct from submerged fermentation (SmF) since microbial growth and product formation occur at or near the surface of the solid substrate particle having low moisture contents. Hence, it is crucial to provide optimized water content to the fermenting substrate. It has been reported that α-amylase production by *B. licheniformis* M27 in SSF was highest in basal wheat bran medium with 65% (w/w) initial moisture content (Ramesh and Lonsane 1990). In the present study, it was observed that 10 mL of distilled water was sufficient to moisten 100 g CMC to give high enzyme titres. It appears, therefore, that 10% moisture content of the medium volume was optimum for cellulase production by *B. subtilis* and *B. circulans*. Tendargy (1998) compared cellulase production in SmF and SSF systems. While the production cost in SSF *in situ* was much less than SmF, the enzyme in SSF crude product was concentrated; thus it could be directly used in agro-biotechnological applications as a feed additive. It has been argued that with appropriate technology, improved bioreactor design and operation controls, SSF may become a competitive method for cellulase production (Pandey et al. 1999).

Most microorganisms grow optimally within a wide pH range. In the present study, maximum cellulase activity was recorded between pH 7.0 and 7.5. Immanuel et al. (2006) 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

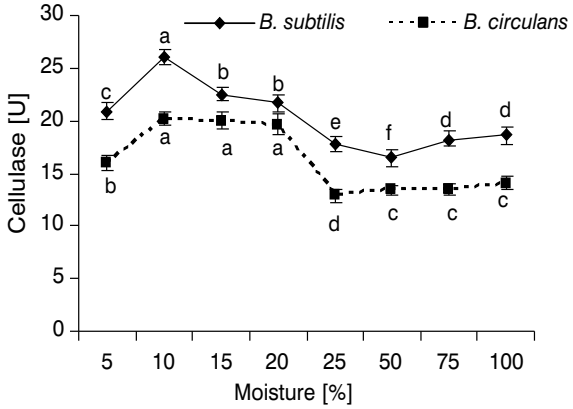

Fig. 1. Effect of moisture on cellulase production by *B. subtilis* CY5 and *B. circulans* TP3; Error bars show standard deviation among three replicates; Means with different letters are significantly different ($P < 0.05$)

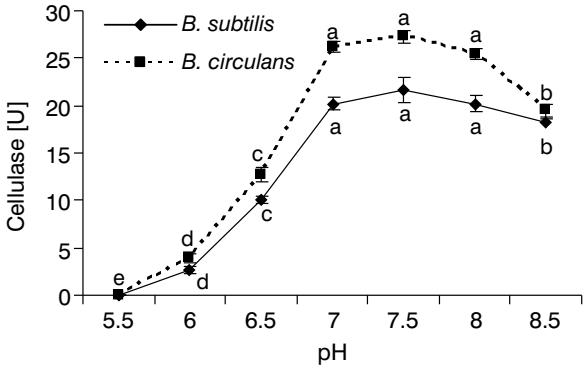

Fig. 2. Effect of pH on cellulase production by *B. subtilis* CY5 and *B. circulans* TP3; Error bars show standard deviation among three replicates; Means with different letters are significantly different ($P < 0.05$)

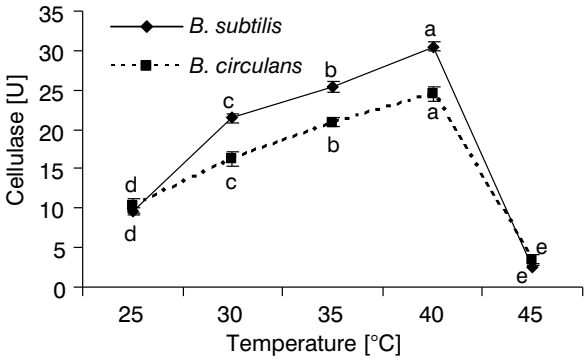

Fig. 3. Effect of temperature on cellulase production by *B. subtilis* CY5 and *B. circulans* TP3; Error bars show standard deviation among three replicates; Means with different letters are significantly different ($P < 0.05$)

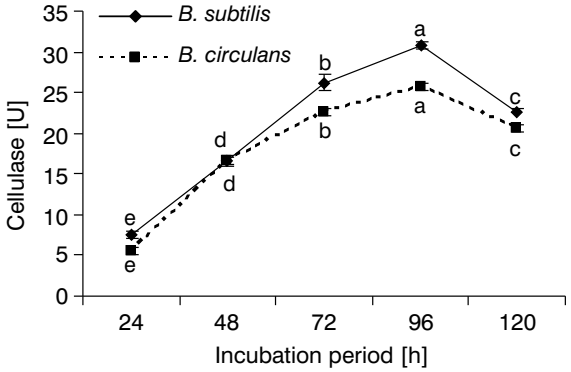

Fig. 4. Effect of incubation period on cellulase production by *B. subtilis* CY5 and *B. circulans* TP3; Error bars show standard deviation among three replicates; Means with different letters are significantly different ($P < 0.05$)

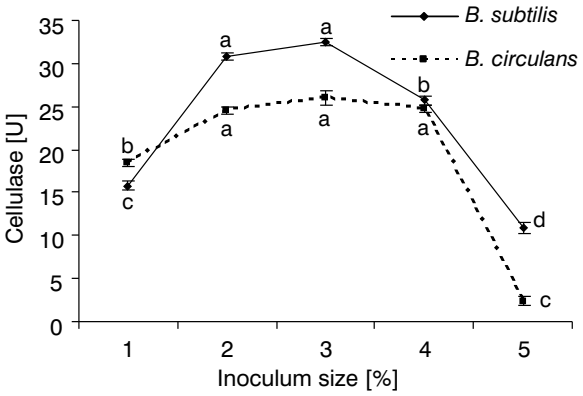

Fig. 5. Effect of percentage of inoculum size on cellulase production by *B. subtilis* CY5 and *B. circulans* TP3; Error bars show standard deviation among three replicates; Means with different letters are significantly different ($P < 0.05$)

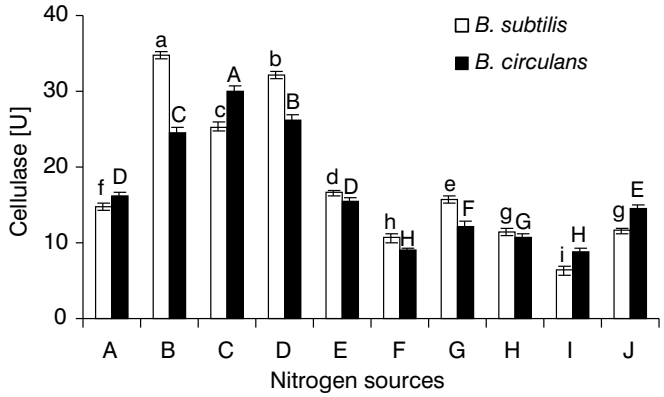

Fig. 6. Effect of different nitrogen sources (A: arginine; B: L-asparagine; C: beef extract; D: tryptophane; E: tyrosine; F: ammonium nitrate; G: ammonium chloride; H: ammonium sulphate; I: potassium nitrate; J: sodium nitrate) on cellulase production by *B. subtilis* CY5 and *B. circulans* TP3; Error bars show standard deviation among three replicates; Means with different letters are significantly different ($P < 0.05$)

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. Coral et al. (2002) reported maximum CMCase activity at pH 7.5 by *Aspergillus niger* (Z10, wild type strain) among the tested pH range between 4.0 and 9.0. Similarly, the optimal pH of 6.0 to 7.0 for maximum protease-resistant cellulase activity in *A. niger* was reported by Akiba et al. (1995). 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 *Cellulomonas flavigens*.

Like pH, temperature is one of the most important parameters essential for the success of a fermentation reaction. Microorganisms grow slowly at a temperature below or above the normal growth temperature because of a reduced rate of cellular production. If the growth temperature is too high but not lethal, there may be a premature induction of target protein expression. For cellulase production by *B. subtilis* CY5 and *B. circulans* TP3, 40ºC 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 40ºC at neutral pH. Further increase in pH and temperature reduced the enzyme activity considerably. Coral et al. (2002) reported the optimum temperature for CMCase activity as 40ºC in *Aspergillus niger* Z10 strain. Rajoka et al. (1998) and Rajoka (2004), however, observed suppressed exoglucanase production at temperatures higher than 30ºC 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.

The culture used to inoculate the fermentation medium must be in a healthy, active state and of optimum size, possibly minimizing the length of log phase, thus in its high rate for substrate conversion. The inoculum quantity of normally used is between 3% and 10% of the medium volume (Lincoln 1960, Meyrath and Suchanek 1972, Hunt and Stieber 1986). A relatively large inoculum volume may be used to generate the maximum production in as short a time as possible, thus increasing the vessel productivity. The physiological condition of the inoculum, when it is transferred to the next culture stage, can have a major effect on fermentation performance. The optimum transfer time must be determined so that the inoculation with an ideal culture can be achieved. Lincoln (1960) stressed that bacterial inocula should be transferred in the logarithmic growth phase when the cells are still metabolically active. Inoculum age is particularly important in the sporulating bacteria, because sporulation is induced at the end of the logarithmic phase and the use of an inoculum containing high percentage of spores would result a long log phase in subsequent fermentation. Keay et al. (1972) reported the use of 5% inoculum for protease production by *Bacillus*. For the production of raw starch hydrolysing amylase by *Bacillus*, 2% inoculum was recommended (Avendano and Cornejo 1987). To determine the optimum inoculum dose and the time of inoculum transfer in the present experiment, the inoculum was transferred after 24 h of growth, i.e., in its log phase. It was observed that 3% inoculum at the age of 24 h was the best.

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. Optimum fermentation period was found to be 48 h for amylase production by *B. subtilis* (Takasaki 1985). Sidhu et al. (1997) reported that 48 h of fermentation was optimum for α-amylase production by *B. stearothermophilus* MK 716. In the present investigation, however, maximum cellulase production by *B. subtilis* CY5 and *B. circulans* TP3 was obtained at 96 h fermentation.

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. In the present study, the amino acid L-asparagine at 0.2% level proved to be the best for *B. subtilis* and the complex nitrogen compound beef extract at the same concentration, for *B. circulans*. Inorganic nitrogen sources, including ammonium nitrate, ammonium chloride, ammonium sulphate, potassium nitrate and organic nitrogen sources namely, the amino acids, arginine and tyrosine were the poor nitrogen sources for CMCase synthesis. On the contrary, Spiridonov and Wilson (1998) found that NH_4 compounds are the most favourable nitrogen sources for protein and cellulase synthesis. Rajoka (2004) reported KNO_3 and NH_4NO_3 as the best nitrogen sources for cellulase synthesis in *C. flavigena*.

The nutritional and physical conditions for growth and metabolite production by an organism depend on its genetic make up. However, the production can be improved by standardizing the culture parameters. After the optimization experiments in this investigation it was observed that the cellulase yield doubled even in its wild state. However, the organisms showed very simple nutrient requirement.

CONCLUSION

This investigation led us to conclude that moisture, pH, temperature, and nitrogen sources play a most crucial role in cellulase production by fish gut bacteria, *Bacillus subtilis* CY5 and *B. circulans* TP3. Solid-state fermentation was suitable for increased cellulase production by

these organisms. They could readily utilize the substrate at 40ºC temperature and pH 7–7.5. Organic nitrogen sources were found to be more suitable for optimum cellulase production of than inorganic sources. The amino acid, L-asparagine and beef extract were most suitable for optimization of cellulase production by *B. subtilis* and *B. circulans*, respectively. Further investigations are required to make use of the full potential of these organisms for cellulase production by employing genetic, biochemical and microbial engineering techniques.

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