



# Production, purification, and characterization of cellulase from *Acinetobacter junii* GAC 16.2, a novel cellulolytic gut isolate of *Gryllotalpa africana*, and its effects on cotton fiber and sawdust

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## Abstract

**Purpose:** The study aims to search for potent cellulase producer from the gut of *Gryllotalpa africana* as well as to characterize and determine the effect of the purified enzyme on the cellulosic waste materials.

**Methods:** The potent cellulolytic strain was identified through morphological, biochemical, physiological, and molecular characterization like 16S rRNA and fatty acid methyl ester profile. After the optimization of cellulase production, the enzyme was purified through DEAE-Sepharose column chromatographic separation. The molecular weight of the purified enzyme was determined by SDS-PAGE analysis. The purified enzyme was characterized in terms of its activity and its effect on cotton fiber, and sawdust was also studied.

**Result:** The selected potent strain GAC 16.2 was identified as *Acinetobacter junii* that was capable to produce enhanced cellulase ( $112.38 \pm 0.87$  U/ml) at standardized optimum fermentation conditions. The molecular weight of the purified enzyme was determined as 55 kDa. The utmost activity of the purified enzyme was detected pH 7.0, temperature 50 °C, and in the presence of metal ions like  $Mg^{2+}$  and  $Mn^{2+}$ . The substantive degradation of cotton fiber and sawdust has been observed in a reasonably short period.

**Conclusion:** Purified cellulase from the selected isolate *A. junii* GAC16.2, a gut isolate of *G. africana*, has the potentiality to degrade cellulosic substances. This property can make the isolate a potent candidature for industrial application, as well as an effective biotechnological tool for environmental monitoring through cellulosic waste management.

**Keywords:** *Gryllotalpa africana*, *Acinetobacter junii*, Cellulase, Optimization, Purification, Cotton fiber, Sawdust

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## Introduction

Cellulose is the most abundant renewable organic molecule on the earth (Klemm et al. 2005). Its enzymatic degradation by cellulase is a promising approach for its effective utilization. The resultant reducing sugars could be used for the production of desired value-added byproducts (Sukumaran et al. 2005; Octave and Thomas 2009). This enzyme is extensively consumed in paper and pulp industries, in textile industry, for the production of biofuel, as an anti-biofilm agent, for waste management in pharmaceutical industries, for pollution control, and also in food as well as in feed industries (Sreena et al. 2016; Aarti et al. 2018; Thomas et al. 2018; Sreena and Sebastian 2018).

Insects are the most species-rich and diverse group of arthropods (Moran and Telang 1998). They harbor a rich and complex microbial community in their guts as symbionts that differ in taxa due to their altered feeding habits. In the course of evolution, these symbiotic associations between insects and different microorganisms have been deeply rooted (Moran and Telang 1998). These symbionts exhibit beneficial activities for their hosts. One such activity is the production of various important hydrolytic enzymes including cellulase. Thus, they play an indispensable role in the digestion of ingested food and are a “hot spot” for useful genes and proteins (Dillon and Dillon 2004; Dharne et al. 2006). Though only a few detail studies have been made concerning the enzyme-producing insect gut bacteria, it requires further observations (Dillon and Dillon 2004).

*Gryllotalpa africana* (order: Orthoptera) is an important soil-inhabiting agricultural pest. Their natural habitat is moistened humus-enriched biotopes. In summer, they make galleries near the ground surface, and overwinter they reside at underground holes having a depth of 0.8–1.2 meter. These insects normally feed on a wide range of economically important plants, particularly potato, wheat, maize, oats, sugar beet, barley, rice, soya, vegetable crops, and grasses (Heinrichs and Barrion 2004; James et al. 2010). It is one of the important major soil pests in eastern Gangetic plains of West Bengal, India. They cause 5–6% plant damage along with 10–15% tuber damage to potato plants (Konar et al. 2005).

Studies regarding the microbial cellulase production have been concerted mostly on fungi, but there is an increasing interest in bacteria (Sukumaran et al. 2005). Further, the report on cellulase production of proteobacteria is very scanty. Members under the genus Acinetobacter are common free-living saprophytic archaea. Owing to their metabolic versatility, they are capable to adapt in varied habitat including the gut of insects (Sand et al. 2011; Malhotra et al. 2012; Vilanova et al. 2012).

Keeping in view the essentiality of the facts, the present investigations were carried out to search for new

potent cellulase producers from the gut of *G. africana* and assess the fermentation parameters for optimization of enzyme production by the potent isolate in submerged condition. The characterization of the purified cellulase and its efficiency on cotton fiber and sawdust was also studied.

## Materials and methods

### Collection of insects

To check the presence of the cellulase-producing gut bacteria of *Gryllotalpa africana* (order: Orthoptera), the insects were collected from the different potato fields and agro-waste dumps located at the Gangetic plain of West Bengal, India. The sites from where the insects were collected are Hatgobindapur (23.2578° N, 87.9788° E), Memari (23.1745° N, 88.1034° E) Polempur (23.2044° N, 87.8435° E), Sagrai (23.1514° N, 87.8169° E), Debipur (23.1422° N, 88.1585° N) of Purba-Bardhaman district and Arambag (22.8728° N, 87.7911° E), Gurap (23.0348° N, 88.1218° E), Singur (22.8150° N, 88.2345° E), Talandu (23.0182° N, 88.3567° E), and Chinchura (22.9012° N, 88.3899° E) of Hooghly district of West Bengal, India. A total of 20 adult insects were collected, two from each location.

### Isolation of gut bacteria

The insects were retained in starvation for 2 days to exclude allochthonous microbial communities from their gut. Then, they were used as a source of screening for cellulolytic microorganisms (Zhang and Jackson 2008). The wings of the insects were excised and then the body surfaces were washed thoroughly with 70% ethanol. The insects were aseptically dissected within the laminar airflow cabinet. The guts were removed immediately after dissection. Portions of the gut were cleaned, cut into fragments, split open by longitudinal incision, and transferred to sterile Petri plates. It was then thoroughly flushed with sterilized chilled phosphate-buffered saline solution (0.89% NaCl, pH, 7.0). Subsequently, the gut fragments were homogenized with the same buffer solution in the ratio of 10:1 (volume:weight). The homogenates were used as inoculum for microbial culture.

### Microbial culture

The gut homogenate (0.5 g) of each of the test insects was used after 10 serial dilutions. Carboxymethyl cellulose (CMC)-agar medium (CMC 0.2%, peptone 0.5%, beef extract 0.5% NaCl 0.5%, agar 2%, Merck, pH 7.0) was used for screening of the cellulolytic bacteria. Samples (0.1 ml) were taken from each dilution and within a laminar airflow cabinet and poured aseptically on sterile CMC-agar plate in duplicate. The inoculated CMC-agar plates were incubated at 35 °C for 48 h. The well-grown colonies with a different morphology from the incubated

CMC-agar plates were isolated. Thereafter, they were streaked several times on the same medium to obtain pure cultures. The pure cultures were maintained on CMC-agar slants at 4 °C for further use.

#### Screening of isolates for qualitative enzyme production

For the screening of potent cellulase producers, the isolates were grown in the CMC-agar plate at 35 °C for 48 h. After that, the plates were flooded with 1% Congo red dye and destained with 1 M NaCl (Teather and Wood 1982). Finally, the promising isolates were screened based on their clear zone (halo) forming ability.

#### Screening of isolates for quantitative enzyme production

For the quantitative assay, the selective isolates were grown in M2 medium broth (Table 1) of initial pH 7.0. In a 100 ml Erlenmeyer flask, a 25 ml medium was taken, and it was inoculated with 4% inoculum ( $1.2 \times 10^7$  CFU/ml), v/v. The inoculated flask was incubated at 35 °C for 3 days at shaking condition (100 rpm). After incubation, the content was centrifuged at 8000 rpm for 10 min at 4 °C. The cell-free supernatant was used for enzyme assay.

#### Enzyme assay

The enzymatic activity of cellulase was measured according to the method of Denison and Koehn (1977) using 1% CMC in phosphate buffer (pH 7.0) as a substrate. The hydrolyzed end product from the CMC substrate due to the cellulolytic activity was measured at 540 nm by the dinitrosalicylic method using glucose as the standard and expressed as U/ml. A mixture of pre-inoculated production medium and the reaction mixture

was used as a control. One enzyme unit (U) is defined as the amount of enzyme capable to release 1 mM of glucose from the substrate in 1 min at 38 °C. The concentration of soluble protein was measured by the method of Lowry et al. (1951).

#### Characterization and identification of the selected isolate

The selected isolate was grown on the CMC-agar plate after 48 h of incubation. The colony characterizations were made through visual observation. Micro-morphological studies were done using a compound microscope and a scanning electron microscope (SEM), Model Hitachi-530, Japan (Dietz and Mathews 1969). Physiological and biochemical characterization of the selected isolate was made according to the Society of American Microbiologists (1957) and Bergey's Manual of Systematic Bacteriology (Garrity 2005).

The DNA of the selected isolate was isolated (Rainey et al. 1996). The 16S rRNA gene was amplified with primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500r (5'-AGAAAGGAGGTGATCCAGCCA-3'), separated on 1% agarose gel, eluted, and purified using a QIA quick gel extraction kit (Qiagen, USA). The purified PCR product was sequenced with four forward and three reverse primers, namely, 8-27f, 357f (5'-CTCCTACGGGAGGCAGCAG-3'), 704f (5'-TAGCGGTGAAATGCGTAGA-3'), 1114f (5'-GCAACGAGCGCAACC-3'), 685r (5'-TCTACGCATTTCACCGCTAC-3'), 1110r (5'-GGGTTGCGCTCGTTG-3'), and 1500r (*E. coli* numbering system). The 16S rDNA gene sequence was determined by the dideoxy chain termination method using the Big-Dye terminator kit at an ABI 310 Genetic Analyzer (Applied Biosystems, USA).

A sequence similarity search was done using GenBank BLASTN (Altschul et al. 1997). Sequences of closely related taxa were retrieved and aligned using the program CLUSTAL\_X (Thompson et al. 1997), and the alignment was adjusted manually. For neighbor-joining analysis (Saitou and Nei 1987), distances between the sequences were calculated using Kimura's two-parameter model (Kimura 1980). Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein 1985).

Fatty acid composition of the cell membrane was determined by fatty acid methyl ester (FAME) analysis according to MIDI version 6.1, Method RTSBA6, Matches Library RTSBA6 6.10 (Morey et al. 2013).

#### Submerged fermentation

Four days old culture of the selected isolate was used as a source of inoculum for performing various experiments. Batch experiments were carried out in a 100-ml Erlenmeyer flask containing 25 ml medium at 35 °C for 3 days at shaking (100 rpm) condition using 4%

**Table 1** Composition of media trialed for cellulase production

Media	Composition	(gm/l)	References
M1	CMC	02	Kar et al. 2008
	Peptone	05	
	NaCl	05	
	Beef Extract	05	
M2	CMC	10	Saha et al. 2006
	KH <sub>2</sub> PO <sub>4</sub>	4	
	Na <sub>2</sub> HPO <sub>4</sub>	4	
	Tryptophan	2	
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2	
	CaCO <sub>2</sub>	0.001	
M3	CMC	10	Coral et al. 2002
	NaNO <sub>3</sub>	2.0	
	KH <sub>2</sub> PO <sub>4</sub>	1.0	
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.5	
	KCl	0.5	
M4	CMC	1.1	Kocher et al. 2008
	Yeast Extract	0.1	
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.5	
	KH <sub>2</sub> PO <sub>4</sub>	10	
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.1	
	NaCl	0.2	

inoculum ( $1.2 \times 10^7$  CFU/ml) and an initial pH of 7.0. After fermentation, the cell-free culture broth was used as a crude enzyme for assay. The strategy adopted to optimize one parameter at a time. The optimized parameter of an experiment was considered for the designing of subsequent experiments keeping all other conditions constant. All the experiments were done in triplicate.

To obtain maximum cellulase production from the selected isolate, the effects of various fermentation factors were optimized. To perform medium suitability tests, different media (M1, M2, M3, and M4) were taken into consideration (Table 1). Among the various physical factors, incubation time (24–84 h) with an interval of 12 h, inoculum volume (1–9%) with an interval of 1%, pH (5.0–8.5) with an interval of 0.25, temperature (30–44 °C) with an interval of 2 °C, and aeration (static to 200 rpm) with an interval of 50 rpm, were optimized. For the nutritional factors, the suitability of the various supplementary carbon sources (sucrose (Sac), fructose (Fru), maltose (Mal), lactose (Lac), dextrose (Dex), mannitol (Man), and malt extract (Mal ex)) were trialed. The effects of several concentrations (0.4–2%, w/v) of selected supplementary carbon sources were also evaluated. To determine the most effective substrate concentration for enzyme production, CMC was tested at different concentrations (0.2–2.8%, w/v). Different inorganic nitrogen sources (ammonium sulfate (AS), potassium nitrate (PN) and ammonium chloride (AC), sodium nitrate (SN), and urea) were also taken into consideration. The effective suitable concentration of selected inorganic nitrogen sources was also checked within a range of 0.4–2%, w/v. Vitamins such as ascorbic acid (ASC), pyridoxine (PYR), thiamine (THI), biotin (BIO), niacin (NIA), and riboflavin (RIB) were also trialed at different concentrations (up to 60 µg/ml). The effect of supplementation of casein hydrolysate and molasses (up to 0.01 %, w/v) was also tested. The effect of different surfactants like SDS, EDTA, Tween 80 and Triton X-100, and their effective concentration (up to 0.2 µg/ml) was also determined.

### Purification

After centrifugation (8000 rpm for 10 min), the supernatant was subjected to ammonium sulfate precipitation with uninterrupted stirring at 4 °C. The precipitated proteins were recovered by centrifugation (10000 rpm for 20 min at 4 °C) and dialyzed (using LA401, HiMedia) against 20 mM potassium phosphate buffer (pH = 6.75) at 4 °C with three changes during 24 h. The partially purified dialyzed sample was used for the DEAE-Sephacel column (Sigma). The dialyzed proteins were eluted using a linear gradient of 0.05–1.0 M NaCl (in phosphate buffer) with a flow rate of 1 ml/min. Using fraction collectors, the eluted fractions were collected. After collection, respective fractions were screened for

protein estimation (Lowry et al. 1951) and measuring total activity by the dinitrosalicylic acid method (Denison and Koehn 1977).

### Characterization of the purified enzyme

The molecular weight of the purified enzyme was characterized by carrying out 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). The molecular ladder (10–250 kDa) (Thermo Fisher Scientific) was used to estimate the molecular weight (M) of the purified enzyme.

To ascertain the optimum pH value of the purified cellulase from the selected isolate, for its activity, several buffers like acetate buffer (pH 4 and 5), citrate buffer (5 and 6), phosphate buffer (pH 6–8), Tris-HCL (pH 8 and 9), Glycine-NaOH buffer (pH 9 and 10), sodium bicarbonate-NaOH buffer (pH 10 and 11), and KCl-NaOH buffer (pH 12) were used. The activity assay was performed by using 2% CMC with these different pH buffers and incubated at 50 °C for 20 min according to Miller (1959).

The influence of temperature on the purified enzyme activity was determined at a wide range of temperatures (20–80 °C) in phosphate buffer (pH 7.0) for 20 min using 2% CMC as a substrate. The activity was determined by the DNS method (Miller 1959).

To check the stability of the purified enzyme upon variable pH conditions, various buffers like acetate buffer (pH 4 and 5), citrate buffer (5 and 6), phosphate buffer (pH 6–8), Tris-HCL (pH 8 and 9), Glycine-NaOH buffer (pH 9 and 10), sodium bicarbonate-NaOH buffer (pH 10 and 11), and KCl-NaOH buffer (pH 12) were tested (Miller 1959).

To test the thermal stability of the purified enzyme, residual activity was determined in different temperature setup (20–80 °C) in 100 mM phosphate buffer (pH 7.0) (Balasubramanian et al. 2012).

The purified enzyme from GAC 16.2, the most promising cellulolytic microbe isolate, was investigated against different substrates (1%) for the substrate specificity test. The substrates were CMC, xylan, avicel, filter paper, and chitin. Reaction mixtures were performed in phosphate buffers (pH 7.0) for 20 min at 50 °C followed by the DNS method (Miller 1959).

The effects of metal ions, inhibitors, and surfactants on the activity of the purified cellulase were also tested. The metal ions employed in this study were  $K^+$ ,  $Na^+$ ,  $Ca^{2+}$ ,  $Mn^{2+}$ ,  $Mg^{2+}$ ,  $Fe^{2+}$ ,  $Ni^{2+}$ ,  $Hg^{2+}$ , and  $Co^{2+}$  with a concentration of 1 mM and 5 mM. Among the inhibitors, phenylmethanesulfonyl fluoride (PMSF),  $\beta$ -mercaptoethanol ( $\beta$ ME), dithiothreitol (DTT), EDTA, iodoacetic acid (IAA), and urea were taken into account at the concentration of (5 mM). Surfactants like SDS, Tween 20, and Triton X-100 were employed at 1%, v/v.

Purified enzyme with 20 mM of phosphate buffer (pH 7.0) mixed separately with metal ions, inhibitors, and surfactants to make different reaction mixtures and individually incubated at 37 °C for 30 min. Relative activity was estimated by the DNS method (Miller 1959).

#### Effect of cellulase on cotton fiber and sawdust

In one case, pieces of sterilized cotton fibers (CF) were dipped into 200 µl purified enzyme (in 20 mM potassium phosphate buffer, pH 6.75) at 37 °C for varied exposures (12–48 h). In another case, the sterilized sawdust (SD) was primarily washed and allowed to exposure (24–48 h) in the enzyme. The amount of reducing sugar was estimated for both of the enzyme-treated CF and SD (Denison and Koehn 1977). Control sets (without enzyme treatment) were also prepared simultaneously. The SEM studies were made after 10 min of gold metallization (Model Hitachi-530, Japan) following Dietz and Mathews (1969).

#### Statistical analysis

The standard error ( $\pm$ ) of mean was calculated from three determinations using Microsoft Office Excel 2013. The significance level of the variables was determined by one-way ANOVA at the level of 95% ( $P < 0.05$ ) using the software IBM-SPSS (V.23).

## Results

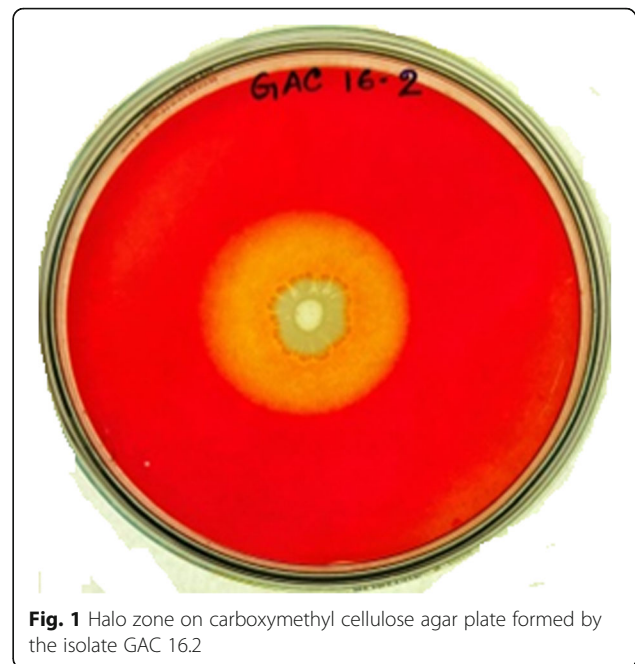
#### Isolation and Screening for cellulase producers

Out of 43 isolates, 5 isolates were primarily screened based on the halo zone forming capability in the CMC-agar plate treated with Congo-red and NaCl. Based on DNS assay, the isolate GAC 16.2 was chosen as a potent cellulase producer having production capability  $0.26 \pm 0.09$  U/ml (Figs. 1 and 2).

#### Characterization and identification of the selected isolate

The colony of GAC 16.2 was grown on the CMC-agar plate. It was observed as off-white, circular with convex elevation, and having an entire margin. The cells were found singly or in aggregate, ellipsoidal  $3\text{--}4 \mu\text{m} \times 2\text{--}2.5 \mu\text{m}$  (Fig. 3), Gram-negative, confluent growth between 15–50 °C, and tolerance of pH between 4.0–10.0 and NaCl up to 5%, and utilization of citrate as sole carbon source, and acid production from xylose, glucose, fructose, sucrose and meso-inositol, and nitrate reduction were found positive (Data not shown).

16S rDNA sequence (1407 bases) of the isolate GAC 16.2 showed a 100% pairwise similarity with *Acinetobacter junii* LMG 998<sup>T</sup> (AM410704). The phylogenetic tree also suggested the closest relationship of GAC 16.2 with the type strain of *Acinetobacter junii* (Fig. 4). This strain (GAC 16.2) has also been deposited to Microbial Type Culture Collection (MTCC), India (Accession no.



**Fig. 1** Halo zone on carboxymethyl cellulose agar plate formed by the isolate GAC 16.2

MTCC 11818), and the 16S rDNA sequence has been submitted to NCBI (GenBank Acc No. KJ174319).

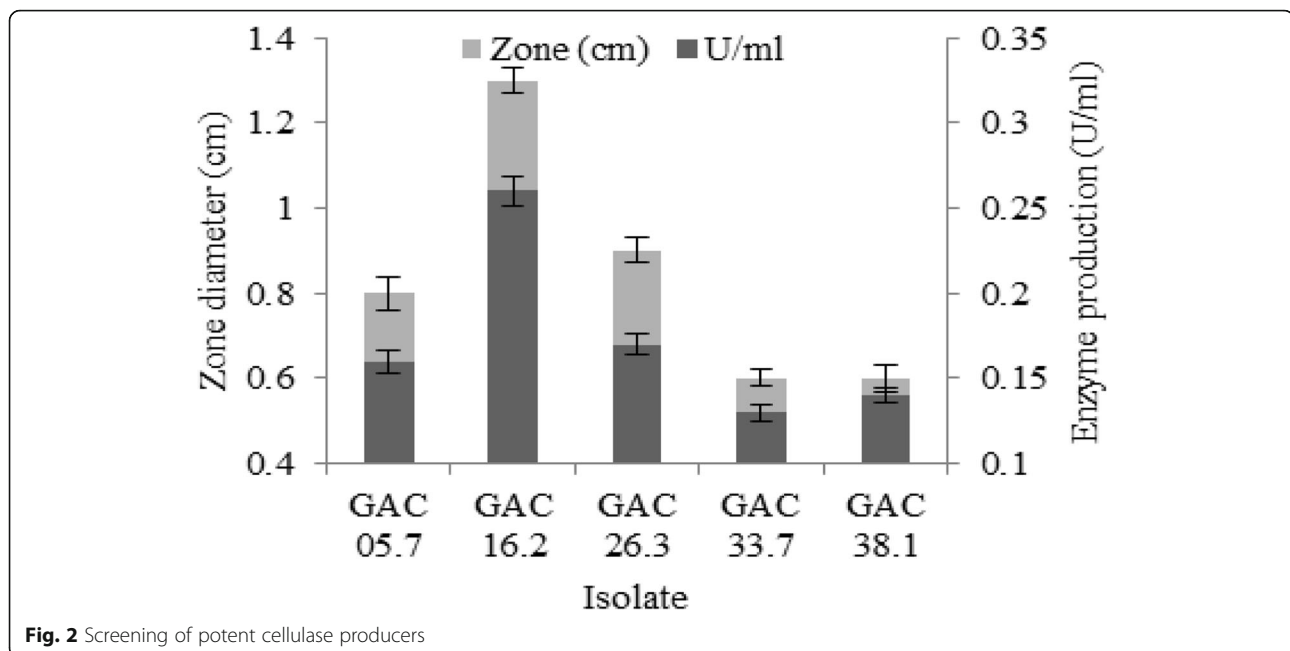
From the FAME analysis of isolate GAC 16.2, contain of saturated and unsaturated fatty acids found to be 30.38% and 69.39% respectively (Fig. 5, Table 2). FAME similarity index of GAC 16.2 was very close to *A. calcoaceticus* (index 0.210) and *A. haemolyticus* (index 0.209).

#### Optimization for cellulase production

From the experimental result, medium M1 was found the most suitable for enzyme production ( $0.26 \pm 0.09$  U/ml), followed by M3, M2, and M4 with production  $0.156 \pm 0.013$ ,  $0.132 \pm 0.012$ , and  $0.114 \pm 0.011$  U/ml, respectively (Fig. 6a).

The enzyme production ability of the isolate increased with the fermentation period up to 72 h ( $0.35 \pm 0.01$  U/ml); thereafter, it declined (Fig. 6b). The experiment revealed (Fig. 6c) that the enzyme production increased with the increase of inoculum size up to 8% ( $0.626 \pm 0.021$  U/ml). The optimum initial pH of the production medium was found as 6.75 for cellulase production ( $0.65 \pm 0.03$  U/ml) by the isolate GAC 16.2 (Fig. 6d). The isolate showed optimum enzyme production ( $0.69 \pm 0.015$  U/ml) at 38 °C which decreased sharply on either side of that temperature (Fig. 6e). It was found that the isolate GAC 16.2 produced best (Fig. 6f) at 150 rpm ( $0.82 \pm 0.041$  U/ml).

Among the several carbon sources, lactose supplementation effected a sharp increase in production ( $49.95 \pm 1.72$  U/ml) but no such boosting effect in the case of sucrose and mannitol that was recorded (Fig. 7a). The optimum concentration of lactose was further checked



(Fig. 7b) and found as 1.4% ( $55.23 \pm 1.86$  U/ml). For assessing the effect of substrate (CMC) concentration on enzyme production, it was found that the production increased slowly with the increase of substrate concentration up to 2% ( $57.19 \pm 1.54$  U/ml) and declined sharply thereafter (Fig. 7c). In the experiment of nitrogen source variation, SN was found much effective ( $92.24 \pm 1.11$  U/ml) in comparison to AS, PN, and AC (Fig. 7d). Further, the optimum concentration of SN was ascertained at the level of 1.6% for cellulase production ( $96.38 \pm 2.4$  U/ml) (Fig. 7e).

Among the vitamins, ASC, PYR, THI, BIO, NIA, and RIB are found as positive for cellulase production (Fig. 7f). However, BIO boosted cellulase production

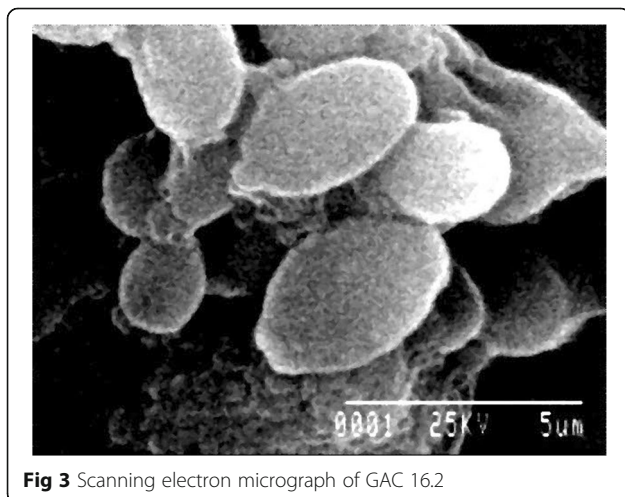
maximally ( $108.61 \pm 0.63$  U/ml). Further, the medium supplementation with molasses and casein hydrolysate found to increase enzyme production by  $109.55 \pm 0.78$  U/ml and  $112.38 \pm 0.87$  U/ml respectively (Fig. 7g). In the present study, the surfactants did not show any positive role in enzyme production (Fig. 7h).

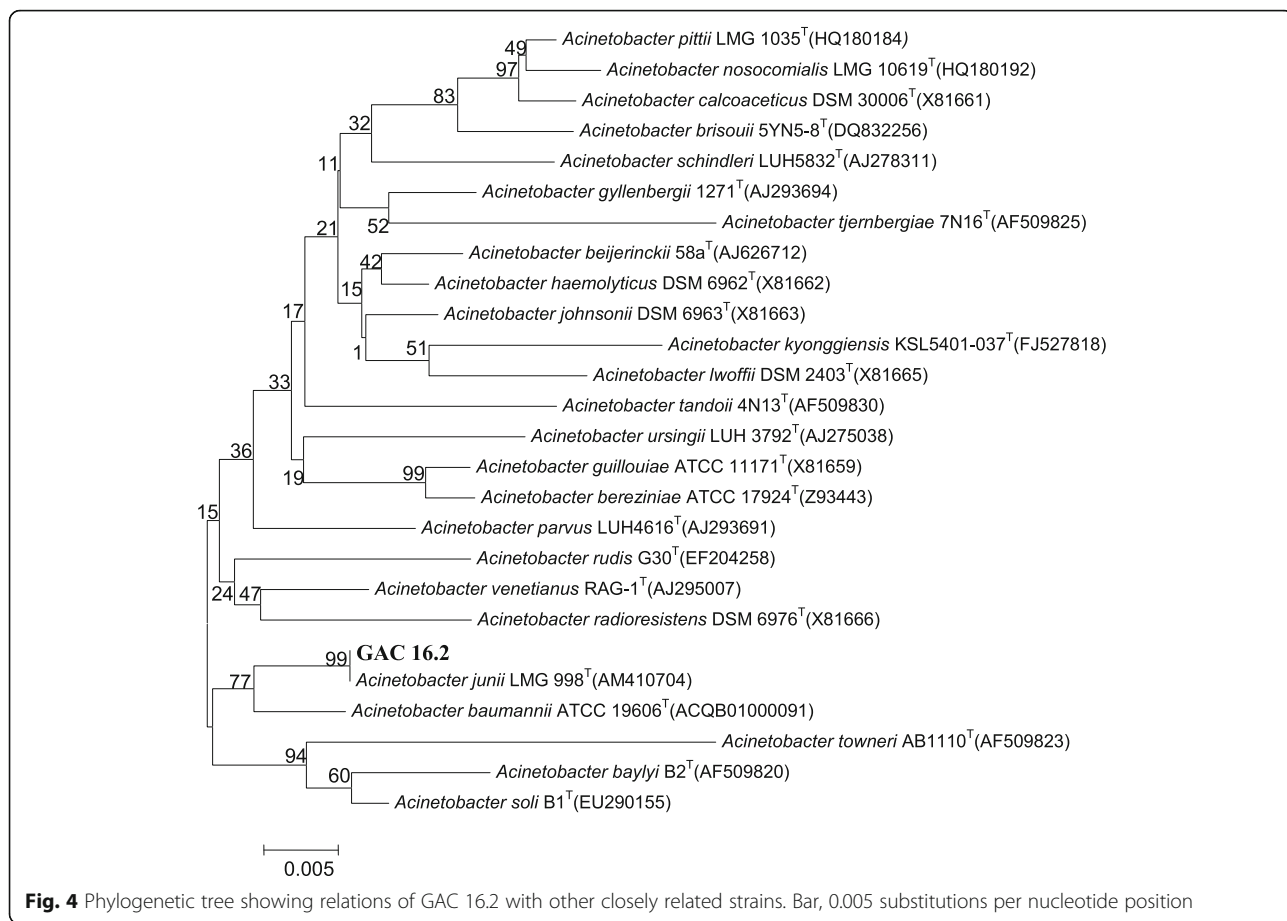
#### Purification

Ammonium sulfate precipitation of the enzyme was standardized, and maximum activity was observed at 40–80% saturation. Cell-free fermented broth exhibited a specific activity of 0.43 U/mg, whereas the dialyzed enzyme showed a specific activity of 2.59 U/mg with 6.02-fold enhancement. After ion-exchange chromatography (DEAE-Sepharose column), all the fractions were assayed for cellulase activity, and fraction F4 found best (3.95 U/mg). The specific activity was successively amplified at every stage of purification, and ultimate augmentation was 9.2-fold (Table 3).

#### Characterization of the purified enzyme

The purified cellulase from GAC 16.2 was found as a single band with a molecular weight (M) of about 55 kDa on SDS-PAGE gel (Fig. 8), and no subunit was observed, and therefore, the purified enzyme was considered to be homogenous. The optimum pH and temperature for the utmost activity of the purified enzyme were found to be 7 (Fig. 9a) and 50 °C (Fig. 9b) respectively. Cellulase from GAC 16.2 exhibited its stability more than 60% in response to pH ranges from 6 to 9, and temperature ranges from 40–60 °C (Fig. 9a, b).



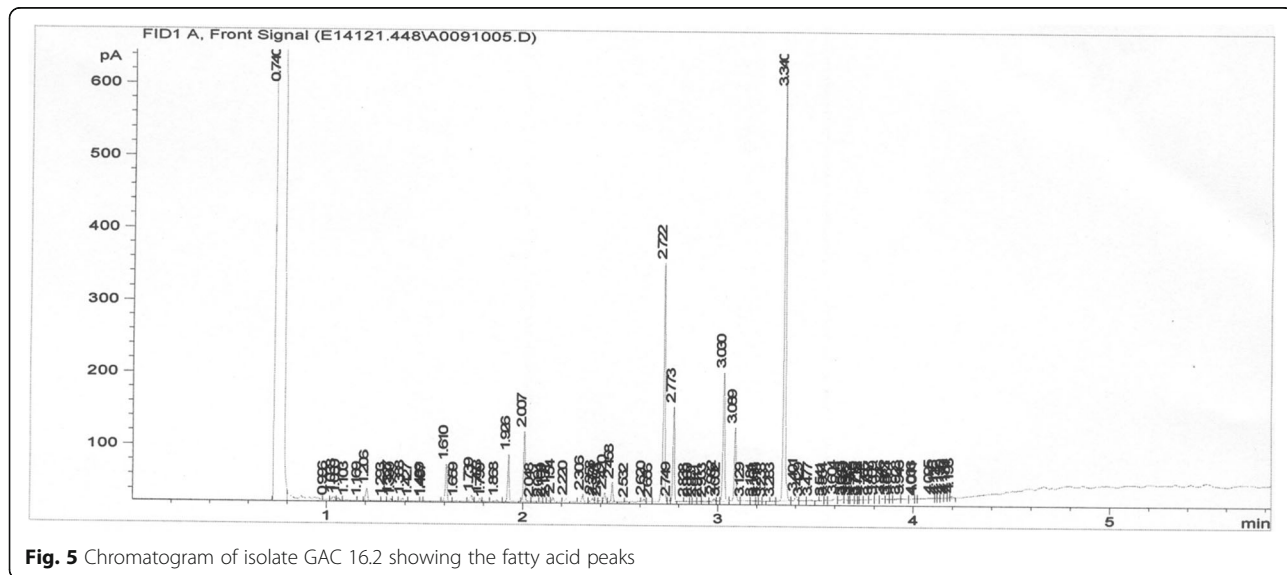


Beyond this range, stability decreased at the level of less than 40%.

In course of evaluation of substrate specificity, purified enzyme represented its utmost activity against CMC (100%) and moderate activity towards filter paper, xylan

but least activity in contrast to avicel, and no reaction output against chitin (Table 4).

The effect of metal ions on purified cellulase reflected that at the 1 mM concentration of different metal ions, Mn<sup>2+</sup>, Co<sup>2+</sup>, and Mg<sup>2+</sup> activated the cellulase activity;



**Table 2** Cell membrane fatty acid content of GAC 16.2

Fatty acid	Content (%)	Fatty acid	Content (%)	Fatty acid	Content (%)	Fatty acid	Content (%)	Fatty acid	Content (%)
Straight chain		Branched chain		Unsaturated		Hydroxyl		Sum in feature	
C 10:0	1.30	ISO 14:0	0.05	14:1 ω5C	0.22	10:0 2OH	0.03	1	0.61
C 11:0	0.25	ISO 16:0	0.04	15:1 ω6C	19.53	10:0 3OH	0.03	2	0.22
C 12:0	3.44	ISO H 18:1	0.02	16:1 ω5C	0.09	11:0 2OH	0.08	3	19.53
C 13:0	0.19	ISO I 19:1	0.05	17:1 ω8C	0.15	12:0 2OH	3.78	4	0.09
C 14:0	0.33	ISO 19:0	0.31	18:1 ω9C	T*	12:0 3OH	5.59	6	0.15
C 15:0	T*	Anteiso 13:0	0.04	Cyclo19:0ω10c/19 ω6	0.02	13:0 2OH	0.23	7	T*
C 16:0	7.22	Anteiso 15:0	0.15	20:1 ω9c	0.12	14:0 2OH	0.03	9	0.02
C 17:0	5.93	Anteiso 17:0	0.08	11methyl 18:1 ω7C	0.10	18:0 3OH	0.05		
C 18:0	0.55								
C 19:0	0.05								
C 20:0	0.02								

Sum in feature 1 comprises 13:0 3OH/15:1 iso H; sum in feature 2 comprises 14:3 OH/16:1 iso I; sum in feature 3 comprises 16:1 ω7C/16:1 ω6C; sum in feature 4 comprises 17:1 anteiso B/iso I; sum in feature 6 comprises 19:1 ω11C/ 19:1 ω9C; sum in feature 7 comprises 19:1 ω7C/19:1 ω11c; and sum in feature 9 comprises 16:0 10-methyl

T\* trace amount

however, Ni<sup>2+</sup> and Hg<sup>2+</sup> remarkably inhibited the enzyme activity. However, at the level of 5 mM, only Mg<sup>2+</sup> and Mn<sup>2+</sup> exhibited their positive effects (Table 5). Among the inhibitors, cellulase activity was inhibited significantly by EDTA and moderately by IAA, PMSF, and urea accordingly (Table 6). All the tested surfactants have an inhibitory effect on enzyme activity (Table 7).

#### Effect of cellulase on CF and SD

In both cases, the effect of the enzyme treatment was pronounced with increased time (Figs. 10 and 11). The efficient enzyme activity has supported by the analysis of the end product (Table 8).

All sets of experiments found statistically significant at the level of  $P < 0.05$ .

#### Discussions

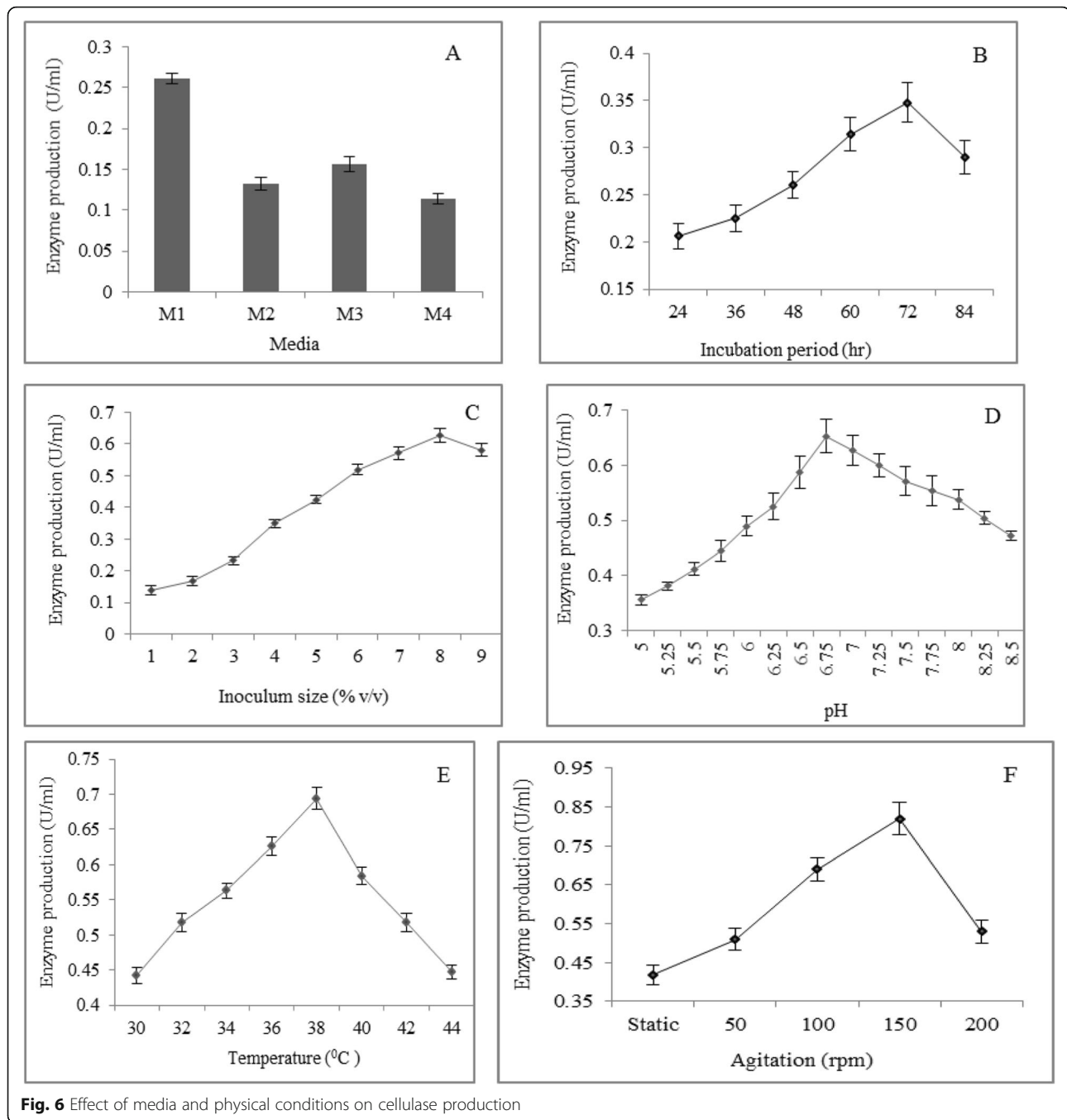
Cellulose exists as crystalline or amorphous microfibrils in plant cell walls. In the gut, the cellulose fibers first need to get fragmented into simpler sugar residues to make it accessible to insects, a process in which bacteria are typically involved through enzymatic digestion (Russell et al. 2009; Watanabe and Tokuda 2010). The important pest *G. africana* dwells in humus-enriched moistened soil and consumes a wide range of economically important plants including potato (James et al. 2010). The pest consumes invertebrates, including insects and earthworms as food alternatively (Banerjee et al. 2017). Literature regarding their gut digestive enzyme is not available. However, Banerjee et al. (2016, 2017) reported two potent enzyme-producing bacteria *Rhodococcus opacus* GAA 31.1 and *Kitasatospora cheerisanensis* GAP 12.4 from their gut capable to produce

amylase and protease, respectively. So, the experiment was designed through conceiving the idea that the gut of this agriculturally important major pest may contain potential sources of cellulolytic microflora that support in the digestion of the consumed food enriched with cellulosic material.

A potent cellulolytic isolate, GAC 16.2, was ultimately screened from the gut content of *G. africana*. Through the study of morphological, physiological, biochemical, and molecular characterization and specific study of cell membrane fatty acid analysis of the selected isolate, GAC 16.2 was identified as *Acintobacter junii*. Dhayal et al. (2014) isolated a strain of *A. junii* from the larvae of *Anopheles stephensi*. There is also a report of isolation of *Acintobacter* spp. from the gut of *Glossina palpalis palpalis* (Geiger et al. 2009). There is also a report about the involvement of *A. junii* in fresh cattle manure composite culture (Ghosh et al. 2018). The *A. junii* F6-02, a soil isolate of Taiwan, is also capable to produce xylanase (Lo et al. 2010).

The isolated GAC 16.2 tolerates a long range temperature (15–50 °C) and pH (4.0–10.0). An endophytic bacterium, *Lentibacillus populi*, isolated from poplar, is also reported to grow within a wide range of temperatures (15–50 °C) and pH at a range of 6.0–9.0 (Sun et al. 2016). Besides this, Nakajo et al. (2006) reported that *Enterococcus faecalis* can grow on broad ranges of pH (4.0–11). The members within the genus are also habituated to grow at a wide range of temperatures and pH. Hrenovic et al. (2014) isolated a strain of *A. baumannii* which grows at low pH (3.37) and tolerates high temperature at (50 °C). Hrenovic et al. (2016) reported the growth of *A. baumannii* beyond 42 °C. The



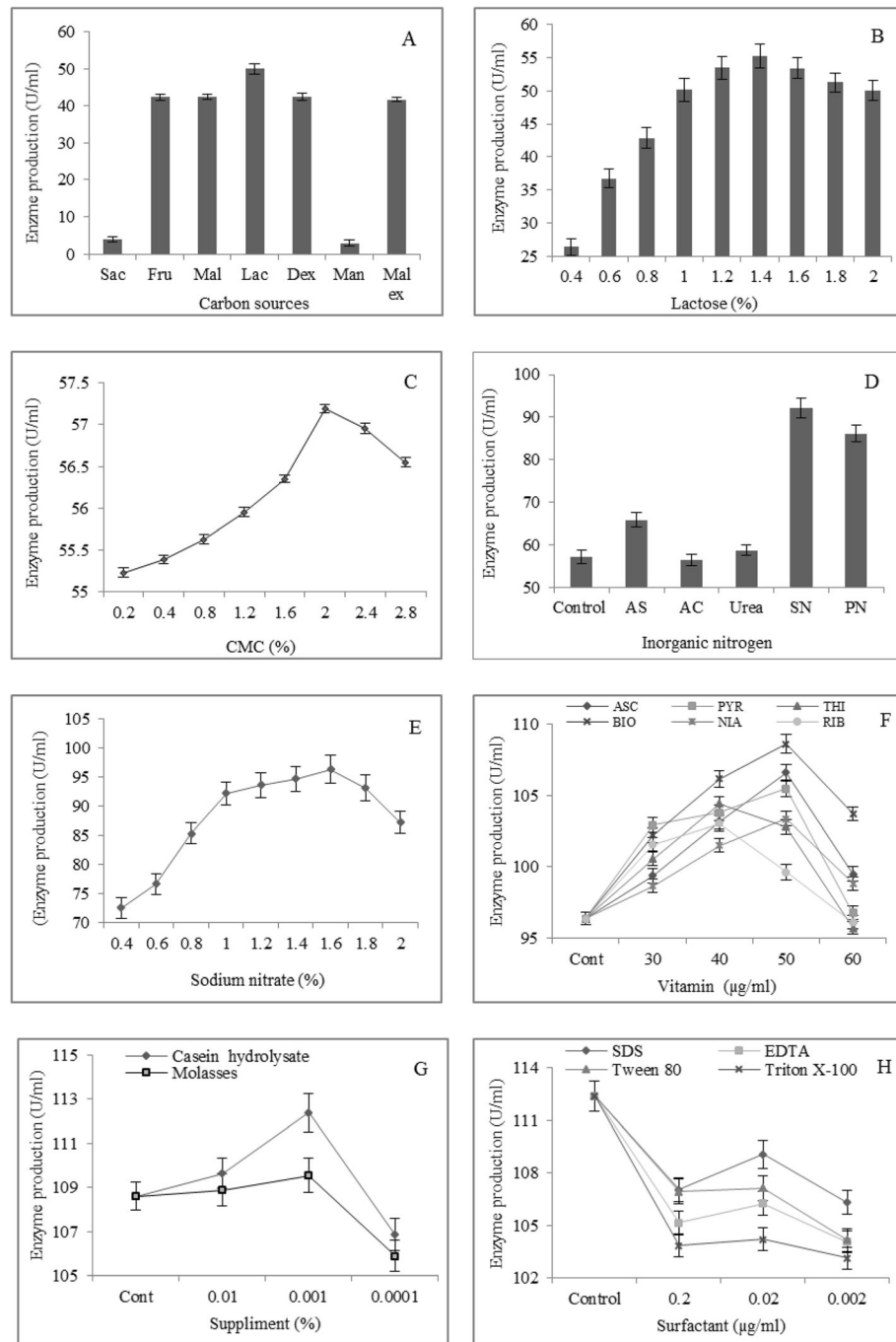


**Fig. 6** Effect of media and physical conditions on cellulase production

growth of *A. seohaensis* sp. nov. growth was observed at pH 5.5–8.0 and temperature 10–40 °C (Yoon and Kim 2007).

Common phenomenon or regulation for the growth and production ability of microorganisms is not available (Roy and Sen 2013). Thus, optimization of fermentation conditions is of great significance in microbial production including the enzyme. The isolate GAC 16.2 showed maximum cellulase production ( $0.26 \pm 0.09$  U/ml) on the M1 in comparison to the other tested media.

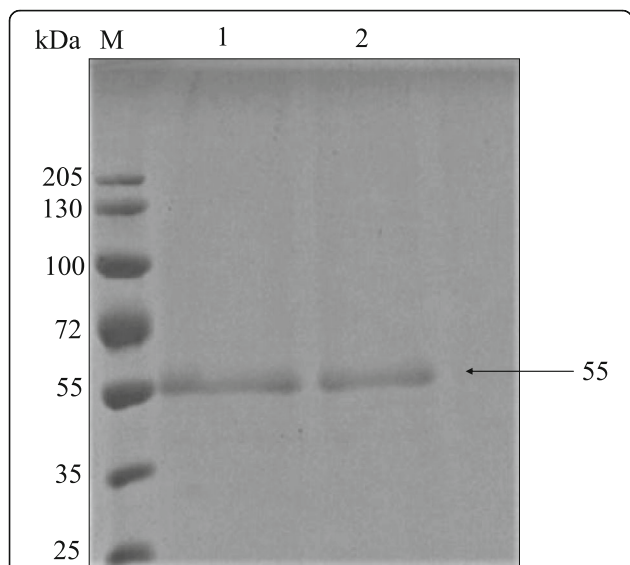
The increase in cellulase production on the M1 medium can be explained by the availability of the required nutrients than that of other tested media for the isolate. Among the cellulolytic bacterial community, *Bacillus*, *Micrococcus*, and *Cellulomonas* are common, and their enzyme production was observed up to 0.034 U/ml (Immanuel et al. 2006). Other cellulase producers like *Brevibacillus* and *Geobacillus* are found to produce maximally 0.02 U/ml and 0.058 U/ml, respectively (Rastogi et al. 2009). The enzyme production ability of the isolate



**Fig. 7** Effect of nutrients and surfactants on cellulase production

**Table 3** Summary of cellulase purification of GAC 16.2

Sample	Volume [ml]	Total protein [mg]	Protein Conc. [mg/ml]	Total activity [Units]	Yield [%]	Specific activity [Unit/mg]	Purification [fold]
Cell-free supernatant	100	261.6	2.61	112.38	100	0.43	1
Dialyzed fraction	21	26.37	1.25	68.28	60.75	2.59	6.02
Column chromatographic fraction	9.5	10.14	1.06	40.05	35.64	3.95	9.2



**Fig. 8** Molecular weight analysis of the purified cellulase by *A. junii* GAC 16.2 in SDS-PAGE

GAC 16.2 increased with the fermentation period up to 72 h ( $0.35 \pm 0.01$  U/ml); thereafter, it declined. This pattern is observed in cellulase producers, viz., *Bacillus pumilus* EWBCM1 (Shankar and Isaiarasu 2011) and *B. subtilis* (Bai et al. 2012). The optimum incubation period depends primarily on nutrient availability and other cultural conditions (Wanderley et al. 2004). Nutrient availability decreases with the increase in the age of the culture. The production increased with the increase of inoculum size up to 8% ( $0.626 \pm 0.021$  U/ml), and this data is corroborated with the finding of Omojasola and Jilani (2009).

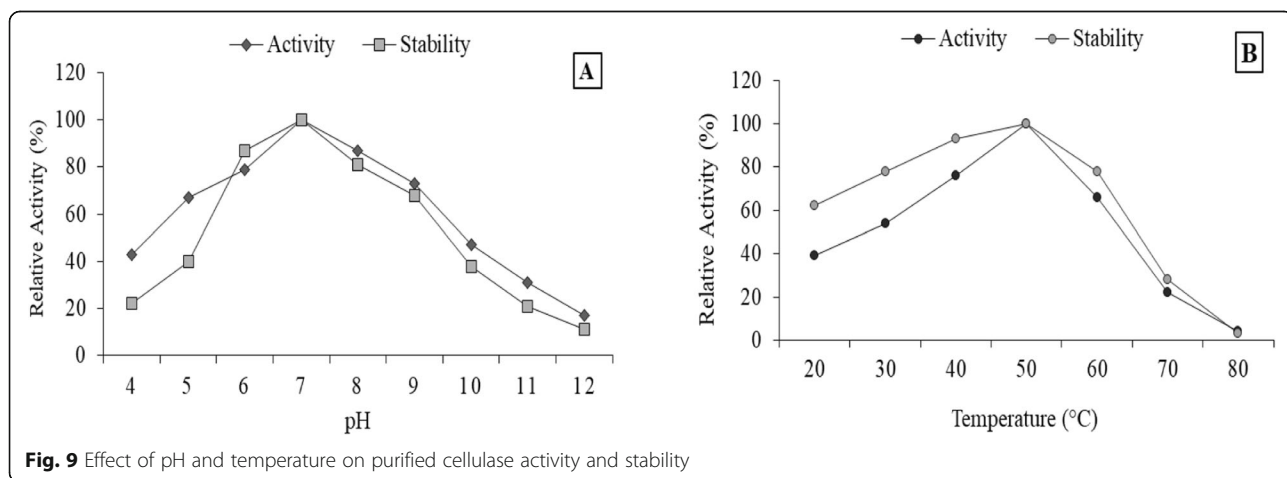
The pH of the medium influences the fermentation capability of an organism. The isolate GAC 16.2 produced cellulase maximally ( $0.65 \pm 0.03$  U/ml) at the production medium having an initial pH of 6.75. Like GAC 16.2, some cellulase producers preferred pH near the

**Table 4** Substrate specificity of the cellulase from GAC 16.2

Substrate	Relative activity (%)
CMC	100
Filter paper	76.2
Xylan	53.7
Avicel	24.3
Chitin	None

neutral for enzyme production (Younis et al. 2010; Bai et al. 2012). The selected isolate showed optimum enzyme production ( $0.69 \pm 0.015$  U/ml) at 38 °C which decreased sharply on either side of that temperature. At lower temperatures, the transport of substrate across the cells is limited and that affects the yield. Moreover, at elevated temperature, the energy requirement is high for growth due to the thermal denaturation of metabolic enzymes resulting in the inhibition in product formation (Aiba et al. 1973). But, the isolate does not require extra energy because it grows almost in ambient temperature.

Under agitation, the oxygen from the air becomes soluble in the fermentation broth. Thus, to provide sufficient oxygen to the fermenting organism, it requires agitation to accelerate the dissolution of oxygen into the medium. Aeration also increases nutrient availability as well as decreases loads of products from the vicinity of the producers (Brock and Madigan 1988). At increased agitation, the enzyme may get inactivated, but agitation at 150 rpm is suitable for its optimum activity (Singh and Kumar 1998). However, the working isolate could produce maximally at 150 rpm which is much less than the majority of the producers, indicating energy conservation. When a medium culture is shaken or stirred, the cellulose fibrils do not form a well-organized pellicle which is produced under static conditions. Cellulose mass will decrease with an increase in shaking speed which may reach up to 160 rpm (Pa'e et al. 2007). This may also be due to *A. junii* that is difficult to adapt to



**Fig. 9** Effect of pH and temperature on purified cellulase activity and stability

**Table 5** Effect of metal ions on the cellulase of GAC 16.2

Metal ion	Relative activity (%)	
	1 mM	5 mM
None	100	100
K <sup>+</sup>	96	89
Na <sup>+</sup>	99	91
Ca <sup>2+</sup>	102	94.5
Mn <sup>2+</sup>	114.5	102.5
Mg <sup>2+</sup>	124.5	107
Co <sup>2+</sup>	107	98
Fe <sup>2+</sup>	101	92
Ni <sup>2+</sup>	38	21.1
Hg <sup>2+</sup>	31.5	19.5

the harsh environment. Also, the original habitat of cellulase producing bacteria within insect gut is not at the much shaken condition.

Among various supplementary carbon sources, lactose presents a notable elevation in production ( $49.95 \pm 1.72$  U/ml). The optimum concentration of lactose was further checked and found as 1.4% ( $55.23 \pm 1.86$  U/ml). Cellulase production in *Acremonium cellulolyticus* and *Microbacterium* sp. was also enhanced by lactose (Fang et al. 2008; Sadhu et al. 2011). The exact cause(s) behind this synergistic effect of lactose with CMC on cellulase production is still unknown. However, the mechanism of lactose induction possibly lies with the intracellular level of galactose-1-phosphate that triggers the signaling for enzyme production. Since cellulose is insoluble, the enzyme itself cannot trigger the induction. It can be induced in the presence of soluble saccharide. Further cellulase production is intricately regulated by the activation and repression mechanisms of soluble sugars (Ilmen et al. 1997). Lactose is the only known economically feasible additive inducers of the cellulase gene (Pandey et al. 2011). It was also found that the production increased slowly with the increase of substrate (CMC) concentration up to 2% ( $57.19 \pm 1.54$  U/ml) and declined sharply thereafter. High substrate concentration can cause product inhibition, resulting in a substantial

**Table 6** Effect of inhibitors on the cellulase of GAC 16.2

Inhibitor	Relative activity (%)
None	100
EDTA	18.1
βME	70.6
DTT	68.3
IAA	38.6
PMSF	46.4
Urea	57.5

**Table 7** Effect of surfactants on the cellulase of GAC 16.2

Surfactants	Relative activity (%)
None	100
SDS	66.1
Tween 20	73.6
Triton X-100	78.3

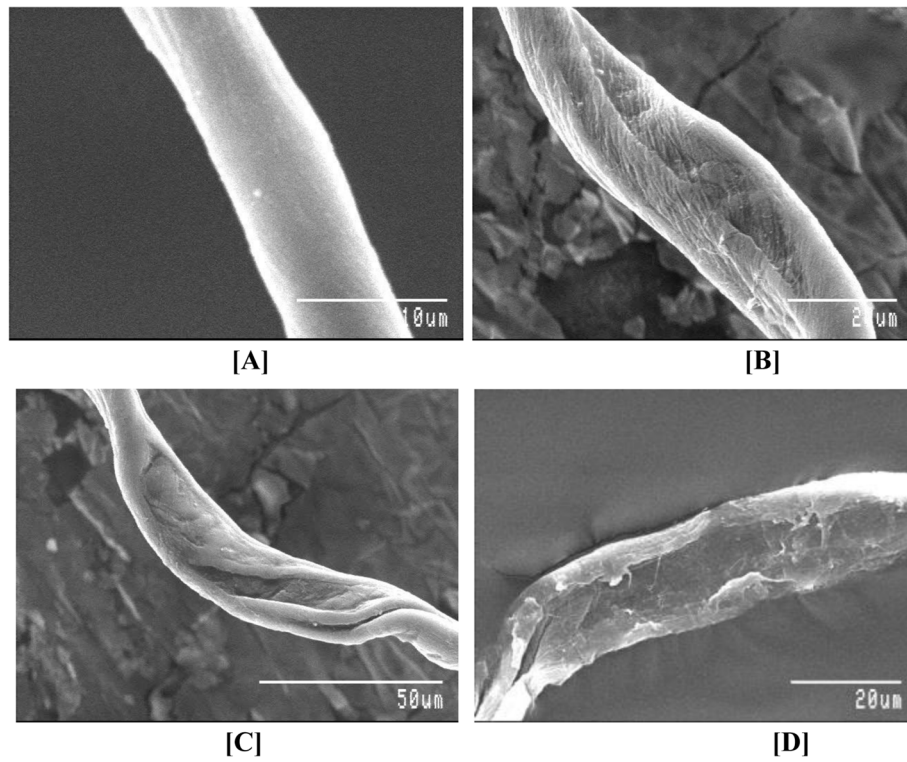
reduction in enzyme formation (Irshad et al. 2013). As a source of nitrogen, sodium nitrate was found much effective ( $92.24 \pm 1.11$  U/ml) compared to ammonium sulfate, potassium nitrate, ammonium chloride, and urea. This finding corroborates with the enzyme production of *Cellulomonas flavigena* (Rajoka 2004).

The metabolic activities of microorganisms are much influenced by vitamins as it acts as a prosthetic group for many enzymes. The vitamins pyridoxine, thiamine, niacin, and riboflavin were found to play a positive role for cellulase production. Mostly the vitamins are found optimally between 40 and 50 µg/ml (Banerjee et al. 2016; Banerjee et al. 2017). However, biotin enhances cellulase production maximally ( $108.61 \pm 0.63$  U/ml). It can be substantiated through biotin requirement by major cellulolytic gut bacteria for their growth (Baldwin and Allison 1983). There is no available report about the effect of ascorbic acid, thiamine, niacin, and riboflavin on cellulase production.

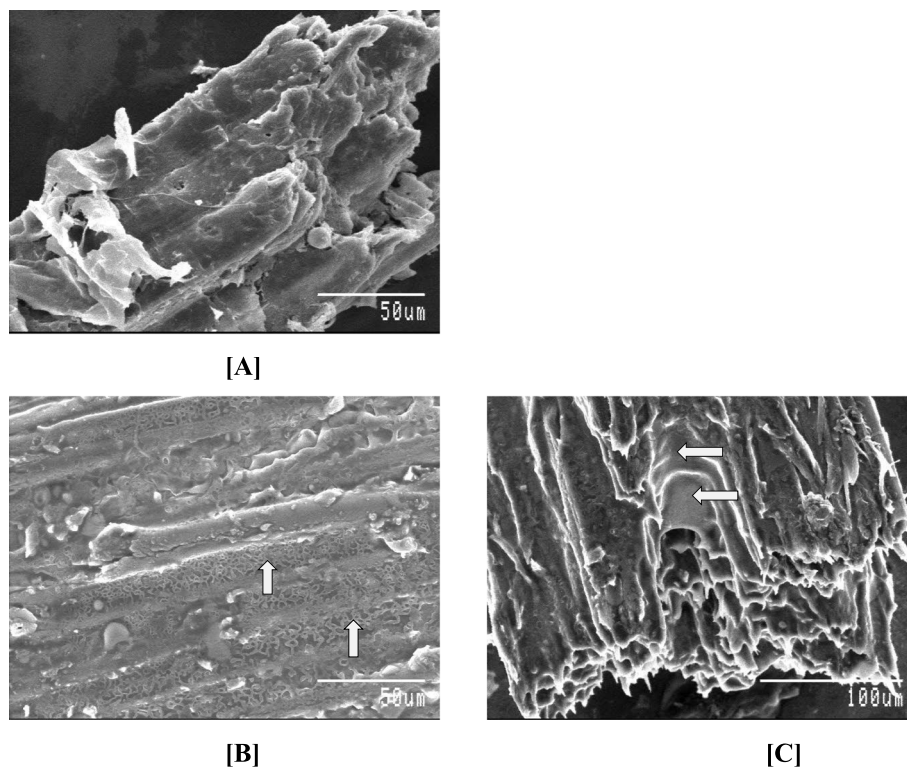
Supplementation of the medium with molasses and casein hydrolysate is found to increase enzyme production. Since the supplemented compounds are crude, such additions may persuade the necessity of certain imprecise features of the producer, resulting in enhanced enzyme production. Investigation in relation to improved cellulase production by the supplementation of molasses and casein hydrolysate till now has not been elucidated.

Commonly, surfactant alters the cell membrane permeability while facilitating the release of extracellular enzymes (Ahuja et al. 2004). In the present study, no enhancement of enzyme production was found due to the surfactant. It might have happened that the cellulase adsorbed by the cellulose form an inactive complex involved in blocking the release of the enzyme (Howell and Mangat 1978). There are reports on such nonproductive bindings (Steiner et al. 1988; Shankar and Isaiarasu 2011). Converse et al. (1988) attributed to the reduction in enzyme production by cellulase adsorption and steric inactivation of the enzyme. Such postulations help to explain the release of a higher amount of enzyme but with less activity.

The molecular weight of the purified cellulase produced by GAC 16.2 was found to be 55 kDa. This is higher than the cellulase of 45 kDa from *Acinetobacter indicus* KTCV2 (Karlapudi et al. 2018) and 48 kDa from *Bacillus* sp. (Sriariyanun et al. 2016). It was found to be



**Fig. 10** Effect of cellulase on cotton fiber with varied exposure. Control (a), 12 h (b), 24 h (c), 48 h and (d)



**Fig. 11** Effect of cellulase on sawdust with varied exposure. Control (a), 24 h (b), and 48 h (c)

**Table 8** Production of glucose from enzyme treated cotton fiber and sawdust

Sample	Treatment period (h)			End product
	12	24	48	
Control	0.00	0.00	0.00	Glucose (mg/ml)
Cotton fiber	0.08	0.23	0.61	
Saw dust	0.02	0.05	0.11	

lower than the cellulase of 58.6 kDa from *Paenibacillus barcinonensis* (Asha et al. 2012), 62 kDa from *Bacillus mycoides* (Balasubramanian et al. 2012), and 67 kDa from *Trichoderma longibrachiatum* (Pachauri et al. 2017). The most optimum pH of the enzyme produced by GAC 16.2 for its utmost activity was found at 7.0. Similar behavior of cellulase was also reported by Yin et al. (2010) and Balasubramanian et al. (2012). The purified cellulase performed best at 50 °C, and such character is treated as an industrial enzyme. Cellulase from *B. mycoides* S122C also exhibited its highest activity at 50 °C (Balasubramanian et al. 2012).

Metal ions can form complexes in association with proteins and other molecules related to enzymes. They may act as donors or acceptors of the electron as structural regulators (Riordan 1977). The activity of purified cellulase from GAC 16.2 is stimulated in the presence of metal ions such as Mg<sup>2+</sup>, Mn<sup>2+</sup>, and Co<sup>2+</sup>. Such stimulation in the activity has also been reported by Asha et al. (2012) that the support of Co<sup>2+</sup> on the activity of cellulase has been documented by Yin et al. (2010). Inhibition in the cellulase activity of GAC 16.2 by Ni<sup>2+</sup> and Hg<sup>2+</sup> metal ions also substantiates with the findings of Yin et al. (2010). The inhibitors, mainly EDTA, IAA, and PMSF, display a significant inhibition in the enzyme activity which is related to the observation of Yin et al. (2010).

Insect gut symbionts, the less explored natural resources, may be the true pathfinders to meet the tremendous demand for biotechnological tools, the enzymes. The cellulase produced by the selected isolate GAC 16.2 can efficiently degrade the cellulosic cotton fiber and sawdust within a reasonably short duration. The detailed study of cellulase production by *Acinetobacter* is not available. Though, Selvam et al. (2014) optimized the production of cellulase from alkali-treated coffee pulp and pineapple waste using *Acinetobacter* sp. TSK-MASC at solid-state fermentation.

In conclusion, the isolate *Acinetobacter junii* GAC 16.2 has been found as a potent cellulase producer. The isolate could enhance cellulase production by 400-fold at standardized fermentation conditions. The enzyme may be considered as an effective tool for industrial degradation of the cellulosic waste material particularly cotton fiber and sawdust. However, the isolate has the

potentiality to act as an efficient agent for environmental monitoring through organic waste management. Moreover, this study reveals the scope for utilization of the potential microbial wealth of the gut of *G. africana* in biotechnological applications.

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#### Authors' contributions

SB performed and analyzed the experimental research work and primarily drafted the manuscript. RNR designed and supervised this work and also configured the manuscript. TKM helped to finalize the manuscript. The authors read and approved the final manuscript.

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#### Availability of data and materials

All data generated or analyzed during this study are included in this article.

#### Ethics approval and consent to participate

Not applicable

#### Consent for publication

Not applicable

#### Competing interests

The authors declare that they have no competing interests.

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