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International Journal of Applied Sciences and Biotechnology

A Rapid Publishing Journal

ISSN 2091-2609

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CODEN (Chemical Abstract Services, USA): IJASKD

Vol-3(2) June, 2015

Available online at:

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Impact factor*: **1.422**
Scientific Journal Impact factor#: **3.419**
Index Copernicus Value: **6.02**

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OPTIMIZATION STUDIES ON CELLULASE PRODUCTION FROM *BACILLUS ANTHRACIS* AND *OCHROBACTRUM ANTHROPI* (YZ1) ISOLATED FROM SOIL

Mohammad Badrud Duza and S.A. Mastan²

¹Research Scholar, Dept. of Biotechnology, Acharya Nagarjuna University, Guntur – 522510

²Matrix-ANU Advanced Aquaculture Research Centre (MAAARC), Acharya Nagarjuna University, Nagarjuna Nagar-522 501, Guntur (Dt.), Andhra Pradesh

Corresponding Author: mail:shaikmastan2000@yahoo.com

Abstract

The present study was carried out to demonstrate the optimization of growth conditions of bacteria with high cellulase activity. Cellulose degrading bacteria were isolated from soil samples collected from different areas of Guntur district, A.P. The bacteria were isolated using serial dilution and pour plate methods. The isolated bacteria were identified by morphological, biochemical and molecular procedures. The isolated bacterial species were screened for cellulase production in sub-merged fermentation process. The two tested bacterial species showed maximum yield for cellulase production. These two bacteria were identified as *Bacillus anthracis* and *Ochrobactrum anthropi* (YZ1). Supplementation of glucose, peptone, tyrosine and EDTA to the fermentation medium is favoured enzyme secretion. The optimum pH and temperature for the activity of crude enzyme was 8 and 45°C, respectively for *Ochrobactrum anthropi* (YZ1) while for *Bacillus anthracis*, it was 8 and 40°C, respectively. 14% of inoculum level and 96 h of incubation period showed the maximum yield by both the species bacteria for cellulase production. The results of present study indicated that favorable fermentation conditions and the selection of a suitable growth medium played a key role in the production of cellulase from newly isolated *Bacillus anthracis* and *Ochrobactrum anthropi* (YZ1).

Keywords: Cellulase production; Bacteria; Soil.

Introduction

Celluloses are a group of hydrolytic enzymes most abundant biomass on earth (Tomme *et al.*, 1995). It is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bio-resource produced in the biosphere (Jarvis, 2003; Zang and Lynd, 2004). Several microorganisms including both bacteria and fungi have been found to produce a variety of cellulases for the degradation of cellulose (Bahkali, 1996; Magnelli and Forchiassin, 1999; Shin *et al.*, 2000; Immanuel *et al.*, 2006). Primarily, cellulases are classified into three main groups: the exoglucanases, endoglucanases (cleaving β -1, 4-glycosidic bonds from chain ends and internally within chains, respectively) and β -glucosidases (cleave the final β -1,4 linkages of cellobiose or small polysaccharides). Bacteria and fungi have been found to produce and secrete these enzymes freely in solution; Bacteria which have high growth rate as compared to fungi have good potential to be used in cellulase production. The cellulolytic property of some of the bacterial genera namely *Cellulomonas*, *Cellvibrio*, *Pseudomonas* sp., *Bacillus* sp.

and *Micrococcus* sp., was also reported (Immanuel *et al.*, 2006; Nakamura and Kppamura, 1982).

Cellulases have enormous potential applications in industries and are used in food, beverages, textile, laundry, paper and pulp industries etc (Camssola and Dillon, 2007; Koomnok, 2005; Cherry and Fidants, 2003). The use of cellulases in the textile industry are cotton softening and denim finishing; in laundry detergents for colour care, cleaning; in the food industry for mashing; in the pulp and paper industries for drainage improvement and fiber modification, and they are even used for pharmaceutical applications (Cherry and Fidants, 2003). They are also used in the formation of washing powders, extraction of fruit and vegetable juices, and starch processing (Camssola and Dillon, 2007). As lytic enzymes, they are of also major importance is the protoplast production for tissue culture and plant metabolites production. The demand for more thermo stable, highly active and specific cellulases is on the increase; therefore, cellulase systems of local microbes have been investigated, keeping in view of the importance and application of the cellulases in industries. In the present

study, efforts have been made to screen the native bacterial species as source for hyper-producers of cellulase.

Materials and Methods

Sample Collection

Bacteria were isolated from soil samples collected from different sources such as soil near to timber depot. Some samples are collected from soil exposed near pulp and paper industries. The samples were collected into sterilized polythene bags and were brought to the laboratory. Bacterial isolates were isolated by serial dilution method. 1 g of soil was transferred to 10 ml of distilled water in test tubes. Dilutions were made up to 10^{-6} and 0.1 ml of soil suspension was spread on to the sterilized Nutrient agar media (NAM). pH of the medium was adjusted to 7. After autoclaving at 121°C and 15 lbs pressure, 20 ml of sterile medium were transferred to sterile Petri plates and allowed for solidification. After solidification of the medium 0.1 ml of soil suspension was spread with the help of spreader and incubated at 37°C for 48 h. The bacterial cultures grown on the medium were sub-cultured for repeated times. Pure culture was transferred on to the agar slants and maintained at 4°C for further studies.

Identification of Cellulolytic Bacteria

Identification of cellulolytic bacteria was carried out by morphological, biochemical tests and 16S RNA sequencing study (Apun *et al.*, 2000).

Biochemical Characterizations

The parameters investigated included Indole test, Methyl red test, Vogues-Proskauer test, Citrate utilization test, Catalase test, Oxidase test, Gelatin test, Casein hydrolysis test, Motility test, Amylase test, Nitrate reduction test, Carbohydrate fermentation test by standards methods (Buchanan and Gibbons, 1974). The various media was prepared in sterile distilled water and pH was adjusted accordingly.

Screening of Cellulolytic bacteria

Pure cultures of bacterial isolates were individually transferred in CMC agar plates. After incubation for 48 h, CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. One molar NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having the largest clear zone were selected for identification and cellulase production in submerged system.

Estimation of Cellulase Activity

Cellulase activity was assayed using dinitrosalicylic acid (DNS) reagent (Miller, 1959) by estimation of reducing sugars released from CMC solubilized in 0.05 M phosphate buffer at pH 8. The culture broth was centrifuged at 14000 ×

g for 10 min at 4°C and the clear supernatant served as crude enzyme source. Crude enzyme was added to 0.5 ml of 1 % carboxymethyl cellulose in 0.05 M phosphate buffer and incubated at 50°C for 30 min. After incubation, reaction was stopped by the addition of 1.5ml of DNS reagent and boiled at 100°C in water bath for 10 min. Sugars liberated were determined by measuring absorbance at 540 nm. Cellulase production was estimated by using glucose calibration curve. One unit (IU) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1 μmol of glucose per minute under standard assay conditions.

Optimization of nutritional conditions for cellulase production

Different nutritional conditions such as; additional 0.5, 1% carbon sources (starch, glucose, sucrose, Dextrose, Manitol, Mannose, Xylose, Fructose), amino acids at concentration of 0.01% (L-Lysine, L-Leucine, L-Histidine, Tyrosine, Tryptophan, Asparagine, Glycine, D-Alanine, Cysteine, Arginine, Phenylalanine), various nitrogen sources (Soybean Meal, Beef extract, Gelatin, peptone, tryptone, yeast extract) at concentration of 0.5% and 1%, vitamins (Riboflavin, Biotin, Pyrodoxine HCl, Folic acid, Nicotinic acid, Thamine HCl) were optimized for cellulase production by newly isolated strains.

Effect of pH on activity and stability of crude cellulases

The optimum pH for the crude enzyme was determined by incubating crude enzyme with substrate (1% CMC) prepared in appropriate buffers; 0.05 M citrate buffer (pH 3.0 to 6.0), 0.05 M sodium phosphate buffer (pH 6.0 to 8.0), 0.05 M Tris-HCl (pH 8.0 to 9.0) and 0.05 M glycine-NaOH (pH 9.0 to 11.0). Crude enzyme mixture in those buffers was incubated for 30 min at 50°C. Cellulase activity was assayed by DNS method. The pH stability was determined by incubating crude enzyme mixture in above-mentioned buffers at room temperature for 30 min and enzyme stability was determined by using DNS method.

Effect of temperature on activity of crude cellulases

The effect of temperature on activity of endoglucanase was determined by incubating crude enzyme with 1 % CMC in 10mM phosphate buffer (pH 8.0) at temperatures between 20 to 60°C. Enzyme activity was assayed by DNS method at different temperatures as described above.

The optimization parameters like Effect of incubation time (24-168 h), Effect of inoculum level (4-16%), Effect of Agitation rate 970-170rpm), Effect of metabolic inhibitor (AgNO₃, EDTA, NAF, KMnO₄, β-ME, PMSF, Iodoacetate), Effect of antibiotics (Penicillin, Streptomycin, Neomycin, Framyicitin, Cephalosporin, Chloremphenicol) has been tested.

Results and Discussion

Isolation of Cellulase Producing Bacteria

There are two bacteria species, out of 17 isolates (TS5SRP and TS16MCN) were isolated from different soil samples were screened as cellulase producing bacteria, as shown in Table 1.

Identification of Isolated bacterial strains

These bacterial isolates were characterized on the basis of colony characteristics, microscopic appearance and biochemical tests. Morphological and biochemical

identification results were given in Table 2. Molecular characterization of these strains was done by 16S rRNA gene analysis. Further these amplified 16S rRNA gene sequences of the bacterial strains was blasted using online tool (MEGA 4). The taxonomical identification was done by the phylogenetic tree construction and the comparison of these bacterial strain sequences with other homologous bacterial sequences. After morphological, physiological, biochemical and taxonomical identification, these two bacterial isolates were identified as *Bacillus anthracis* (TS5SRP), *Ochrobactrum anthropi* –YZ1 (TS16MCN).

Table 1: Screening of different enzymes producing bacterial strains

S. No	Organism tested	Result			
		Amylase	Protease	Cellulase	Lipase
1	TS1MCP	-ve	+ve	-ve	-ve
2	TS2MCN	-ve	-ve	-ve	+ve
3	TS3BP	+ve	+ve	-ve	-ve
4	TS4BP	-ve	+ve	-ve	-ve
5	TS5SRP	+ve	-ve	+ve	-ve
6	TS6MCN	+ve	-ve	-ve	-ve
7	TS7BN	-ve	+ve	-ve	-ve
8	TS8MCN	-ve	+ve	-ve	-ve
9	TS9MCN	-ve	+ve	-ve	-ve
10	TS10MCN	+ve	-ve	-ve	-ve
11	TS11BP	-ve	-ve	-ve	+ve
12	TS12BN	-ve	+ve	-ve	-ve
13	TS13SP	+ve	-ve	-ve	-ve
14	TS14SN	+ve	+ve	-ve	-ve
15	TS15MCN	+ve	-ve	-ve	-ve
16	TS16MCN	+ve	-ve	+ve	-ve
17	TS17BP	+ve	-ve	-ve	-ve

Table 2: Morphological and biochemical characters of strains isolated from soil sample

S. No	Strain Code→ Name of the Test	TS1MCP	TS2 MCN	TS3BP	TS4BP	TS5 SRP	TS6MCN	TS7BN	TS8MCN	TS9MCN	TS10MCN	TS11BP	TS12BN	TS13SP	TS14SN	TS15MCN	TS16MCN	TS17BP
1	Gram Staining	+(GP)	-	+(GP)	+(GP)	+(GP)	-	-	-	-	-	+(GP)	-	+	-	+(GP)	-	+(GP)
2	Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Urease	-	-	+	-	+	-	-	-	-	-	+	-	-	-	-	+	-
4	H ₂ S test	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-
5	Oxidation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	Methyl Red	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+
7	Indole test	-	+	-	+	+	-	+	-	+	+	-	-	-	-	+	-	-
8	Vogues-Proskauer(VP) test:	-	-	+	-	+	-	+	-	-	-	+	+	+	-	-	-	-
9	Catalase:	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	Fermentation test	-	-	+GP	+	+	+	+	-	+	+	+GP	+	+	+	+	+	+
11	Nitrate reductase	+	+	-	-	-	+	-	-	+	-	-	+	+	-	-	-	+

Strain identification by 16S rRNA sequencing

16S ribosomal RNA gene sequencing of isolates TS5SRP and TS16MCN were performed. Result showed that TS5SRP was identified as *Bacillus anthracis* strain and TS16MCN was identified as *Ochrobactrum anthropi* strain. These partial sequences were submitted to GenBank with accession number KF525355.1 and KF525345.1 respectively. (Appendix: 1).

Assay of Cellulase activity

Estimation of enzyme activity for the positive isolates has conducted. Concentration of enzyme in crude sample was calculated by reacting with the enzyme with substrate comparing the resultant O.D with standard graph. The enzyme activity was expressed in International Unit (IU). 1U/ML = Amount of enzyme which releases 1micro mole glucose under assay conditions. The results of cellulase assay are shown in Table 3.

Table 3: Determination of Lipase activity from Cellulase producing Bacteria

S No	Strain tested	Name of the Strain	Cellulase activity IU/mg
1	TS5SRP	<i>Bacillus anthracis</i>	4.2
2	TS16MCN	<i>Ochrobactrum anthropi</i>	3.6

Cellulase optimization

Among seventeen bacterial species isolated from soil samples two species were capable of producing cellulase. These two bacterial species, capable of producing cellulase are *Bacillus anthracis* (TS5SRP) and *Ochrobactrum anthropi* (TS16MCN), were subjected for optimization, production and purification studies. Different growth parameters such as carbon source, nitrogen source, vitamin source, pH, temperature, incubation time, inoculums level, vitamins, amino acids, metabolic inhibitor, antibiotics and agitation rate were optimized. The carbon sources used in the study were starch, glucose, sucrose, dextrose, mannitol, mannose, xylose, and fructose. Nitrogen sources were supplemented in the medium in the concentration of 0.50 and 1.0%. Nitrogen sources used during the optimization studies were soybean meal, beef extract, gelatin, peptone, tryptone, yeast extract. The vitamins supplemented to the medium include Riboflavin, Biotin, Pyridoxine HCl, Folic acid, Nicotinic acid, Thiamine HCl. The pH of the medium was varied from 3 to 11. The incubation temperature was maintained in the range of 20-60°C. The inoculums level was varied from 4 to 16%, inoculums level was optimized. The agitation rate of orbital shaker was maintained in the range of 70-170rpm, the suitable rpm was selected after optimization studies. The medium was also supplemented

with metabolic inhibitors such as AgNO₃, EDTA, NAF, KMnO₄, β-ME, PMSF, iodoacetate and best source for metabolic inhibition was selected. Antibiotics were also added to the medium Penicillin, Streptomycin, Neomycin, Framyicitin, Cephalosporin and Chloramphenicol were used in the optimization studies. Based on the results, the fermentation media has been designed and the production of cellulase was carried out. The culture used for inoculation in the fermentation medium must be in healthy, active state and of optimum size, possibly in the log phase, thus it will be in its high rate for substrate conversion.

Among all carbon sources used in this study, glucose showed maximum cellulase production of 4.12 IU and 4.84 IU by two bacterial species namely *Ochrobactrum anthropi* –YZ1 (TS16MCN) and *Bacillus anthracis* (TS5SRP) at 1.0% of carbon source supplementation. In case of 0.50% of glucose as carbon source supplementation medium, *Ochrobactrum anthropi* (TS16MCN) produced maximum of 3.91 IU and *Bacillus anthracis* (TS5SRP) produced 4.63IU, the results were presented in Fig.1. Nitrogen source was optimized with different compound in different percentage and among them, Peptone and tryptone were proved to be best for *Ochrobactrum anthropi* (TS16MCN) produced highest of 4.29IU and 4.48IU in 0.50% and 1.0% of Peptone supplementation. Bacterial species *Bacillus anthracis* (TS5SRP) produced 4.69IU and 4.82IU in case of addition of tryptone in 0.50% and 1.0% respectively. Results for the effect of nitrogen source were given in Fig.2.

Amino acids have profound effects on cellulase production as they are the building blocks of proteins. In this study, it was observed that cellulase activity in the culture was significantly high with tyrosine. In the presence of amino acids, cellulase synthesis might be increased, which resulted in increase in enzyme activity. Among the 11 amino acids added as supplement in the medium (Fig.3), *Ochrobactrum anthropi* (TS16MCN) produced highest enzyme activity of 4.66IU by utilizing tyrosine. *Bacillus anthracis* (TS5SRP) yielded more of 0.30IU than the *Ochrobactrum anthropi* (TS16MCN), i.e. it produced 4.96 IU of cellulase in the production medium. Vitamins were considered as minor sources of nutrition. These are responsible for proper growth and nourishment of bacterial isolate and facilitate them to produce enzymes. While carrying the optimization studies of cellulase enzyme vitamins were optimized (Fig. 4) and in presence of Pyridoxine HCl, bacterial isolates *Ochrobactrum anthropi* (TS16MCN), and *Bacillus anthracis* (TS5SRP) showed cellulase production of 4.55 IU and 4.94 IU. It was comparatively higher than the enzyme activity in medium supplemented with other vitamin sources.

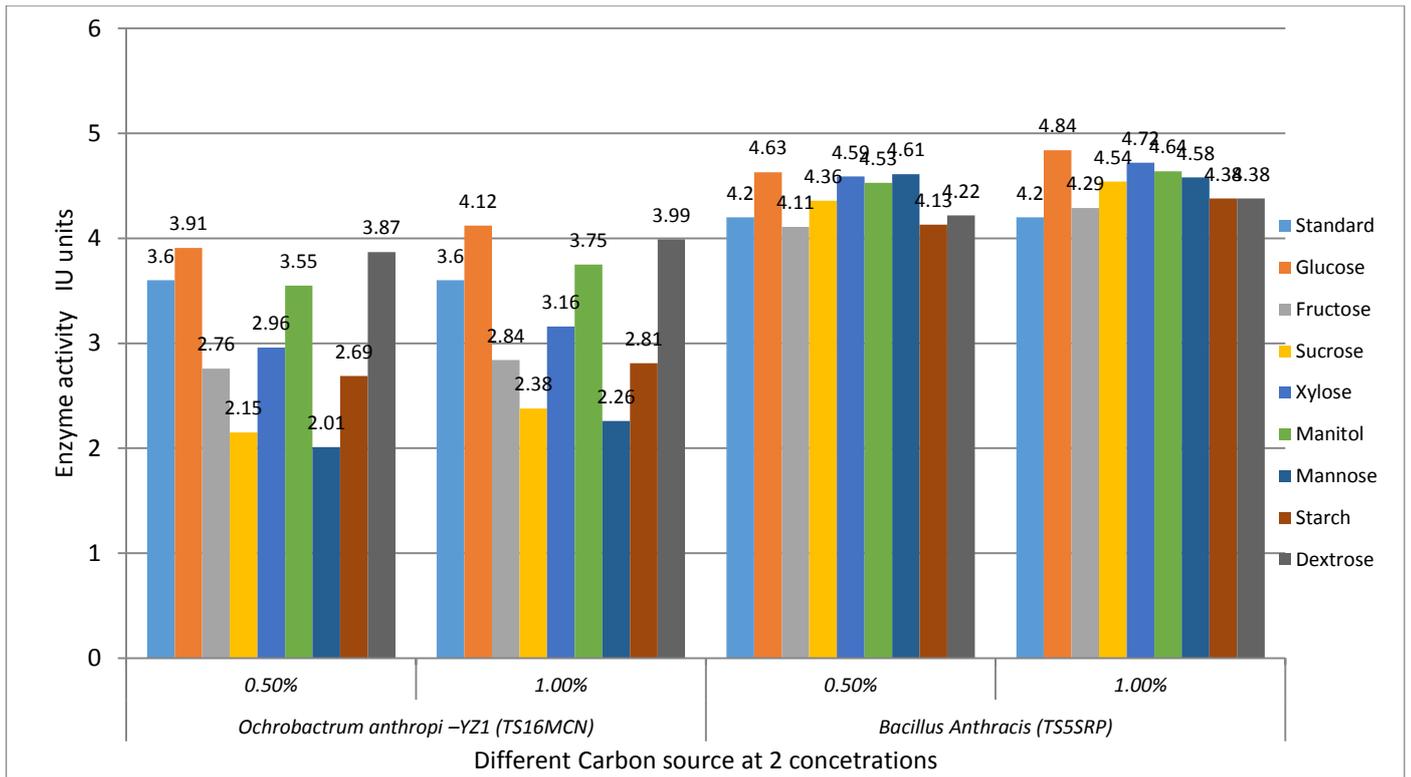


Fig.1: Optimization of carbon source for cellulase production

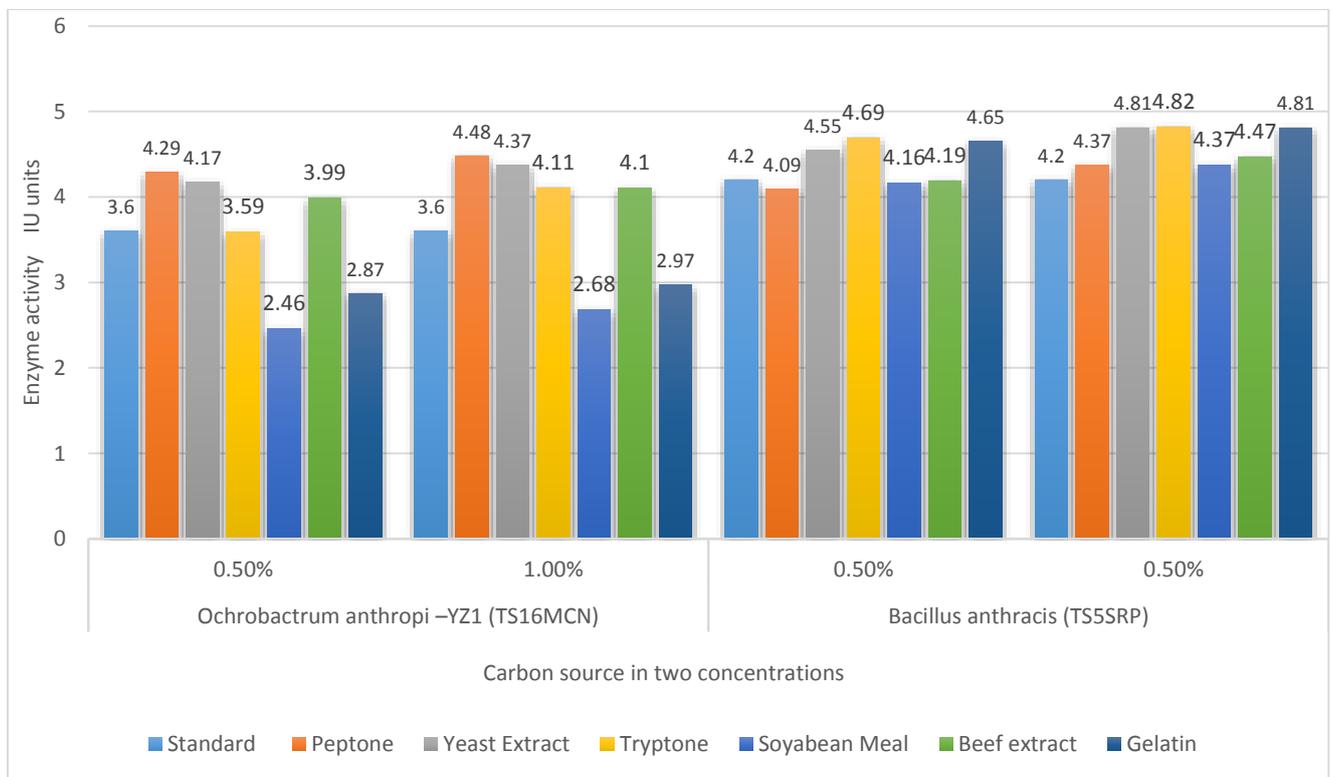


Fig.2: Optimization of nitrogen source for cellulase production

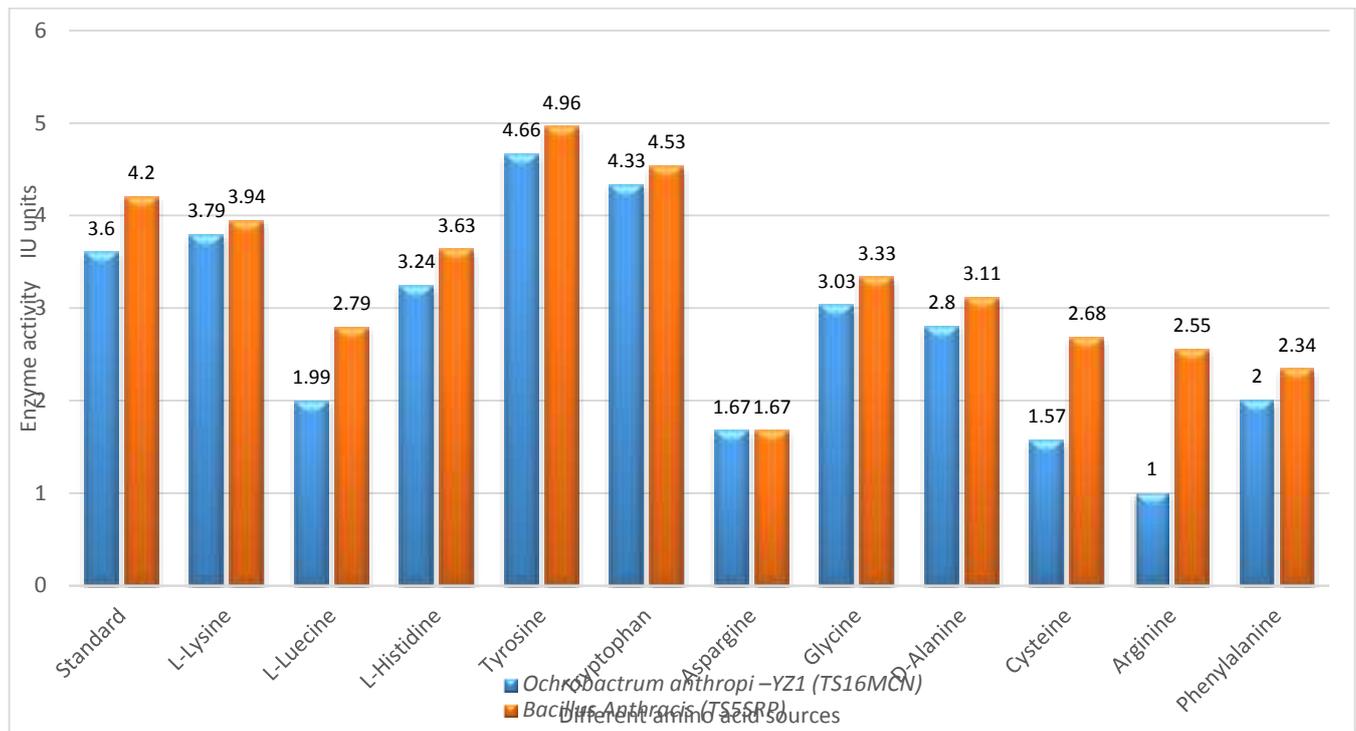


Fig.3: Optimization of amino acids source for cellulase production

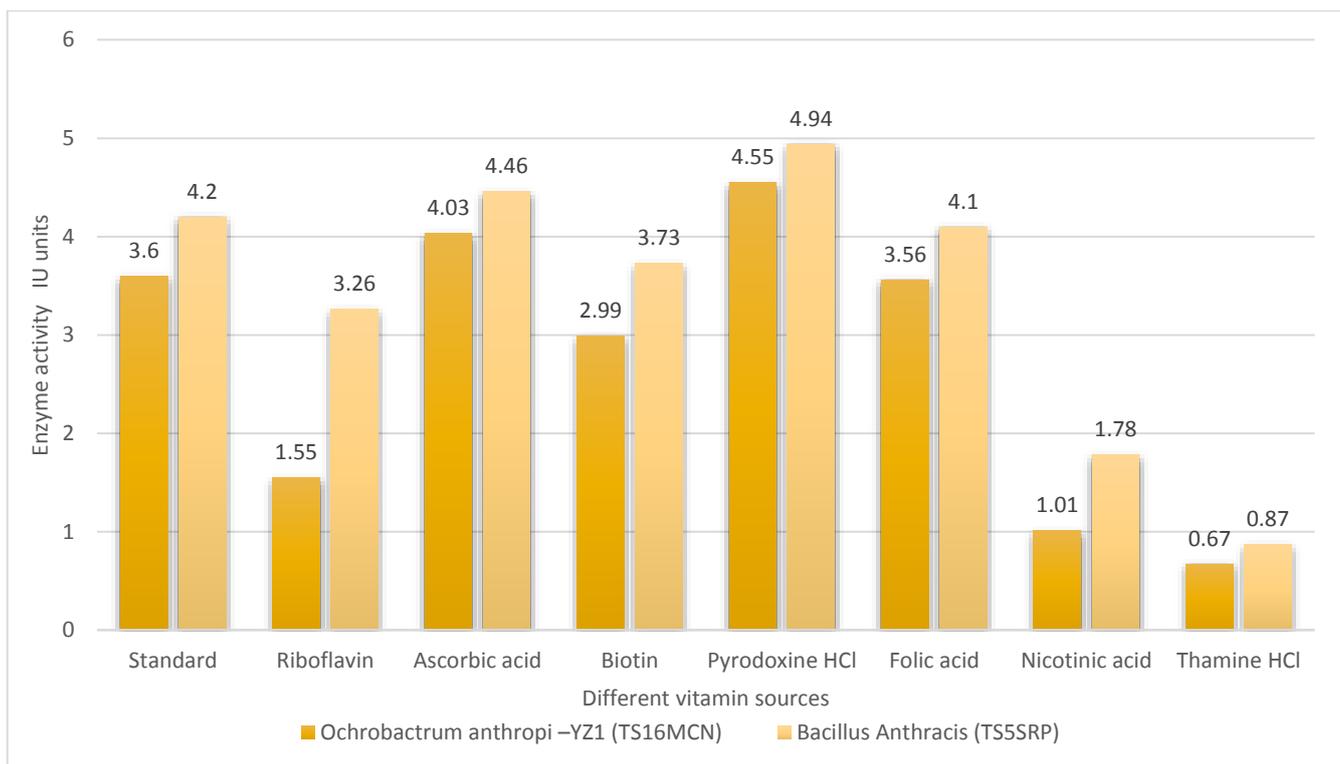


Fig.4: Optimization of vitamin source for cellulase production

In order to determine all effects on cellulase production, the selected bacterial isolates were grown in CMC broth and incubated at various parameters like varying pH (3 to 11), temperature (20 to 60°C) and incubation period (24 to 168 h at 37°C) and enzyme activity was determined by measuring cellulase activity. The pH of the production

medium greatly influences the enzyme production, as enzymes are sensitive to very low and high pH values. From the results given in Fig.5, it was illustrated that slightly alkaline pH of 8 was most suitable for production. At pH 8 bacterial species, *Ochrobactrum anthropi* (TS16MCN) yielded 4.32IU and *Bacillus anthracis* (TS5SRP) produced

4.69IU of cellulase enzyme. Thus pH of 8 was most suitable for cellulase production. Temperature for cellulase production was optimized by maintaining production medium at different temperature ranging from 20 to 60°C (Fig.6). The optimum temperature found to be 40°C. Both

Ochrobactrum anthropi (TS16MCN) and *Bacillus anthracis* (TS5SRP) species produced 4.63IU and 4.01IU of cellulase, which were highest among all other production medium maintained at different temperatures.

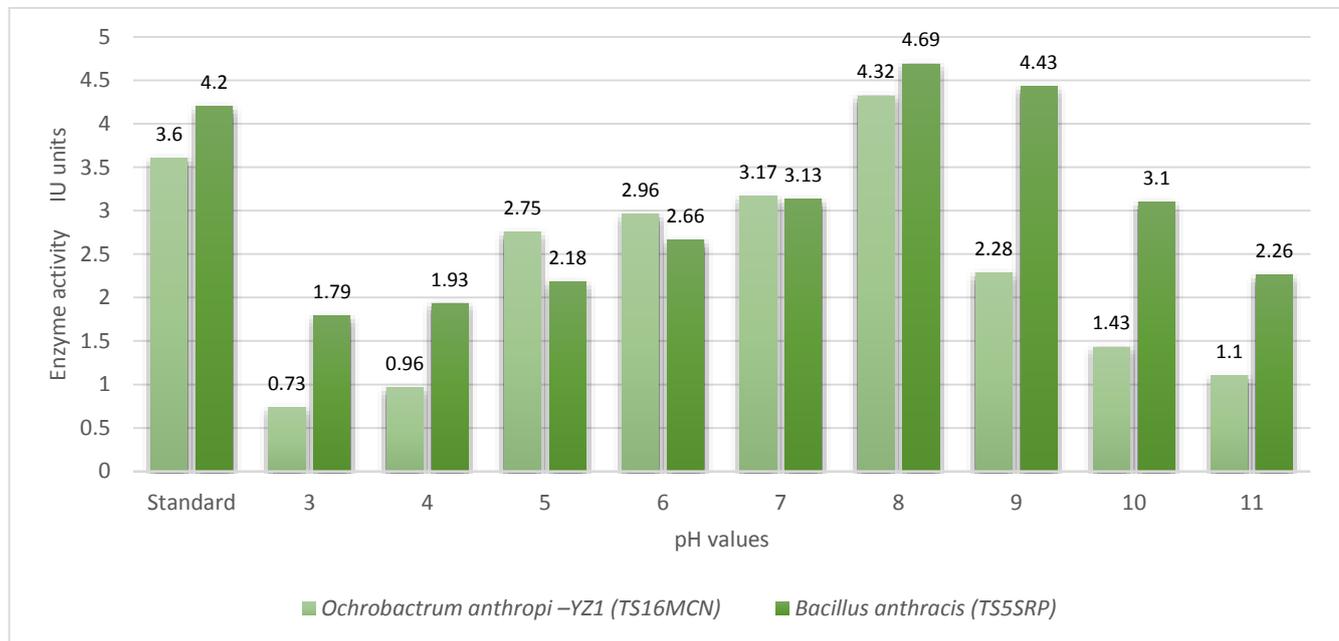


Fig.5: Optimization of pH for cellulase production

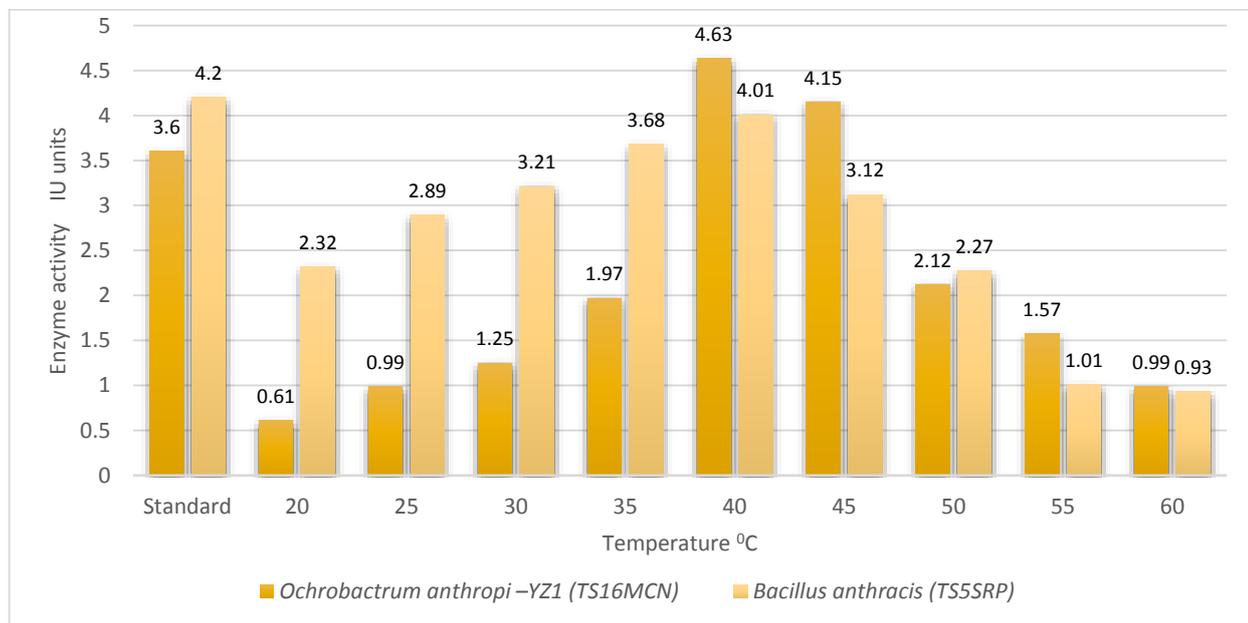


Fig.6: Optimization of temperature for cellulase production

The other essential factors like inoculums level (Fig.7), incubation time (Fig.8) and agitation rate (Fig.9) were also optimized to determine the enzyme activity. The maximum enzyme activity (4.77 IU) was noted at the 14% of inoculums size of bacterial isolate *Bacillus anthracis* (TS5SRP). *Ochrobactrum anthropi* (TS16MCN) produced

maximum of 4.33IU when 14% inoculums was added in the production medium. The inoculums size of 14% was optimum for cellulase production. The time of incubation plays vital role in enzyme production. Bacterial species produce enzymes during stationary phase of growth phase. After overcoming the exponential phase cells grow and

multiple, consume all the nutritional content in the medium. The incubation time for bacterial species and that of mycelium greatly varies. Bacterial species selected for cellulase production showed maximum enzyme activity

when they were incubated for 96h, *Ochrobactrum anthropi* (TS16MCN) yielded 4.33IU and *Bacillus anthracis* (TS5SRP) produced 4.99IU of cellulase.

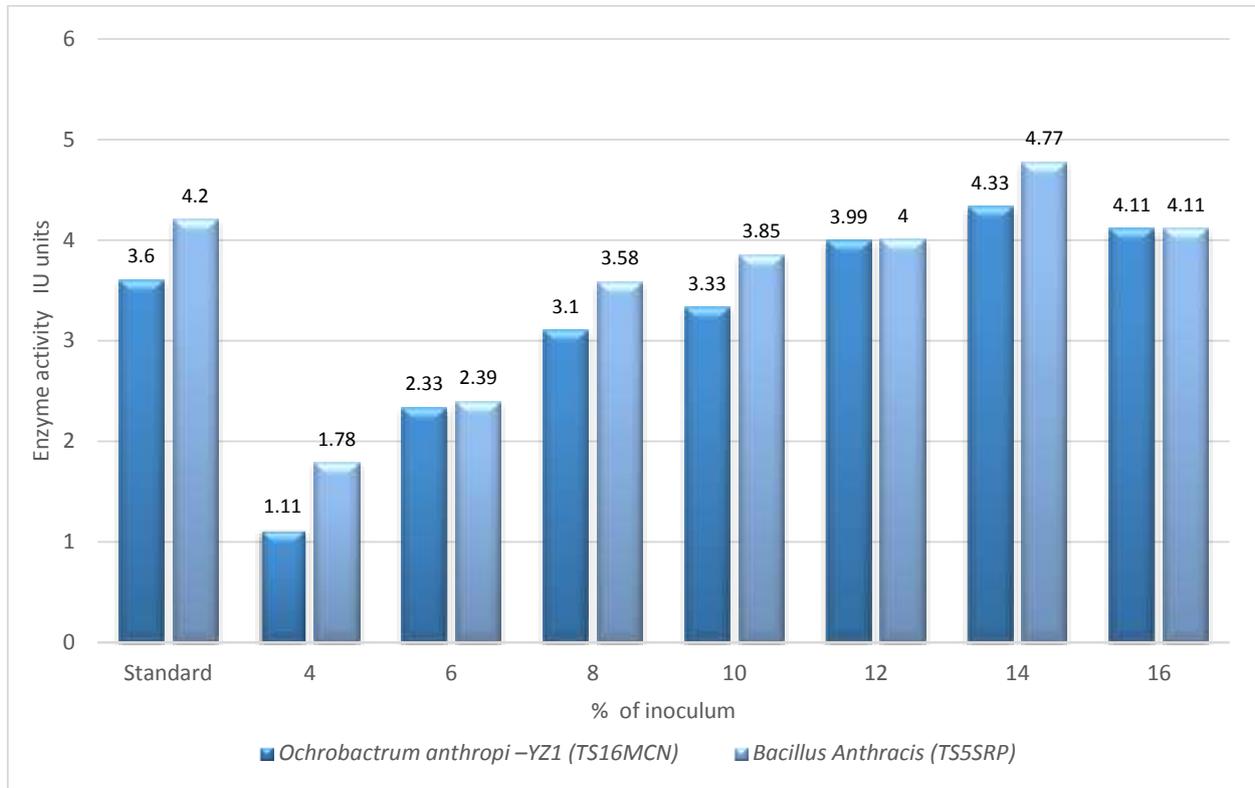


Fig.7: Optimization of inoculum level for cellulase production

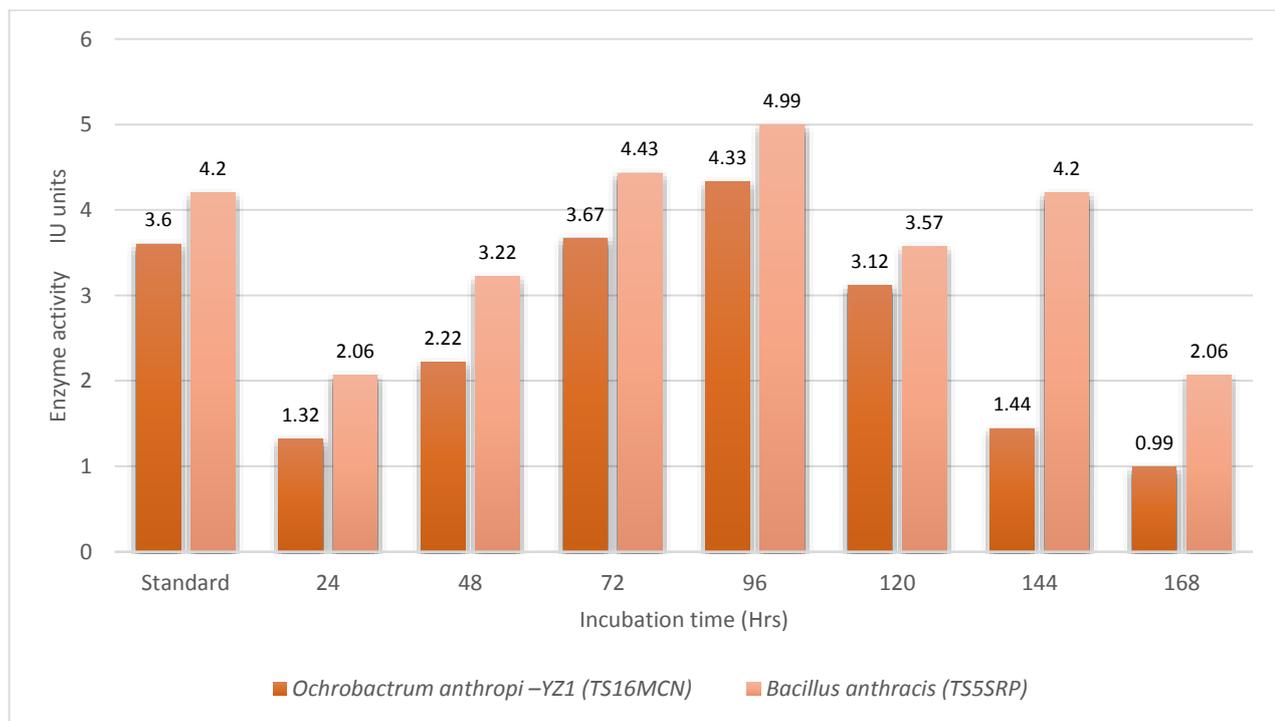


Fig.8: Effect of Incubation time for cellulase production

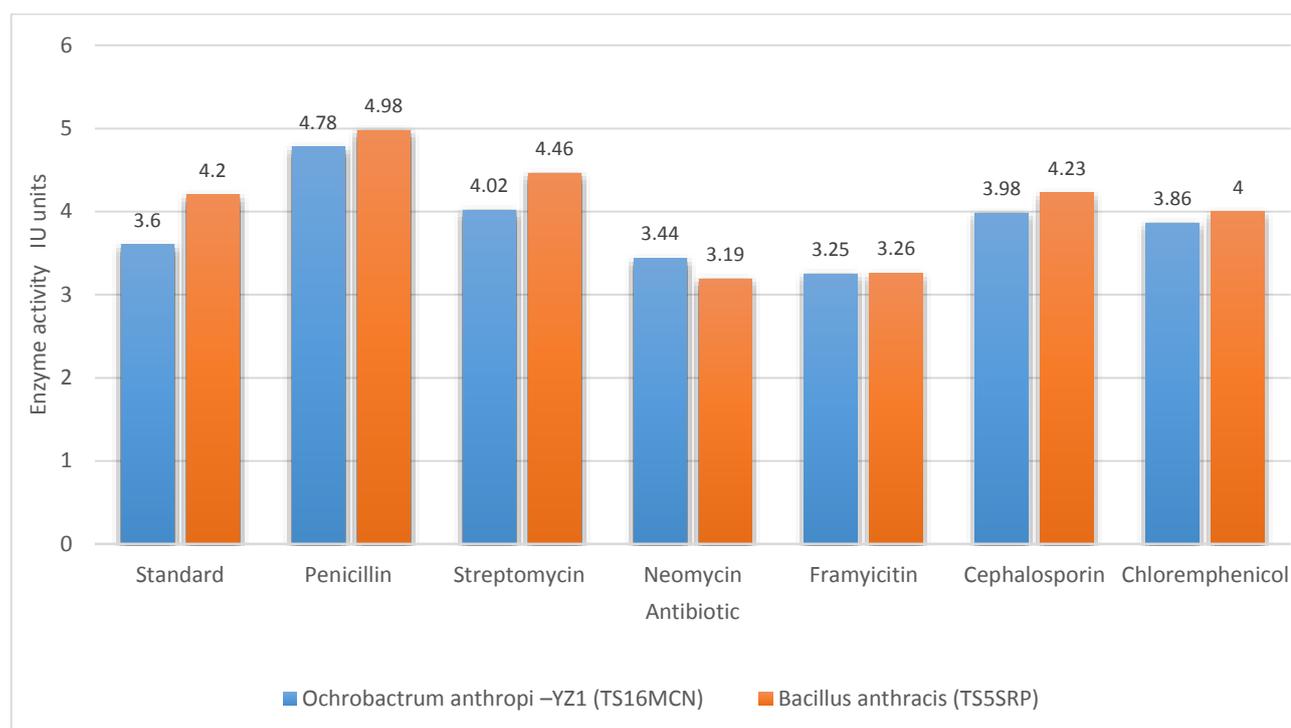


Fig.9: Effect of antibiotics for cellulase production

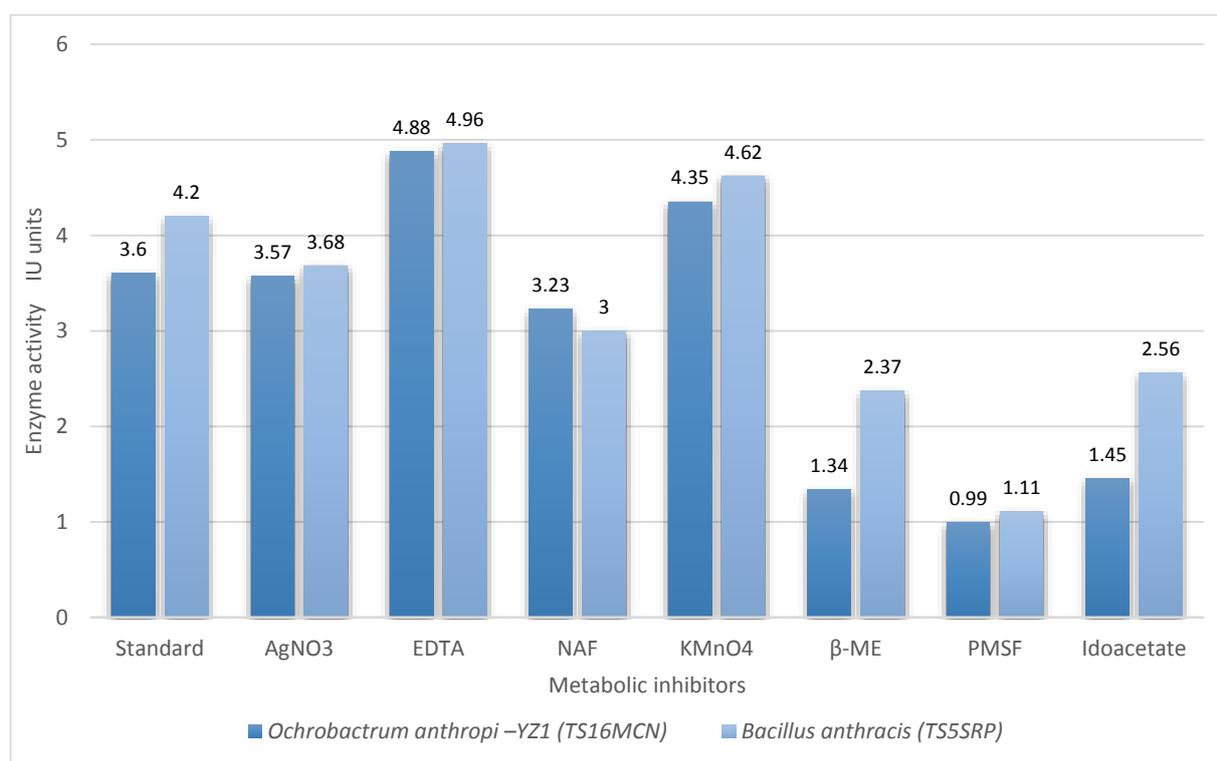


Fig.10: Effect of metabolic inhibitors for cellulase production

The other essential parameters like agitation rate and addition of antibiotics and metabolic inhibitor are also studied in optimization study. In agitation study both *Ochrobactrum anthropi* (TS16MCN) and *Bacillus anthracis* (TS5SRP) has shown the maximum production at 150rpm (Fig.11). In presence of penicillin as an antibiotic

Ochrobactrum anthropi (TS16MCN) and *Bacillus anthracis* (TS5SRP) has shown maximum production of cellulase (12). In presence of EDTA metabolic inhibitor both species shown high yield among all inhibitors and the results were presented in Fig.10.

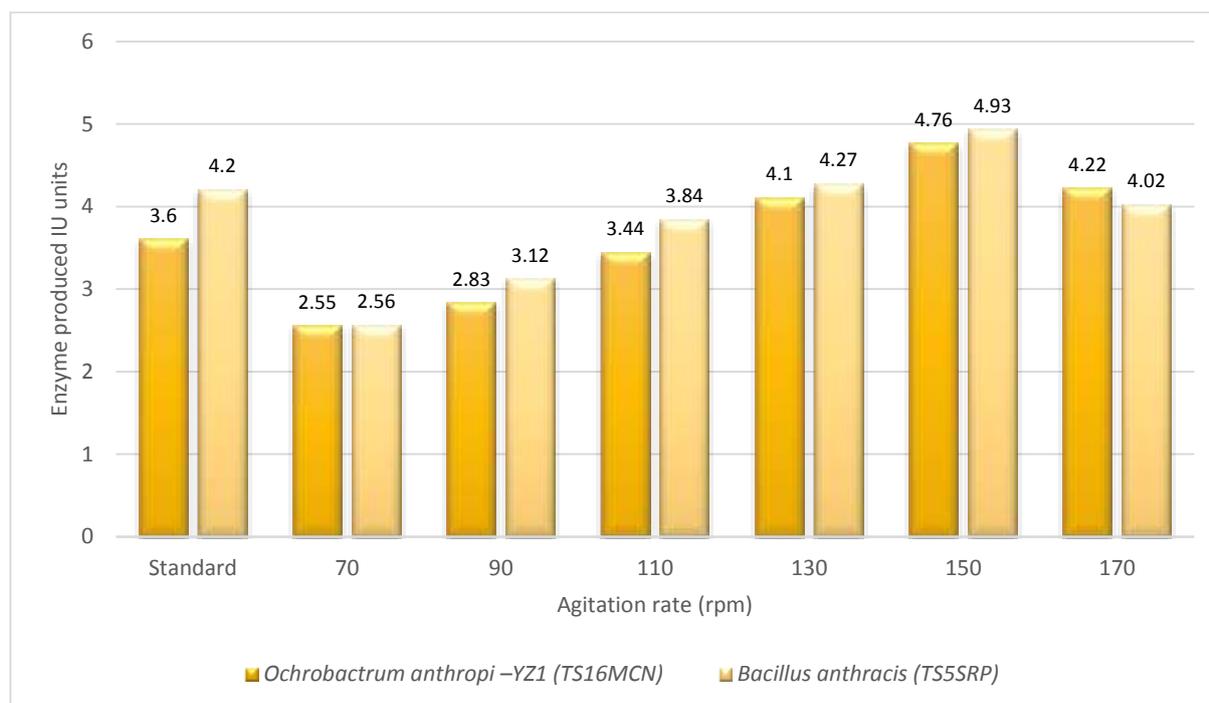


Fig.11: Effect of agitation rate for cellulase production

Singh *et al.*, (2012) have isolated cellulase producing bacteria from soil sample from different sites of Allahabad. On the basis of maximum hydrolytic zone formation on CMC agar media it was observed that 10 isolated were found positive for cellulase production. Cellulase was produced by *Bacillus circulans* with banana peel as solid substrate. The optimum condition for cellulose production was 25°C and 7.0 pH. The crude and purified enzyme showed enzyme activity of 0.56U/ml and 0.58U/ml, respectively. Fatima *et al.*, (2013) screened fifty five *Bacillus* isolates and identified using morphological, biochemical and molecular characterization using 16s rDNA analysis, from compost and alkaline clay soil from Sudan. Puspita *et al.*, (2012) have isolated cellulolytic bacteria from peat soil from South Sumatra, Indonesia. Amir *et al.*, (1989) isolated cellulolytic bacterium, *Streptococcus* sp. which was isolated from soil. Cellulase enzyme production was carried out in 250 ml Erlenmeyer flask using potato waste as a substrate in sub-merged fermentation. The strain produced maximum cellulase with initial medium pH of inoculums size of 2% and incubation temperature of 35°C for 48 h of fermentation period. The isolated bacterium, *Cellulomonas* sp. can be used as potential producer of effective cellulase which would be beneficial in industrial application. A similar observation has also been noticed in the present study.

Conclusion

The present work was carried out to optimize the nutritional and environmental parameters for improving cellulase production by the cellulolytic bacteria. The cellulase producing bacterial *species* was isolated from soil and characterized by various staining procedures, biochemical

analysis. From present study, the result showed that cellulase producing bacteria can grow at optimized condition. Thereby different optimized conditions were tested for two isolated and significant increase in cellulase production was achieved. The use of these microorganisms for the production of enzymes offers a promising approach for its large scale production and as a possible food supplement or in pharmaceutical industry. Further research on production, characterization and purification of cellulase through these optimised conditions such as pH, temperature and various substrate utilizations would reveal those strains with higher cellulase production potential.

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Appendix: 1

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ORIGIN

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181 ACCCGCGTCG CATTAGCTAG TTGGTGAGGT AACGGCTCAC CAAGGCAACG ATGCGTAGCC
241 AACCTGAGAG GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGG
301 CAGCAGTAGG GAATCTTCCG CAATGGACGA AAGTCTGACG GAGCAACGCC GCGTGAGTGA
361 TGAAGGCTTT CGGGTCGTAA AACTCTGTTG TTAGGGAAGA ACAAGTGCTA GTTGAATAAG
421 CTGGCACCTT GACGGTACCT AACCAGAAAAG CCACGGCTAA CTACGTGCCA GCAGCCGCGG
481 TAATACGTAG GTGGCAAGCG TTATCCGGAA TTATTGGGCG TAAAGCGCGC GCAGGTGGTT
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601 CTTGAGTGCA GAAGAGGAAA GTGGAATTCC ATGTGTAGCG GTGAAATGCG TAGAGATATG
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721 GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT GAGTGCTAAG
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841 GTACGGCCGC AAGGCTGAAC TCAAAGGAAT TGACGGGGGC CCGCACAGCG GTGGAGCATG
901 TGGTTTAA
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ORIGIN

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121 TTGGTGGGGT AAAGGCCTAC CAAGGCGACT ATCCATAGCT GGTCTGAGAG GATGATCAGC
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241 CAATGGGCGC AAGCCTGATC CAGCCATGCC GCGTGAGTGA TGAAGGCCCT AGGGTTGTAA
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361 GCCAACATCC GCGGTAATAC AAAGGGGGCT AGCGTTGTTC CGATTTACTG GCGGTAAAGC
421 GCACGTAGGC GGGCTAATAA GTCAGGGGTG AAATCCCGGG GCTCATCCCC AGAACTGCCT
481 TTGATACTGT TAGTCTGAG TATGGTAGAG GTGAGTGGA TCCGAGTGT ACAGGTGAGA
541 TTCGTAGATA TTCAGAGGAA CACTTGTGTC GAAGGCAGCT CACTGGACCA CTACTGACGC
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