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RESEARCH ARTICLE

EFFECT OF NUTRITIONAL AND ENVIRONMENTAL FACTORS ON CELLULASE PRODUCTION BY STREPTOMYCES ALBADUNCUS FROM THE GUT OF EARTHWORM, EISENIA FOETIDA

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ABSTRACT

Earthworms are eco-friendly and play a variety of roles in agro ecosystem. The gut of earthworm is the factory to manufacture the beneficial microbial densities and their products. The excreted nutrients enrich thousand times more than the surrounding soil. Experiments have proven that crops grown in earthworm inhabitant soils had increased the yields from 25% to over 30% than in earthworm free soils. Researchers had reported that bacteria living in the gut of worms would breakdown many hazardous chemicals such as hexachloro cyclohexane (HCH) into detoxified forms maintaining the biological buffering of the soil. Most of the agricultural lands are recorded for lower abundance of earthworms (or) leading to abandon the croplands due to the lack of favorable conditions influencing the growth of plants. Investigation of the interaction of actinomycetes with soil invertebrates is one of the ways to study the development in biogeocenoses. The earthworm gut is favorable for the development of actinomycetes due to neutral pH, optimal humidity and temperature. Cellulose is considered as one of the most important sources of carbon on this planet. Cellulose degradation and its subsequent utilization is important for global carbon sources. The value of cellulose as a renewable source of energy has made cellulose hydrolysis as the subject of intense research and industrial interest. The bioconversion of cellulosic materials has been receiving attention in recent years. It is now a subject of interest for the contribution and to the development of a large-scale conversion processes beneficial to mankind. Agricultural waste and in fact all celluloses can be converted into products that are of commercial importance such as ethanol, glucose and single cell protein. Cellulase enzyme has been reported as one of the commercialized products from the bioconversion of cellulosic materials. The role of microbial activity in the earthworm gut, cast and soil is very essential for the degradation of organic wastes for the release of nutrients to plants. During vermicomposting organic matter undergoes, physico-chemical and bio-chemical changes by the combined effect of earthworm gut flora and also other microbial activities. Earthworm transforms the constituents of organic waste into a more useful vermicompost initially by grinding and digestion by aerobic and anaerobic microflora. Much of the research on vermicomposting had been focused on the changes in the chemical parameters.

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INTRODUCTION

A great deal of research on the enzymatic degradation of cellulose and hemicellulose from different substrates has been developed in the last two decades. Degradation of cellulosic biomass from agricultural wastes by bacteria / fungi/ actinomycetes plays a vital role in carbon recycling. Treatment of cellulose by cellulolytic enzymes for practical purposes has attracted the continuing interest of biotechnologists.The role of microbial activity in the earthworm gut, cast and soil is very essential for the degradation of organic wastes for the release of nutrients to plants. During vermicomposting organic matter undergoes, physico-chemical and bio-chemical changes by the combined effect of earthworm gut flora and also other microbial activities. Earthworm transforms the constituents of organic waste into a more useful vermicompost initially by grinding and digestion by aerobic and anaerobic microflora. Much of the research on vermicomposting had been focused on the changes in the chemical parameters. Earthworm, the friend of the farmer, naturally provides nutrients of the soil by biological conversion involving the beneficial microorganisms and contributing the nutrients as well as biocontrol products. Earthworm populations have gone down significantly with the

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use of pesticides/ chemical fertilizers which influence the proliferation of microorganisms. Efforts need to be placed for replacement of earthworm populations and/or total number and types of gut microbial population to the soil by different treatments. Cellulase enzyme has been reported as one of the commercialized products from the bioconversion of cellulosic materials. This production from the renewable cellulosic resources can give us many benefits especially for decreasing air pollution and the production of biofuel the ethanol. This adds macro-economic benefits for rural communities especially women and society at large. Despite realizing these benefits at bench scale, commercialization and widespread application of degraded lignocellulosic biomass must be developed and enlarged.

Some features like degree of crystallinity, lignification and capillary structure of cellulose etc., of natural cellulosic materials known to inhibit their degradation / bioconversion. The crystallinity and lignification will limit the accessibility and susceptibility of cellulose to cellulolytic enzymes and other hydrolytic agents. However, many physical, chemical and microbial pre-treatment methods for enhancing bioconversion of cellulosic materials have been reported. Pre-treatment of cellulose opens up the structure and removes secondary interaction between glucose chains. Cellulolytic enzyme activities of isolated microorganisms were determined using cotton wool, carboxy methyl cellulose and other cellulosic compounds as substrates. All cellulolytic microorganisms showed high growth rates at 30^0C-35^0C. Cellulolytic microorganisms showed high growth rate at acidic pH 4.5. Cellulose degrading organisms have been exploited for the conversion of cellulolytic materials into soluble sugars or solvents useful in several biotechnological and industrial applications.

MATERIALS AND METHODS

Production of Cellulase by egas isolate

Mineral salts medium (100 ml in 500 ml Erlenmeyer flask) was inoculated with 1ml of 5 day old EGAS culture and incubated at 30^0C with vigorous aeration in a shaker at 150 rpm for 7 days. Cells were separated by centrifugation at 8,000 x g for 20 minutes at 4^0C. The cell free culture filtrate was used as crude enzyme source. Filter paper assay method was employed to determine cellulase activity of the filtrate (Mandels and Weber, 1976). Filter paper activity was a measure of total cellulolytic activity resulting from combined action of different enzyme components present in the culture filtrate. In this method, the Whatmann No. 1 filter paper as a cellulosic substrate was incubated with EGAS + isolate as a source of enzyme. The liberation of reducing sugar was measured by DNS method. One unit of cellulase was defined as the amount of enzyme releasing 1 Micromole of reducing sugar per ml per minute. Protein concentration was measured by Lowry method (1951).

Effect of Environmental factors on Cellulase activity

The factors such as pH, temperature, time, metal ions, inhibitors and surfactants that play an important role on enzyme activity were tested while comparing the results with controls.

Effect of pH on cellulase activity

The effect of pH on activity of cellulase was measured on day 5 by incubating 0.5 ml of culture filtrate containing CMC (0.5%) and 1.5 ml of buffer, after adjusting the pH to 3, 5, 7, 9, 11. The buffers used were (50 mM): Citrate buffer (pH 4 and pH 5), Sodium phosphate buffer (pH 6 and pH 7) and Tris – HCL buffer (pH 9 - 11).

Effect of Temperature on cellulase activity

The influence of temperature on enzyme activity was studied by using the reaction mixture containing 1.5 ml of 50 mM of Tris HCl buffer at pH 9.0 and 0.5 ml of crude enzyme preparation and incubated at 30^0C - 55^0C with intervals of 5^0C until a decline in the enzyme activity was observed.

Effect of Time on cellulase activity

The 250 ml Erlenmeyer flask with 100 ml of mineral salts medium inoculated with 1 ml of 5 day old EGAS1 isolate. The flasks were incubated at 30^0C on shaker at 250 rpm for 1 - 7 days. After every 24 hrs, 5 ml of the culture medium was centrifuged at 8,000 x g for 20 min at 4^0C. The cell free culture filtrate was used as crude enzyme source. Exo-β-glucanase and Endo-β-glucanase enzymes were assayed as described by Mandels and Weber (1969) while β-glucosidase activity was assayed as described by Herr (1979).

Effect of Metal ions and inhibitors on cellulase activity

Effect of four metal ions such as $CuSO_4$, $MnSO_4$, $ZnSO_4$, $CaCO_3$ and two inhibitors like EDTA, NaN_3 was determined by incubating crude enzyme with 1 mM concentration of each metal ion and inhibitor.

Effect of surfactants on cellulase activity

Mineral salts medium 50 ml was amended with 1% cellulose and dispensed to each of 250 ml Erlenmeyer conical flasks. Only one of the selected surfactants (Triton X- 100, Tween-20, Tween- 80, Sodium Dodecyl Sulphate) was added to each of the flasks at 0.015% level. It lowers the surface tension of a liquid allowing easier spreading and lowering of interfacial tension between two liquids or between a liquid and a solid. The flasks devoid of surfactants were the controls. All flasks were added with EGAS1 isolate and incubated for 15 min at 65^0C. After cooling 0.3 ml of DNS reagent was added and boiled for 5 min at 100^0C. Extracellular protein content and activity of individual enzyme components of cellulase system in the culture filtrate of EGAS1 isolate was read at 540 nm by spectrophotometer (Sudeep George, 2000, Challapandi, 2008).

Optimization of Cellulase production in *Streptomyces Albaduncus* as influenced by the Nutrients

Factors influencing cellulase production under laboratory conditions were studied for EGAS1 isolate, as it had exhibited highest cellulolytic activity in terms of Filter Paper Units (Feng Xu, 2007).

Effect of different carbon sources on cellulase production

For maximum growth and high cellulase production, mineral salts medium was used, to determine the effect of supplementation of different carbon sources. For cellulase production carbon sources used were carboxy methyl cellulose, cellulose, cellobiose and glucose added at 1% (W/V) to 50 ml mineral salts medium in 250 ml Erlenmeyer conical flasks. After sterilization, the flasks were aseptically inoculated with EGAS1 isolate and grown in axenic conditions. Samples were withdrawn from all the flasks with growing culture for measurement of activity of individual enzyme components of cellulase in the culture filtrate.

Effect of Carboxy Methyl Cellulose concentration on cellulase production

In earlier reports, cellulase production by organism was studied at only one level of CMC concentration (0.5% w/v). This experiment was conducted to determine effect of CMC supplementation at different levels to the medium on cellulase yield by the organism. Mineral salts medium was amended with CMC ranging from 0.5%, 1.0%, 1.5%, 2.0% and 2.5% (w/v) concentration and inoculated with EGAS1 isolate. Activity of individual enzyme components of cellulase system in the culture filtrate was determined as specified in the section 3.7.

Effect of nitrogen source on cellulase production

Mineral salts medium amended with 1% cellulose was dispensed to 250 ml Erlenmeyer conical flasks at the rate of 50 ml medium per flask. Only one nitrogen source either urea or peptone or tyrosine or yeast extract or ammonium sulfate at concentration of 0.3% (W/V) nitrogen was added to each flask. The flask with cellulose-amended medium devoid of nitrogen source had served as control. All flasks were aseptically inoculated with EGAS1 isolate and incubated at 30^0C for growth. The flasks were processed for measurement of cellulase system in the culture filtrate (Challapandi, 2008).

Cellulase Production by *Streptomyces Albaduncus* (EGAS1)

After identification of actinomycete culture, the efficiency of the organism for cellulase production was determined using the mineral salts medium. Cellulolytic activity of EGAS1 isolate was determined according to filter paper assay method (Mandels & Weber, 1969). The amount of soluble reducing sugar that was (glucose) released into the production medium was determined. The cellulase activity was expressed in terms of Filter Paper Units (FPU). The volume of EGAS1 isolate filtrate responsible for the release of 1µ mole of glucose per min was considered to be one filter paper unit. Since *Streptomyces albaduncus* had been detected to exhibit highest cellulolytic activity in terms of filter paper units of 1.92 FPU/ml, this organism was further exploited to assess the cellulolytic potential in subsequent experiments. The actinomycete isolate of the present investigation need to be further studied in depth of its cellulolytic potential for actual application in the conversion of waste products into value-added and useful products.

RESULTS AND DISCUSSION

Effect of Environmental factors on Cellulase activity

Environmental factors such as pH, temperature, time period and metal ions/inhibitors had shown significant effect on cellulase activity of *S. albaduncus*.

Effect of pH on cellulase activity

Influence of pH on cellulase activity by actinomycete was examined on day 5. Individual enzyme components of cellulase system i.e., Exo-β-glucanase, Endo-β-glucanase, β-glucosidase had shown maximum activity at pH 9 Fig.5. Hence it was capable of producing alkaline cellulase. As the isolate was found active at pH 7, also it can be used in degradation of organic matter, detergents and sewage treatment. High activities of Exo-β-glucanase (4.1 IU/ml), Endo-β-glucanase, (4.9 IU/ml), and β-glucosidase (1.5 IU/ml) were recorded in the culture filtrate at pH 9 derived from the growth of *S.albaduncus* in the medium.

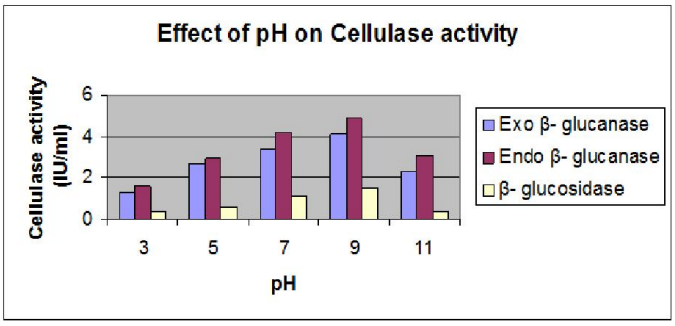

Fig. 5. Effect of pH on Cellulase activity

Effect of Temperature on cellulase activity

Temperature highly influences the metabolic reactions through enzymatic activities, thereby affecting the growth of the organism. Effect of temperature on enzyme production indicated that exo-β-glucanase, endo-β-glucanase and β-glucosidase enzyme was maximum at 45^0C. The extracellular protein, total soluble sugar content and cellulase enzymes were assayed at 5 days of incubation and represented in Fig.6. This culture filtrate also yielded higher activity in terms of Exo-β-glucosidase with 4.4 IU/ml, Endo-β-glucanase with 4.9 IU/ml and β-glucosidase with 1.5 IU/ml.

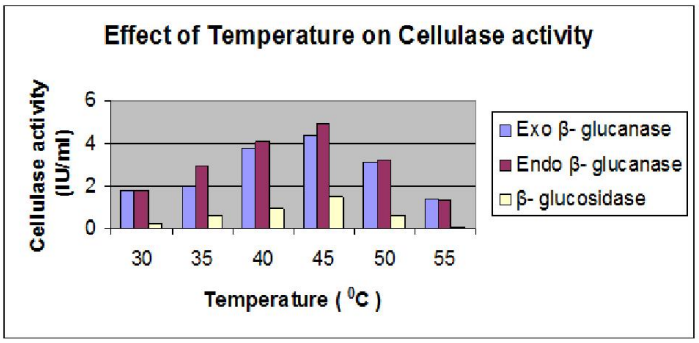

Fig. 6. Effect of Temperature on cellulase activity

Effect of Time on cellulase activity

Time response of cellulase production in actinomycete within 1-7 days was studied. Maximum production of Exo-β-glucanase, Endo-β-glucanase and β-glucosidase was recorded on day 5 and decline was in later stages during the following days i.e., 6th and 7th day (Fig. 7).

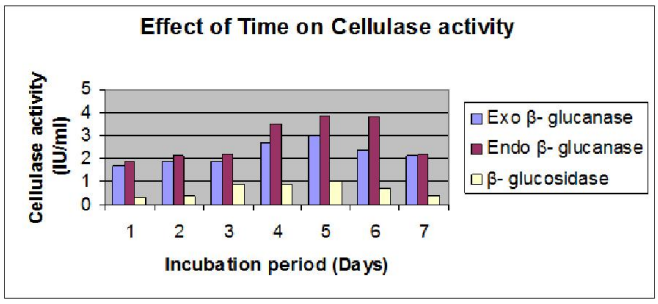

Fig. 7. Effect of Time on cellulase activity

Effect of Metal ions and inhibitors

Effect of different metal ions and inhibitors on cellulase production in actinomycete was studied. Metal ions tested had shown stimulatory effect on microorganisms and it was observed that $CuSO_4$ increased exo-β-glucanase, endo-β-glucanase and β-glucosidase activity. The remaining metal ions showed relatively a decrease in enzyme activity. The culture filtrate had yielded higher activity in terms of Exo-β-glucanase with 8.9 IU/ml, Endo-β-glucanase with 9.2 IU/ml and β-glucosidase with 1.8 IU/ml in the case of the metal ion $CuSO_4$ Fig.8.

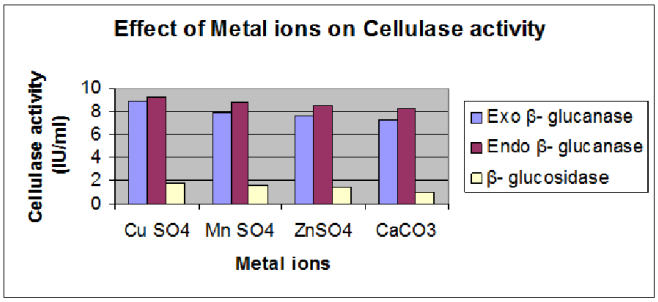

Fig. 8. Effect of Metal ions on Cellulase activity

Inhibitors like EDTA, NaN_3 at 1mM concentration had inhibited the activity of all the three enzymes. The culture filtrate had shown that the activity with NaN_3 in terms of Exo-β-glucanase with 4.0 IU/ml, Endo-β-glucanase with 5.3 IU/ml and β-glucosidase with 0.75 IU/ml (Fig.9).

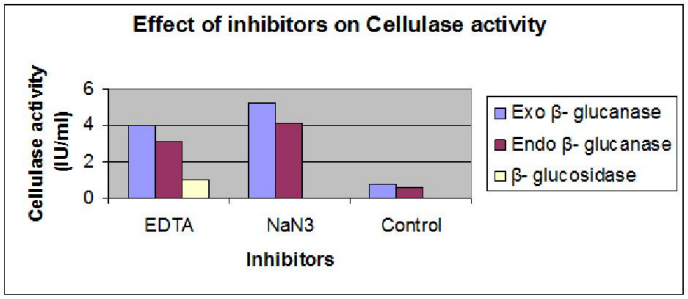

Fig. 9. Effect of inhibitors on Cellulase activity

Effect of surfactants on cellulase production

Influence of surfactants such as Triton-X-100, Tween-80, Tween-20 and SDS on cellulase production by *Streptomyces albaduncus EGAS1* was determined. Cellulase production in the medium amended with surfactants was compared (Fig.10). The medium with Tween-80 had yielded more extracellular protein and highest cellulolytic activity of Exo-β-glucanase with 0.98 IU/ml, Endo-β-glucanase with 1.99 IU/ml and β-glucosidase with 0.86 IU/ml followed by the results of Tween-20, Triton X and SDS.

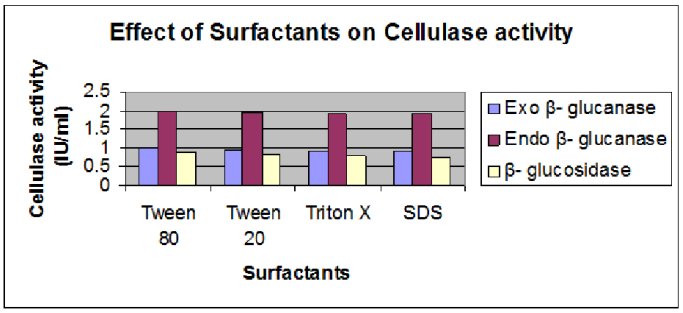

Fig. 10. Effect of surfactants on cellulase activity

Optimization of Cellulase production

Streptomyces albaduncus EGAS1 had exhibited the maximum cellulolytic potential and was further assessed by determining the effects of nutrients on cellulase production.

Effect of different carbon sources on cellulase production

Cellulase production and secretion of extracellular protein on mineral salts medium with different carbon sources at 1% level was compared for the organism. Results of this comparative study were represented in Fig. 11. Of all the carbon sources tested, CMC elicited the production of highest titres of Exo-β-glucanase with 4.6 IU/ml, Endo-β-glucanase with 5.1 IU/ml and β-glucosidase with 1.6 IU/ml Effects of other carbon sources cellulose, cellobiose was intermediate between those exhibited by glucose and CMC. The growth and production of cellulolytic enzymes were highly favoured on Carboxy Methyl Cellulose in comparison to microcrystalline cellulose. The inclusion of glucose in medium at higher concentration in media suppressed the carboxy methyl cellulose and effected pronounced repression of cellulase synthesis.

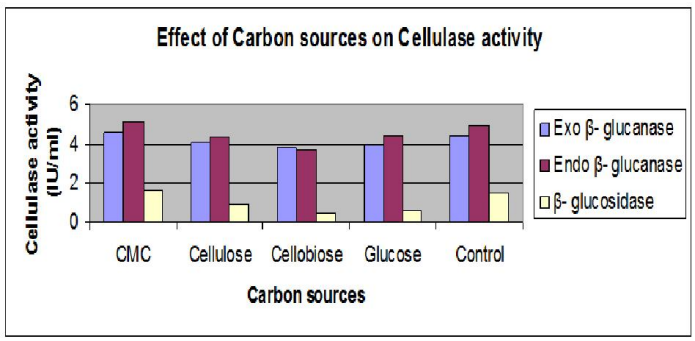

Fig. 11. Effect of different carbon sources on cellulase activity

Effect of Carboxy Methyl Cellulose concentration on cellulase production

The effect of different concentrations of CMC at 0.5%, 1.0%, 1.5%, 2.0% and 2.5% on cellulase production by *Streptomyces albaduncus* EGAS1 was examined Fig.12. Production of cellulase was increased with increase in the concentration of CMC up to 1.5% (w/v). Further increase in the CMC concentration beyond this level did not result in proportionate increase. The titres recorded for exoglucanase production *by Streptomyces* were 1.29, 1.56, 3.95, 3.85, 2.71 IU/ml and corresponding values for endoglucanase and β-glucosidase were 2.16, 2.36, 3.21, 2.29, 1.90 and 1.5, 2.57, 3.31, 2.31, 1.90 IU/ml respectively. Thus it is clearly evident from those results that CMC concentration at 1.5% was optimum for cellulase production. In this study, decreased production of cellulase at initial concentration with concomitant accumulation of glucose was further observed.

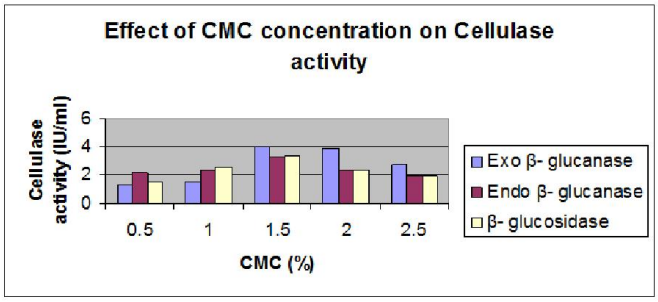

Fig. 12. Effect of CMC concentration on Cellulase activity

Effect of different nitrogen sources on cellulase production

Nitrogen is one of the important elements required for growth of microorganisms. Provision of utilizable form of nitrogen source to organisms is the basic requirement to be fulfilled for optimal growth. In order to find out the best utilizable form of nitrogen source for growth, extracellular protein and cellulase production by actinomycete on mineral salts medium supplemented with different nitrogen sources such as urea, peptone, sodium nitrite and yeast extract were determined. The results obtained from this experiment were represented in Fig.13. Urea appeared as the best nitrogen source followed by peptone for the highest activity of Exo-β-glucanase with 4.8 IU/ml, Endo-β-glucanase with 5.3 IU/ml and β-glucosidase with 1.6 IU/ml.

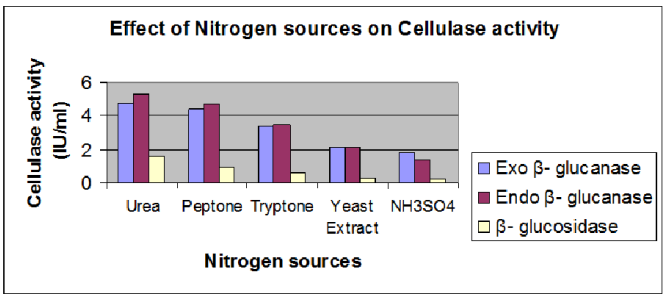

Fig. 13. Effect of different nitrogen sources on cellulase activity

From these results it may be concluded that the isolate can produce a complete set of cellulase enzyme complex, which is essential for rapid hydrolysis of cellulosic materials. The activity of each enzyme component was greatly influenced by factors like pH, Temperature, Time, Metal ions / inhibitors, surfactants and Nutrients which can be best monitored for the maximum activity of the enzyme complex thus the degradation. In general, cellulolytic microorganisms showed high growth rate at acidic pH 4.5. Gava (1988) reported that the majority of actinomycetes isolated from rhizosphere and non-rhizosphere soil grow at a pH range varying from 6.5 to 8.0. An another report, in support of the present result *Streptomyces* AC-29 and AC-92 had presented good growth, characterized by abundant mycelium, in culture media with pH between 5.0 and 5.5. These findings suggest that these are acid tolerant isolates (Moreira and Siqueira, 2002). Research conducted by Coelho and Drozdowicz (1998) in acid soils (pH 4.9) in the Brazilian Cerrado demonstrated the presence of a microbial population sufficiently numerous and rich in actinomycetes. The results of this work indicate the adaptability of these microorganisms under these environmental conditions, showing that they have a good ability to compete and survive in acid soils. Similar observations were observed in our present study. Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001).

Cellulase production in the present studied for the efficiency by the organism for was determined using the mineral salts medium. Cellulolytic activity of EGAS1 isolate was determined according to filter paper assay method (Mandels and Weber, 1969). The results indicated that *Streptomyces albaduncus* exhibit highest cellulolytic activity (1.92 FPU/ml) by the production of cellulase, which is one of the key enzymes for cellulose biodegradation. Cellulase activity in the present study was determined by filter paper using the DNS method, total endoglucanase activity was determined against carboxymethyl cellulose (CMC) followed by the DNS method . Similar method was adopted by Miller, 1959; Ghose, 1987, and the β-glucosidase activity was determined against Þ-nitrophenyl –β-D-glucoside and the liberation of Þ-nitrophenol was accompanied by adsoption spectroscopy at 410 nm. Similar method was used by Claeyssens, 1992. In the present study the effect of environmental factors such as pH, temperature, metal ions/inhibitors and time period on cellulase activity of actinomycetes was studied and it was observed by the EGAS1 a significant effect on cellulase activity. High activities of Exo-β-glucanase (4.1 U/ml), Endo-β-glucanase,(4.9 U/ml),and β-glucosidase(1.5 U/ml) were also recorded at pH 9, yielded higher activity in terms of Exo-β-glucosidase with 4.4 U/ml, Endo-β-glucanase with 4.9 U/ml and β-glucosidase with 1.5 U/ml at temperature 45^0C. Results were similar with the following authors; microorganisms produce cellulases which are released under a variety of environmental conditions, such as high temperature (30^0C-50^0C) and at a range of pH (5-9) (Burns, 1978; Klein, 1989; Sinsabaugh and Linkins, 1989; Deng and Tabatabai, 1994, Jack Doyle *et al,* 2005).

Among the above noted microorganisms the cellulolytic thermophilic microorganisms are of particular interest, because of their ability to produce thermostable cellulases which are generally stable under a variety of severe conditions including highly acidic and alkaline pH as well as temperatures upto

60^0C.(Bhat and Bhat, 1997). As observed Jaradat *et al*., 2008 the *Streptomyces* strain J2 had shown the highest crude enzyme activity (432 U L^{-1}) after 3 days of incubation at pH 7 and 60°C of temperature. Theberge *et al*. (1992) had reported an optimum pH of 5.5 for endoglucanase production by the strain of *Streptomyces lividans*. Solingen *et al*. (2001) had worked an alkaline novel *Streptomyces* species isolated from east African soda lakes that have an optimal pH of 8, highlighting the effect of alkaline environment on the adaptation of these *Streptomyces*. McCarthy (1987), had recorded an optimal temperature for cellulase activity in the range of 40-55^0C for several *Streptomyces* species including *S. lividans, S. flavogrisus* and *S. nitrosporus*. Jang and Chen (2003) described a CMCase produced by a *Streptomyces* T3-1 with optimum growth at a temperature of 50^0C, whereas Schrempf and Walter (1995) described a CMCase production by a *S. reticuli* at an optimum temperature of 55^0C.

The pH profiles for CMCase from *S.drozdowiczii* have shown more than 50% activity in all pH values tested within the range from 3 to 10. Optimum activity occurred at pH 5.0, but another peak at pH 10.0 was observed. Semedo *et al.,* (2000), have found optimum pH 7.0 for CMCase activity produced by *S.drozdowiczii*, with an extra peak with minor activity in pH 11.0. According to George *et al,* (2001) CMCase from culture supernatant obtained from a species of *Thermomonospora* presented optimum activity at pH 5.0 where as Jang and Chen obtained a CMCase produced by *Streptomyces* T3-1 with optimum activity at pH 7.0. Comparison in terms of enzyme activity is difficult to establish because the prokaryotic cellulases may present very different action from those of fungi, with optimum temperature and pH variations among them (Heck, *et al;* 2002). Harchand and Singh (1997) also investigated a profound effect of pH on cellulase activity of *S. albaduncus*. pH and temperature optima for endoglucanase productivity of both isolates were 7-7.5 and 26^0C respectively. Beyond this limit of pH and temperature these isolates did not shown substantial enzyme activity. Similarly, *Streptomyces sp.* F2621 (Tuncer *et al*; 2004) and *S.albogresius* (Van Zyl, 1985) also exhibited maximum endoglucanase activity at 26-30^oC with initial pH of 6.5-7.0.

The present isolate was capable of producing alkaline cellulase. As the isolate was found active at pH 8, it can be used in degradation of organic matter, detergents and sewage treatment. Wakararchuk *et al.*; (1994) had reported that paper industry needs those cellulases, which are active above pH 6.5-7. The results here obtained for the optimum temperature for CMCase activity from *S.albaduncus* are in agreement with others found in literature. Jang and Chen (2003) described a CMCase produced by a thermophilic strain of *Streptomyces* T3-1 with optimum temperature of 50^0C, where as Schrempf and Walter (1995) have cited a CMCase from *Streptomyces reticuli* with optimum at 55^0C, and George *et al.,* 2001 an endoglucanase from *Thermomonospora* with optimum at 50^0C. The present investigation on the effect of temperature on enzyme production indicated that maximum exo-β-glucanase, endo-β-glucanase and β-glucosidase enzyme was shown at 55^0C indicating the thermotolerant nature of the enzyme. In the present study time of cellulase production by *Streptomyces albaduncus* was recorded on day 5 respectively and decline was in latter stages i.e., 6^{th} and 7^{th} day.

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