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RESEARCH ARTICLE

PRODUCTION OF CELLULASE FROM AGROWASTE BY *Acinetobacter species* ISOLATED FROM CLINICAL SPECIMENS

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ABSTRACT

The present investigation carried out on production of cellulase from agrowaste by *Acinetobacter species* isolated from clinical specimens. 4 Clinical samples collected from different labs, out of four clinical specimens, only one sample showed the presence of *Acinetobacter species* after culturing it on the Leeds Acinetobacter Agar Base medium and Blood Agar medium. Agrowaste such as wheat husk, gram husk and tuwar husk were collected for the production of cellulase. In the contemporaneous exploration after the incubation of 48 hours at 37^{o}C it was resolute that the mixed substrate is appropriate for the production of cellulase which showed highest activity of cellulase 0.93nm at the concentration of 4600 micro gm/ mL of matose/ mL of enzyme per minute. However, the single substrate, Gram husk found to be most suitable among wheat husk, tuwar (Pigeon pea) husk for the production of cellulase. The optimum pH for the production of cellulase is found to be 7.0 and the optimum temperature was determined 40^{o}C with 0.66 nm cellulase activity.

Key words: *Acinetobacter sp.,* Cellulase, Agrowaste, Clinical specimens.

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INTRODUCTION

The recognition that environmental pollution is a worldwide threat to public health has given rise to new massive industry for environmental restoration. Despite the massive utilization of lignocellulosic material, there are still ample cellulose containing raw materials and waste products that are not exploited or that could be used more efficiently. The problem in this respect is, however, to develop sustainable processes that are economically profitable. Biological degradation, for economic and ecological reasons, has become an increasingly popular alternative for the agriculture, industrial, organic, as well as toxic wastes. These waste have been insufficiently disposed leading to environmental pollution (Chandra *et al*., 2007). Numerous industrial and agricultural wastes generated due to agricultural practices and food processing, such as rice straw, yam peels, cassava peels, and banana peels, represent one of the most important energy resources. These waste products can potentially be bioconverted into value-added products through the action of enzymes (Nfor *et al*., in press). Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including fungi, bacteria, and actinomycetes during their growth on cellulosic materials. These microorganisms can be aerobic, anaerobic, mesophilic, or thermophilic (Koo, 2001; Kubicek, 1993). Cellulose and hemicelluloses comprise the major part of green plants and this is the main reason for using such terms as "cellulosic wastes" or simply "cellulosics" for those materials that are produced especially as agricultural crop residues, crop processing wastes, fruit and vegetable wastes, animal waste, and so on (Ryu and Mandels, 1980; Wood, 1992). The world is currently encountering the fuel energy deficiency from a continuously increasing utilization rate. By 2030, the expected energy demand will increase 7-fold when compared in 2005 (Razmovaski *et al*., 2012). A promising approach relies on the production of bioethanol from the abundant and mass. Cellulose, the most common natural renewable biopolymer, is commonly degraded by the hydrolytic action of a multi component enzyme system – the cellulase and represents the key stepfor biomass conversion. The enzymatic hydrolysis requires synergistic action of cellobiohydrolase or exoglucan ase (E.C.3.2.1.91), endoglucanase or carboxymethylcellulase (E.C.3.2.1.4) and cellobiase or β-glucosidase (E.C.3.2.1.21) (Cavaco-Paulo 1998). Other countries are seeking the alternative sources or renewable energy in various forms to provide a long-term energy supply. Furthermore, renewable energy is better than fossil energy in that it can reduce the CO_2 content which is a cause of global warming. Cellulose is the most common material which can be used as renewable energy. The main component of cellulose is polysaccharide and they are readily available mainly from agricultural wastes

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such as bagasse, rice straw, rice husk etc. (Lee *et al*., 2008 and Sarkar *et al*., 2012). The use of agricultural wastes can be help to increase the value of these materials, which helps farmers indirectly (Chang, J.S. *et al*., 2010). Cellulase production from bacteria can be an advantage as the enzyme production rate is normally higher due to the higher bacterial growth rate as compared to fungi. Screening of bacteria, optimisation of fermentation conditions and selection of substrates are important for the successful production of cellulase (H. Arffin, *et al*., 2006). The term *'Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex' was coined by Gerner-Smidt *et al*., for a group of four phenotypically similar species. These included *Acinetobacter calcoaceticus, Acinetob acter baumannii* and genomic species (gen. sp.) 3 and gen. sp. 13 which were later named *Acinetobacter pittii* and *Acinetobacter nosocomialis,* respectively (Nemec *et al.,* 2015). *Acinetobacter* is first described in 1911 as *Micrococcus calco-aceticus*. *Acinetobacter spp.* has natural habitats such as water, soil and it has been isolated from foods, arthropods, and environment. In humans, *Acinetobacter* can colonize skin, wounds and the respiratory and gastrointestinal tracts. Some strains of *Acinetobacter* can survive environmental desiccation for weeks, a characteristic that promotes transmission through fomite contamination in hospitals. *Acinetobacter* has been a pathogen of hot and humid climate, where it has been a major cause of infections, particularly in intensive care units (ICUs) and sometimes a cause of community-acquired pneumonia (Munoz *et al.,* 2008). *Acinetobacter baumannii* may occasionally cause skin, soft tissue infections outside of the military population. The organism caused 2.1 % of ICU-acquired skin, soft tissue infections in one assessment. *Acinetobacter baumannii* is an occasional cause of UTI, being responsible for just 1.6% of ICU-acquired UTIs. *Acinetobacter baumannii* has been reported to be a more common cause of ICU-acquired bloodstream infection than of non-ICU-ward infection. *Acinetobacter baumannii* bloodstream infection is 34.0% to 43.4% in the ICU and 16.3% outside the ICU (Begum *et al.,* 2013). *Acinetobacter* is a genus of gram-negative bacteria belonging to the Gammaproteobacteria. They are nonmotile, oxidase negative, highly pleomorphic and usually occur in pairs. *Acinetobacter* is occupied an increasingly important role as an opportunistic pathogen in the hospital environment (Shakibaie *et al.,* 2012). The present study drawed a bead on isolation and identification of *Acinetobacter spp*. from various clinical specimens and to produce cellulase enzyme from agrowaste by *Acinetobacter spp*. for the aspects of economic theory concerned with the welfare of society and priorities to be observed in the allocation of resources.

MATERIALS AND METHODS

Collection of clinical samples

Clinical samples, such as urine, pus, blood, bed swab were collected from the different labs in the Nagpur city, like Vishakha laboratory, Dhantoli, Nagpur, Suvish was laboratory, Ramdaspeth, Nagpur and Government Medical College, Nagpur. All the bacterial strains used in this work were clinical isolates collected over a month of period from the different labs in the Nagpur city.

Collection of Agrowaste

Different types of agrowaste were collected from shops. Agro waste such as wheat husk, gram husk and tuwar (Pigeon pea) husk were collected for the production of cellulase.

Isolation of bacteria from samples

The loopful of sample was took from the each specimen and were streaked asceptically on petriplates containing appropriate media, such as Leeds Acinetobacter Agar Base (specific), Blood Agar and MacConkey Agar media respectively. The inoculated plates were incubated at 37^{o}C for 24 hours in the incubator (Biji *et al.,* 2017).

Identification of Bacteria

Acinetobacter spp was identified on the basis of morphological characteristics such as Gram's staining and motility by hanging drop method. Biochemical characteristics also performed to identify bacteria like IMViC test, sugar fermentation test, TSI, Urease, Catalse and oxidase test. Cultural characteristic was done by inoculating clinical samples on Leeds Acinetobacter Agar Base (specific), Blood Agar and Mac Con key Agar. The identified bacteria was subcultured on Nutrient Agar slant and preserve at 4^{0}C for further use.

Pre-treatment of agricultural wastes

Each wheat, gram and tuwar husk was chopped into small pieces, and then grounded into powder. These materials are used in medium preparation for cellulase production (Poomai *et al.,* 2014)

Production of cellulose

Inoculum

The substrates were weighed and added into boiling test tubes containing distilled water with 1% Sodium sulphate as a source of sodium and sulphur to the bacteria. The substrates were vigoursly mixed, and the flasks are autoclaved at 121^{o}C for 15 min. The flasks are cooled and inoculated with 1mL of *Acinetobacter species* isolate carrying 10^{8} cells mL (0.8 OD at 600 nm) as a seed culture under aseptic condition and incubated at 37^{o}C for 48 hours (Selvam *et al.,* 2014). Same media used for Gram husk in eight different tubes, autoclaved, cooled and inoculated with *Acinetobacter species* culture and incubated at 30^{o}C, 37^{o}C, 40^{o}C, 45^{o}C and 50^{o}C and pH 4, pH 5, pH 6, pH 7, and pH 8 for 48 hours.

Enzyme recovery

The fermented solution from each flask is extracted. Later, the mixture is filtered initially through muslin cloth into the eppendroff tubes and centrifuged at 10,000 rpm for 10 min at 4^{0}C. The supernatant is used as crude enzyme source for further assay (Selvam *et al.,* 2014).

Screening test of cellulose

Confirmation of cellulose-degrading ability of produced enzyme was performed by making well and filled with cellulose enzyme on the CMC media. After incubation of 24 hours a solution of congo red dye was spread on the surface of CMC. The use of Congo-Red as an indicator for cellulose degradation in CMC medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria.

Cellulose enzyme showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies (Lu *et al.,* 2004).

Enzyme activity assay

Before the finding of activity of cellullase, standard protocol was performed to find the concentration of maltose per mL of enzyme per minute as stanadard. Cellulase activity of cell-free supernatant is evaluated using a reducing sugar assay which determined by the 3, 5-dinitrosalicylic acid (DNS) method (Poomai *et., al* 2014). Finally, the determination of enzyme activity was monitored by spectrophotometrically at 540nm.

RESULTS AND DISCUSSION

Isolation of *Acinetobacter species* from clinical specimens
Out of four clinical specimens, one sample which was swab from the bed showed the presence of *Acinetobacter species* after culturing it on the Leeds Acinetobacter Agar Base medium and Blood Agar medium. The bacterial culture showed growth on the media in the form of smooth, rounded, mucoid and opaque colonies. The isolate was characterized through cultural, morphological, and biochemical studies as shown in the following Table no.1 and 2 respectively. In the presence of overall relatedness, the isolated species was identified as *Acinetobacter species*.

Morphological characteristics

Gram staining: Isolate of *Acinetobacter species* was found to be Gram negative.
Motility: Non motile

Biochemical characteristics

Cultural characteristics of *Acinetobacter species*

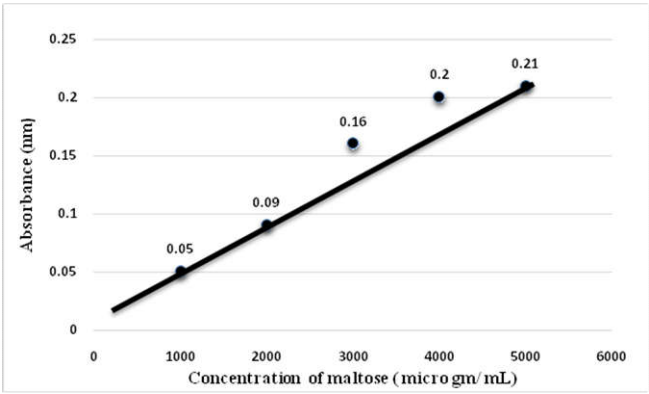
The current study out of four samples, *Acinetobactor species* was found only from the swab from the bed from Government Medical Hospital, Nagpur. Present study found that *Acinetobacter* was Gram-negative, non-motile, strictly aerobic, coccobacillus formed opaque, mucoid colonies, catalase positive, oxidase negative, nitrate negative, citrate positive, showed variable results with urease, Indole negative, MR negative, VP negative (Table no. 1 and Table no. 2) the investigated results matched with the Bergey's manual standard. The investigation was done by Zheng, *et al.,* (2013) on the *Acinetobacter*, and they found that *Acinetobacter* is a glucose-nonfermenting, gram-negative, aerobic, coccobacillus; the most common species of which include *Acinetobacter baumannii* and *Acinetobacter lwoffi.* The work done by Sivaranjani, (2013) studied muitidrug- drug resistant *Acinetobacter* species from various clinical samples in a tertiary care hospital from South India showed presence of Gram-negative cocco-bacilli by microscopy.

Determination of activity of cellulose

Isolate S1 was proceeded for production of cellulase. Production was done by submerged fermentation which was carried out by taking 5 gm of dry substrate in a 250 mL of Erlenmeyer flask to which 100 mL distilled water containing production medium i.e., 1% Na_2SO_4. The contents of the flasks were mixed and autoclaved at 121°C for 15 min. The flasks were cooled to room temperature and then inoculated with 1 mL of 24 hrs grown culture asceptically and incubated at 37°C for two days. After incubation of two days, enzyme from fermented bacterial bran was squeezed through a muslin cloth. Extracts were pooled and centrifuged at 4°C for 10 min at 8000 rpm to separate all the cell debries. The clear supernatant was used in enzyme assay. Present investigation states that the mixed substrates showed optimum cellulase productivity. On the basis of separate substrate, Gram husk showed optimum activity among wheat and tuwar husk.

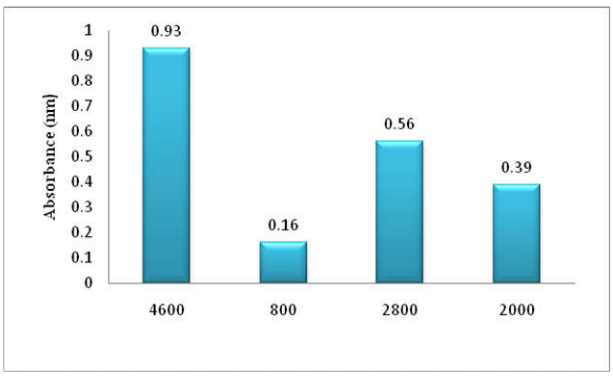
Production of cellulase by *Acinetobacter* species

Standard protocol gave the increasing absorbance with respect to maltose (Table No. 4) and hence the graph plotted for the investigated readings is a straight line (Graph no. 1). Cellulase production was done successfully from the agrowastes by *Acinetobacter species* by submerged fermentation which was produced from the different substrates.



Graph No.1 Showing the concentration versus absorbance of maltose standard

In order to find suitable activity of cellulase, *Acinetobacter species* culture was grown in the Luria Broth medium for 48 h. and 1 mL of culture was inoculated in the different substrates and autoclaved at 37°C for 48 h. Highest cellulase production was determined by the mixed substrates i.e. of 0.93 nm at the concentration of 4600 µg/ mL of maltose per mL of enzyme per minute. In this study investigated that Gram husk is the most suitable substrate for the production of cellulase as it showed the highest enzyme activity i.e. 0.56nm while the activity of enzyme for wheat and tuwar were 0.16 and 0.39 at the concentration 2800, 2000 and 800µg / mL of maltose / mL of enzyme/ minute respectively (Table No.5). The results showed that the mixed medium of substrates was suitable for production of cellulase (Graph No. 2).



Graph No. 2. Showing the activity of cellulose by *Acinetobacter* species from different agrowaste

Table No. 1. Biochemical characterization of *acinetobacter species*

Sample	Indole	MR	VP	Citrate	Urease	Catalase	Oxidase	TSI		
								Acid	Gas	H_2S
S1	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve

Table No. 2. Sugar fermentation by *Acinetobacter species*

Sample	Dextrose		Lactose		Sucrose		Mannitol		Maltose	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
S1	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Table No. 3. Cultural characteristics of *Acinetobacter species*

Sr. No.	Media	Characteristics observed
1.	Leeds Acinetobacter Agar Base	Smooth, rounded, opaque, mucoid colonies were observed.
2.	Mac conkey Agar	Faint pink, tint colonies were observed.
3.	Blood Agar	Translucent to opaque, convex and entire colonies observed.

Table No. 4. Observation table of Maltose as standard

Sr. No.	Test tube	Absorbance	Concentration of maltose (micro gm/ mL)
1.	Blank	0.00	0.00
2.	1	0.05	1000
3.	2	0.09	2000
4.	3	0.16	3000
5.	4	0.20	4000
6.	5	0.21	5000

Table No. 5. Cellulytic potential of *Acinetobacter species* from all the substrate

Sr. No.	Substrates	Concentration (micro gm/ mL)	Absorbance (nm)
1.	Wheat	800	0.16
2.	Tuwar	2000	0.39
3.	Gram	2800	0.56
4.	Mixed	4600	0.93

Table No. 6. Cellulase activity of *Acinetobacter species* through Gram husk on different pH

Sr. No.	pH	Concentration (micro gm/ mL)	Absorbance (nm)
1.	Blank	0.00	0.00
2.	4	4750	0.49
3.	5	6750	0.68
4.	6	7150	0.73
5.	7	8900	0.85
6.	8	4150	0.43

Table No. 7. Cellulase activity of *Acinetobacter species* through Gram husk on different temperature

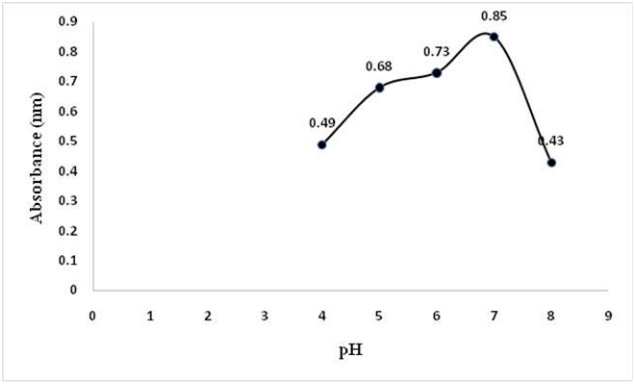
Sr. No.	Temperature	Concentration (micro gm/ mL)	Absorbance (nm)
1.	Blank	0.00	0.00
2.	30°C	5300	0.55
3.	37°C	5600	0.57
4.	40°C	6550	0.66
5.	45°C	5995	0.61
6.	50°C	5300	0.55

The same investigation was done by Poomai *et al.* (2014) studied on cellulase enzyme production from agricultural waste by *Acinetobacter sp*. KKU44 concluded that in order to find suitable substrate (carbon source) from agricultural wastes, *Acinetobacter*s pp. KKU44 was grown in BM, RSM, and RHM at 37 °C, 150 rpm for 72 h. For BM culture, the bacterial culture of 36 h. showed the highest cellulase activity at 83, 93, and 56 U/mL when determined at 50, 60, and 70 °C, respectively. For RSM culture, the bacterial culture of 36 hr. showed the highest cellulase activity at 74, 83, and 56 U/mL when determined at 50, 60, and 70 °C, respectively. For RHM culture, the bacterial culture of 36 h. showed the highest cellulase activity at 74, 83, and 46 U/mL when determined at 50, 60, and 70 °C, respectively. The results showed that baggass is suitable for using as substrate in cellulase production. Selvam *et al.* (2014) also worked on the production of cellulase but they used different substrate the mixed combination of coffee pulp waste (CPW) and pineapple waste (PW) residues for cellulase production using newly isolated *Acinetobacter sp.* TSK-MASC in solid state fermentation. Response surface methodology based Box–Behnken design (BBD) was employed to optimize variables such as pH, incubation time, concentrations of CPW and PW. The higher production (888 U mL) was achieved after 60 h of incubation with 3.0 g L1 of CPW and PW at pH 7.0.

Effect of different pH on cellulase activity

In order to determine the highest cellulase productivity from single substrate gram husk was used and maintained for the

cellulase production at different pH and temperature. In the current investigation it is investigated that gram husk showed variable activity at different pH. The absorbance or the activity shown by gram husk by *Acinetobacter species* after the incubation of 48 h. at the pH 4, 5, 6, 7 and 8 with the concentrations 4750 µg / mL, 6750 µg / mL, 7150 µg / mL, 8900 µg / mL and 4150 µg / mL were observed as 0.49, 0.68, 0.73, 0.85, 0.43 respectively (Table no.6). From the above data the study states that the optimum production of cellulase is carried out at the pH 7.0 having concentration 8900 micro gm/ mL as it gave highest absorbance 0.85 at 540 nm (Graph No. 3). The investigation was done by Sadhu, *et al*. (2013) on production, purification and characterization of a novel thermotolerent endoglucanase (CMCase) from *Bacillus* strain isolated from cow dung and the present investigation investigated that the enzyme hydrolyzed CMC in the pH range of 5.0-9.0, and exhibited highest activity at pH 7.0. However, significantly high activity was also recorded on either side of this point which indicated that the enzyme has characteristically broad range of pH activity.

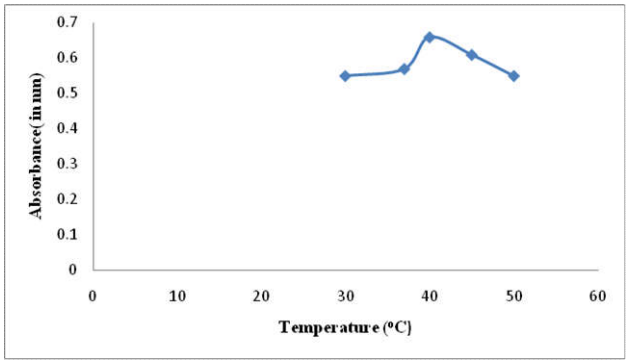


Graph no.3 representing the effect of pH on cellulose production

The enzyme also had a broad range of pH (6.0 – 9.0) stability where it retained more than 80% activity for 1.0 hr, though maximum stability pas found at pH 7.0. Selvam *et al*. (2014) investigated process optimization of cellulase production from alkali-treated coffee pulp and pineapple waste using *Acinetobacter* sp. TSK-MASC and found that the optimum temperature for cellulase activity was found to be 50 ^{o}C at pH 7.0. On the contradictory to the present study, Gomathi *et al.* (2012) worked on the title Submerged fermentation of wheat bran by *Aspergillus flavus* for production and characterization of carboxy methyl cellulase where they found that the maximum enzyme production was found to be 0.654 IU/ml at pH 6 during 3rd day of incubation. However, Sherief *et al. (*2010) reported the initial pH 5-6 for maximum volumetric productivity of cellulases. Likewise, the study done by Zheng *et al*., studied isolation, screening, and identification of Cellulolytic Bacteria from Natural Reserves in the Subtropical Region of China and Optimization of Cellulase Production by *Paenibacillus terrae* ME27-1 they found that, the CMCase produced by the strain ME27-1 was stable at pH 5.0–9.5, and almost 85% residual activity was retained. Only a few studies have reported that CMCase was stable at such a wide pH range.

Effect of different temperature on cellulase activity

Purified enzyme preparation was recorded to showed activity over broad range of temperature (30^{o}C, 37^{o}C, 40^{o}C, 45^{o}C and 50^{o}C) with the optimal activity at 40^{o}C. The absorbances shown by the cellulase produced by *Acinetobacter species* from gram husk at the temperature 30^{o}C, 37^{o}C, 40^{o}C, 45^{o}C and 50^{o}C were 0.55 nm, 0.57 nm, 0.66 nm, 0.61nm and 0.55nm respectively (Table No. 7) having concentrations 5300 micro gm/ mL, 5600 micro gm/ mL, 6550 micro gm/ mL, 5995 micro gm/ mL and 5300 micro gm/ mL respectively. The optimum temperature for cellulase production was 40^{o}C showed highest production of cellulose enzyme - 6550 micro gm/ mL. The lowest activity was seen at 30^{o}C (Graph no. 4). The investigation done by Zheng- *et al.* (2013) found that the best incubation condition was 28^{o}C. The CMCase activity declined when the initial pH and incubation temperature were not optimal. Whereas, R. Waeonukul *et al*. (2009), investigated that there have been diverse reports on the optimal initial pH and temperature for cellulolytic enzyme production by *Paenibacillus* sp. Furthermore, Kumar *et al*. reported that the optimal initial pH and temperature for CMCase production by *P. polymyxa* were 5.5 and 37^{o}C, respectively. Sadhu *et al.* (2013), studied on the cellulase production and the study determined that the purified enzyme preparation was recorded to show activity over a broad range of temperature (20°-70°C) with the optimal activity at 50°C and declined thereafter. Thermo-stability range of the enzyme showed that it was thoroughly stable at 50°C. However, activity of this enzyme gradually declined with increase of temperature from 60 – 70°C. Nevertheless, sufficient activity of the enzyme (more than 75–80%) was present at 60 – 70°C for 1–2 hr. Gomathi *et al.* (2012), investigated that incubation temperature of the fermentation medium is a critical factor has insightful influence on metabolic activities of microorganisms. The effect of different incubation temperature (20- 70^{o}C) on the CMCase production was investigated. The production of enzyme was maximal in flasks incubated at 30^{o}C. As the temperature increased, there was a gradual decrease in the enzyme production. Poomai *et al.* (2014), found that the cellulase enzyme producing bacterial strain has been isolated from rich straw and identified by 16S rDNA sequence analysis as *Acinetobacter* sp. KKU44. The *Acinetobacter sp*. KKU44 strain was able to grow and exhibit high cellulase enzyme activity at high temperature. The optimal temperature for its growth and cellulase production was 37°C. The optimal temperature of cellulose activity was 60°C. The highest cellulase enzyme activity was 120 U/mL at 36 h when grown in LB medium containing 2 % (w/v) bagasse at 37 °C, 150 rpm. On the other hand, Selvam *et al.,* (2014) investigated that The effect of temperature on activity of cellulase was determined at various temperatures ranged between 30 ^{o}C and 80^{o}C.The optimum temperature for cellulase activity was found to be 50^{o}C at pH 7.0 and decreased rapidly as the temperature increased above 60^{o}C.



Graph No. 4. Graph pointing the curve for the effect of temperature on production of cellulase

The investigation done in the current study is useful for the bioconversion of lignocellulosic materials and also has tremendous applications in industries.

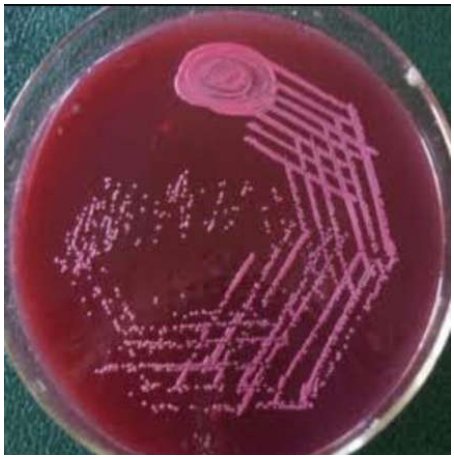


Fig 1: Cultural characteristics of Acinetobacter sps. on Mac conkey agar

Fig. 2. Cultural characteristics of *Acinetobacter species*on Leeds Acinetobacter Agar Base

Fig. 3. Substrates used for the cellulase production

Fig. 4. Assembly for cellulase production

Conclusion

There are wide applications of *Acinetobacter species* in hazardous waste treatment or as producers of economically important bio-products. However some investigations got different footprint with respect to the production of cellulase enzyme. Abundant research works are resulting into revamp perception relevant to cellulase enzyme for the fabrication of valuable by-products such as bioalcohol, compost, organic acid animal feed, etc.

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