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PRODUCTION AND CHARACTERIZATION OF A HALOTOLERANT CELLULASE FROM *BACILLUS AQUIMARIS* VITP4 STRAIN

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ABSTRACT
B.aquimaris VITP4 strain, a moderately halotolerant bacterium, isolated from Kumta coastal area, Karnataka, India produced significant amount of cellulase in Zobell's media. At 5% NaCl concentration, maximum growth and enzyme production was observed. Among the nitrogen sources, ammonium chloride and ammonium nitrate gave maximum production of cellulase. Galactose, lactose and glucose were found to be favourable carbon sources for bacterial growth and cellulase production. The culture supernatant containing the cellulase (26 kDa) was concentrated by membrane filtration (MWCO 10kDa) and was subsequently precipitated using ammonium sulphate. The enzyme showed significant activity in a broad range of temperature (30-70°C) with an optimum at 40°C and activity in the pH range of 4.0-11.0 with an optimum at pH 8.0. Metal ions such as Mg^{2+}, Ca^{2+}, Ni^{2+} and Fe^{2+} enhanced the enzyme activity. However, Hg^{+} and Ag^{+} completely inhibited the enzyme activity even at low concentration. Addition of detergents such as Tween-20, SDS and β-ME resulted in loss of activity, but CTAB (10 mM) did not have any inhibitory effect on cellulase activity. In addition to cellulose, the bacterium was found to be capable of using carboxy methyl cellulose and filter paper as alternate substrates and resulted in equivalent production of cellulase, revealing possible application in paper processing industries.

KEY WORDS
Cellulose, Cellulase, Bacillus aquimaris VITP4 strain, halotolerant, metal ions

INTRODUCTION
A large number of microorganisms are known to produce a wide range of enzymes. Among microorganisms, *Bacillus* species are shown to produce a variety of extracellular hydrolyzing enzymes (Sanchez-porro *et al.*, 2003). Fermentation techniques made the production of microbial enzymes easier and advantageous. Also, screening and optimization of fermentation conditions and selection of suitable substrate plays a vital role in the production of enzyme. Hydrolysis of cellulose to glucose needs the synergistic action of three types of enzymes; endoglucanase(1, 4 – ß – d – glucan – 4 - glucanohydrolase; EC 3 . 2 . 1 . 4), exocellobiohydrolase (1 , 4 – ß – d - glucan glucohydrolase; EC 3.2.1.74) and ß-glucosidase (ß-d-glucoside glucohydrolase; EC 3.2.1.21) (Maxim Kostylev and David Wilson, 2012). These are generally present as multimeric enzyme complexes called cellulosomes. Cellulases that convert cellulose into soluble sugar are in great demand in many industrial processes. *Trichoderma* and *Aspergillus* species are the best known source for the production of commercially available cellulase, but cellulase produced form bacteria has not been commercialised. Cellulases are widely used for dehairing of the animal hides,

bio stoning of denim fabrics, biopolishing of the fabrics, clarification of fruit juices, baking and deinking of paper, in the production of bio ethanol and to improve the digestibility of the animal feed (Ramesh Chander Kuhad, 2011)

Even though extensive research has been carried out on alkaline cellulases from alkalophilic *bacilli (Ito, 1997 ;* Yuichi Nogi *et al., 2005;* Alshelmani *et al., 2013)* , very few reports are available on alkaline cellulases from moderately halotolerant bacteria (Nitin Trivedi *et al*., 2011; Feng Yi *et al*., 2007; Soon Jin Kim *et al*., 2008). Halotolerant microorganisms have no specific requirement for salt, but can grow up to 3M NaCl (Ventosa *et al*., 1998) and the enzymes produced by these organisms can be of great use because of their salt tolerant property. Therefore, in the present study cellulase production by a moderately halotolerant bacterium *Bacillus aquimaris* VITP4 was investigated under various growth conditions and the properties of the produced cellulase were analysed.

MATERIALS AND METHODS

All analytical and media components were purchased from Hi-Media (Bombay, India)

Screening for cellulolytic activity

B. aquimaris VITP4 strain was screened for its cellulase activity by Congo red dye assay. The bacteria were grown on Marine agar plates 2216 (Zobell's marine agar) containing 1% (w/v) cellulose. The plates were incubated for 48 hrs to detect its cellulolytic activity. After incubation, the plates were flooded with 1% Congo red (w/v) for 20 minutes. Then the Congo red solution was decanted and destaining was carried out by flooding the plates with 1M NaCl for 15 minutes.

Bacterial growth kinetics and cellulase production

The growth and cellulase production from *B. aquimaris* VITP4 was studied by culturing the bacteria in 200 ml broth for a period of 96 h at 37°C in Zobell's media with an agitation rate of 150 rpm. The samples were withdrawn at 4h interval and the optical density at 600nm was used to monitor the bacterial growth. The halotolerant nature of the strain was studied by growing them at different concentrations of NaCl (0-15% v/v). The cellulase production was estimated by carrying out enzyme assay as described below. Protein content was determined by Lowry's method using Bovine Serum Albumin as the standard [Lowry *et al*., 1951). In order to investigate the effect of (initial) media pH on cellulase production, the pH of the media was initially adjusted to the required pH using either 0.1 M HCl or 0.1 M NaOH.

Enzyme activity

Cellulase activity was assayed by the procedure described by Miller (Miller, 1959). Accordingly, 500 µl of crude enzyme was added to 500µl of 1% (w/v) cellulose (50mM Tris buffer, pH 8.0). After 60 minutes of incubation the enzyme reaction, at 45°C, was terminated by adding 1ml of Dinitrosalicylic acid reagent. The solution was subsequently heated to 95 ºC. After 15 minutes, the solution was cooled to room temperature and 0.2 ml of Roschell's salt was added. The absorbance at 595 nm was then measured to estimate the amount of reducing sugar released. One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute. Enzyme activity was calculated using glucose standard graph as a reference in the concentration range of 200-1000µg/ml.

Effect of pH on cellulase production

The influence of pH on cellulase production was determined by growing VITP4 at different pH (6-11) at 37°C. After 72 h, cellulase production was quantified.

Effect of carbon and nitrogen sources on cellulase production

As cellulase is an inducible enzyme, various carbon (fructose, galactose, glucose, lactose, sucrose, starch, xylose, maltose and cellulose) and nitrogen sources (peptone, yeast extract, beef extract, ammonium nitrate, ammonium chloride sodium nitrate, ammonium sulphate and potassium nitrate) were used at a concentration of 0.5% (w/v) as a sole source of carbon in the culture media and enzyme production was monitored at regular intervals at 37°C.

Ammonium sulphate precipitation

To the cell free supernatant, ammonium sulphate was added to attain a saturation of 80 % (w/v) at 4°C and kept for overnight; the precipitate was collected by centrifuging the solution at 10,000xg for 20min. The pellet was dissolved in 50mM Tris buffer (pH 8.0). The solution was desalted by dialyzing against 50mM Tris buffer. Enzyme activity and protein content was estimated by following the above said methods.

Molecular weight determination

Molecular weight of the cellulase was determined by SDS-PAGE electrophoresis. Standard molecular weight markers viz, β-Galactosidase (116 kDa), Bovine Serum albumin (66 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (35 kDa), β-lactoglobulin (25 kDa), Lysozyme (18 kDa) medium range markers were used to estimate the molecular weight. Electrophoresis was performed according to the Laemmli method (Laemmli, 1970). The protein bands on the gel were stained with Coomassie Brilliant Blue R-250. Standard molecular weight markers were used to determine the molecular weight of the cellulase.

Influence of pH, temperature, NaCl and additives on cellulase activity

In order to determine the operational range of the purified enzyme, the enzyme along with the substrate was incubated under the following conditions: pH (4.0-11.0) with appropriate buffer system viz, citrate buffer (pH 4.0-6.0), Phosphate buffer (pH 6.5-7.5), Tris-HCl (pH 8.0-10.0) and Glycine-NaOH buffer (pH 10.0-12.0) and temperature (30-70°C). The effect of salt on cellulase activity was investigated by using different concentrations of NaCl (0-15%).

The effect of various metal ions and detergents on purified cellulase activity were determined by preincubating the enzyme with individual metal ions (Ca^{2+}, Mg^{2+},Fe^{2+},Zn^{2+},Ni^{2+} ,Hg^{2+}, Ag^{+},K^{+}) and detergents (CTAB, SDS,Tween-20) for an hour in 50mM Tris buffer (pH 8.0) at 37°C. Enzyme assay was carried to measure the residual activity. The activity assayed in the absence of metal ions or additives was recorded as control.

FPase activity:

To determine the filter paper digestive potential (FPase) of the enzyme, 50 mg of Whatman filter paper (cut into small pieces) was added to 2ml of 50 mM Tris buffer (pH 8.0). To this suspension, 2ml of crude enzyme was added and incubated at 40°C for 60min. After incubation the enzyme reaction was stopped by boiling the reaction mixture at 95°C for 15 min. The supernatant was then used to assay the enzyme activity.

RESULTS AND DISCUSSION

Screening for cellulase production

Bacillus aquimaris VITP4 strain, isolated from Head-Bunder Lake (Coastal saltern, Kumta coast, Karnataka, India), was shown to produce different industrially important enzymes (Pooja and Jayaraman, 2009; Pooja and Jayaraman, 2011; Anupama and Jayaraman, 2011; Thaz and Jayaraman, 2014). In addition to their moderate salt tolerance, the enzymes produced by this bacterium had also been shown to be active in a wide range of experimental conditions, including pH, temperature, metal ions and detergents. In continuation of our efforts to explore the applicability of this bacterium to produce other industrially important enzymes we embarked on

the production of cellulolytic enzymes. Initial experiments with agar plate (with 1% (w/v) Cellulose) revealed (Fig. 1) clear zones around the colonies indicating that the strain is capable of producing cellulolytic enzymes.

Fig. 1. Screening for cellulolytic activity of *B.aquimaris* VITP4 strain on Cellulose agar plate and clear zone around the colonies indicates the hydrolysis of cellulose.

Microbial growth and cellulase production

B.aquimaris VITP4 growth was studied in order to identify the suitable time period for cellulase production (Fig. 2). Cellulase production was not observed in the first four hours of growth and thereafter production increased significantly with a maximum growth at 72h (1.43±0.05 U/ml). The secretion of cellulase enzyme in the stationary phase can be compared with the other reports (Shankar, 2011; Bajaj *et al.*, 2009; Namita Bansal *et al.*, 2012) where the maximum enzyme production was obtained in the stationary phase corresponding to an incubation period of 72 h.

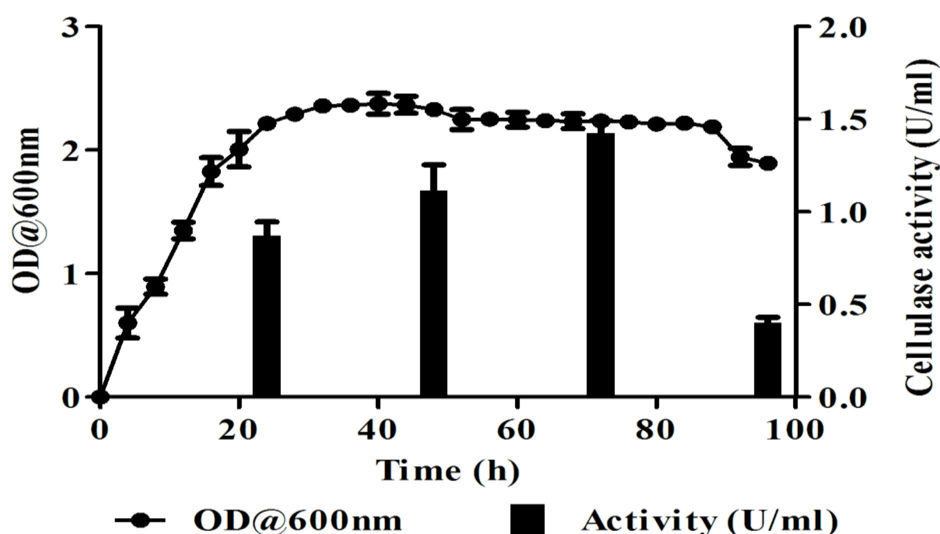

Fig. 2.Growth kinetics of *B.aquimaris* VITP4 strain in Zobell Marine broth with 1% cellulose. Samples were withdrawn at 4 h interval for the determination of cell growth by OD@600nm and cellulase production (U/ml).

Effect of pH on cellulase production

Figure 3 depicts the effect of media (Initial) pH on cellulase production. The bacterium could grow and produce extracellular cellulase in a wide range of pH (6.0 -11.0). Cellulase production was maximum at pH 8.0 indicating that pH has a direct effect on the uptake of mineral nutrients by bacteria from the medium. Similar effects were observed for the production of amylase and protease by this organism (Pooja and Jayaraman, 2009; Anupama and Jayaraman, 2011). Many *Bacillus sp.* were shown to produce cellulase maximally at pH 8.0 (Goyal Varsha *et al*, 2014; Mukesh Kumar *et al,* 2012; Acharya and Anitha, 2011).

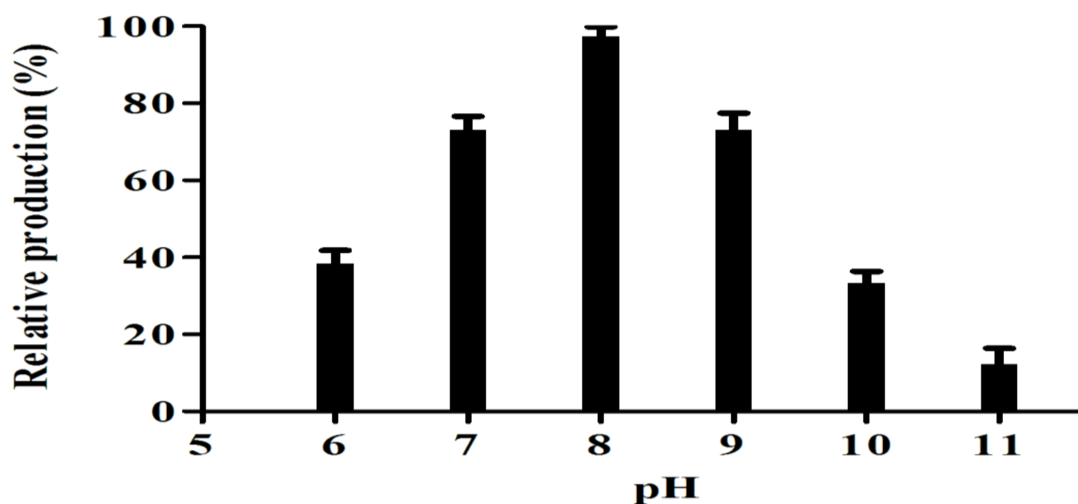

Fig. 3. Effect of medium pH on cellulase production. The *B. aquimaris* VITP4 grown in medium with different pH, samples were taken on 72 h of incubation and cellulase activity was determined. Production at pH 8 was taken as 100%.

Effect of NaCl concentration on cellulase production

Cellulase production by *Bacillus aquimaris* VITP4 was dependent on NaCl concentration [Fig. 4]. The bacterium was able to grow up to a salt concentration of 15% with an optimum growth at 5%. Even cellulase production was highest in the presence of 5% NaCl, indicating a clear one-to-one correlation between bacterial growth and enzyme production. Bacterial growth was observed even at 0% NaCl showing its non dependency on salt for its moderate growth, thus indicating the halotolerant nature of the bacterium. In general halotolerant bacteria growing in the presence of extracellular NaCl concentration will have a different phospholipid and fatty acid composition when compared to non-halophilic bacteria[7]. Halotolerant strain *Salinivibrio sp*. (Wang Chung-Yi *et al.*, 2009) reported a clear dependence on Na^+ ion for maximum enzyme activity and at 5% of NaCl concentration the activity was more, but enzyme was active over a range of 1-15% of NaCl and bacterium was able to grow even in the absence of NaCl.

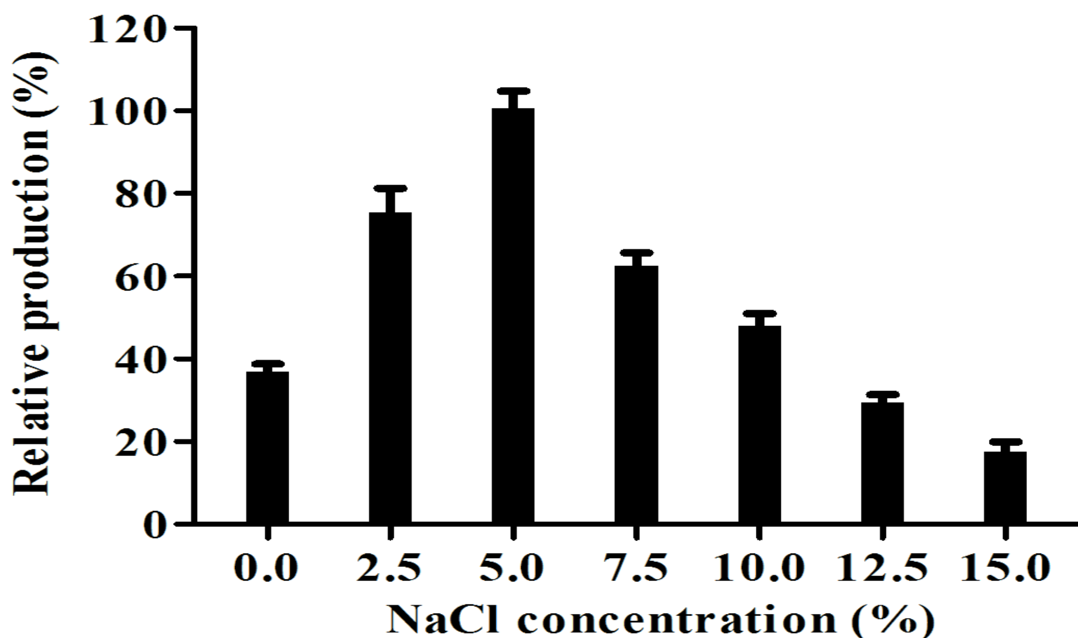

Fig. 4.The cellulase production from *B.aquimaris* VITP4 strain was studied after 72 h of incubation by growing the organism in media containing over a broad range of NaCl concentration (0-15%). The highest production at 5% NaCl concentration is taken as 100%.

Effect of nitrogen and carbon sources on cellulase production

It is well documented that addition of different nitrogen sources increase or decrease (Sreeja *et al*, 2013; Acharya and Anitha 2012; El-Shishtawy *et al*., 2014) cellulase production. In the present study, the enzyme production was affected significantly under different concentration of the organic and inorganic nitrogen sources (Table 1). The inclusion of inorganic nitrogen sources was found to be more influential in supporting the cellulase production by *B. aquimaris* VITP4 strain. Among the different nitrogen sources used, NH_4Cl (1.22±0.04) followed by NH_4NO_3 (1.17±0.04) gave maximum cellulase production. *Bacillus* sp. E13 was shown to result in maximum enzyme production with inorganic nitrogen sources such as NH_4Cl and NH_4NO_3 (Sadhu Sangrila *et al*., 2013) and increased production with NH_4NO_3 was reported in *B. pumilus* EWBCM1 (Shankar, 2011) and *Bacillus* sp. (MTCC10046) (Sadhu Sangrila *et al*., 2013). Among the organic nitrogen sources, cellulase production was maximum with beef extract (1.11±0.02 U/ml) followed by yeast extract (1.01± 0.03 U/ml). Yeast extract was found to be the best nitrogen source for cellulase production in *Cellulomonas cellulans (*Sugumaran *et al*., 2013) and *Bacillus* sp. MPTK 121 (Mukesh Kumar *et al*., 2012).

Table 1.Effect of nitrogen and carbon sources on cellulase production (U/ml) by *B.aquimaris* VITP4 strain. Control was Zobell's marine broth and highest production in the control medium was taken as 100%. Different nitrogen and carbon sources were added at a concentration of 0.5% to the media and were incubated under the shaking conditions of 150 rpm for 72 hours and the supernatant was used as a crude enzyme to calculate the cellulase activity.

Nitrogen / Carbon source (0.5%, w/v)	Cellulase activity (U/ml)
Control	1.38±0.05
Peptone	0.98± 0.03
Yeast extract	1.01 ±0.03
Beef extract	1.11 ±0.02
Ammonium nitrate	1.17 ±0.04
Ammonium chloride	1.22±0.04
Sodium nitrate	0.71 ±0.02
Ammonium sulphate	0.90 ±0.01
Potassium nitrate	0.93 ±0.01
Fructose	0.71±0.04
Galactose	1.03±0.07
Glucose	0.85±0.05
Lactose	0.89±0.02
Sucrose	0.74±0.02
Starch	0.82±0.03
Xylose	0.60±0.02
Maltose	0.81±0.01
Cellulose	0.72±0.07

Among the various carbon sources used, galactose (1.03±0.07) gave the maximum cellulase production. Similar results have been reported for several *Bacillu*s species. *Bacillus brevis* isolated from Indore (India), was found to secrete five times more cellulase on addition of galactose in the culture medium (Singh and Anil Kumar, 2008). Supplementation of galactose, mannose and glucose in media resulted good growth and cellulase production in *B .pumilus* EWBCM1 (Shankar , 2011). Yan *et al*., reported that the soluble carbons sources with reducing groups such glucose, fructose, galactose could induce cellulase production and readily available carbon sources will support the bacterial growth and more cellulase production can be observed in the stationary phase (Hong Yan, 2013).

Influence of pH, temperature and NaCl on cellulase activity

Cellulase activity was observed in the pH ranging from 4.0 - 11.0 with optimal activity at pH 8.0 (Fig. 5) and the enzyme was able to retain more than 60% activity in the pH range of 6.0 to 9.0. Figure 6 shows the activity profile of cellulase enzyme at different temperatures. Optimum activity was obtained at 40°C and the same optimum temperature was reported by many workers. Beyond 50ºC there is sudden decrease in the activity of the enzyme probably indicating inactivation of the enzyme due to thermal denaturation. A number of enzymes with pH and

temperature optima of 8.0 and 40°C were reported previously (Nasir *et al*., 2011). Among the different NaCl concentration tested (0-15%), the maximum enzyme activity was observed at 5% NaCl (Fig. 7).

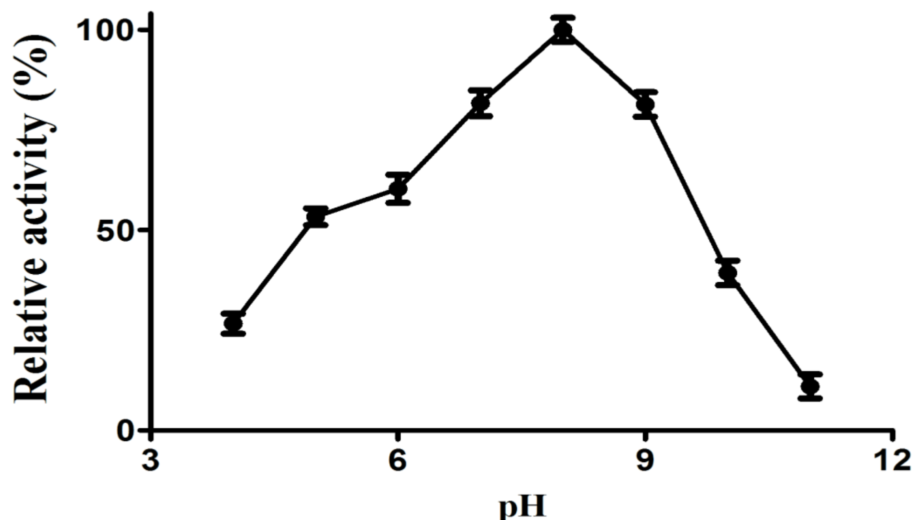

Fig. 5. Effect of pH on cellulase activity. The enzyme activity gradually increased with the increasing pH and showed optimum activity at pH 8.0 and there was a decline in the activity on further increase in pH.

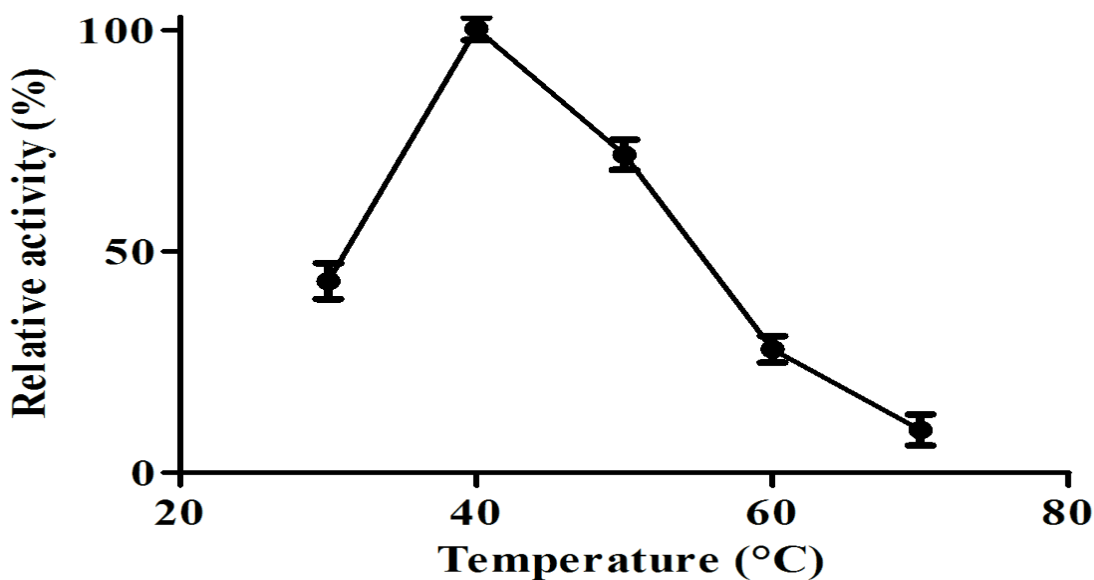

Fig. 6.Effect of Temperature on cellulase activity. The incubation of enzyme at different temperature showed that the optimum temperature for cellulase activity is 40°C. A further increase in temperature resulted in decline in activity.

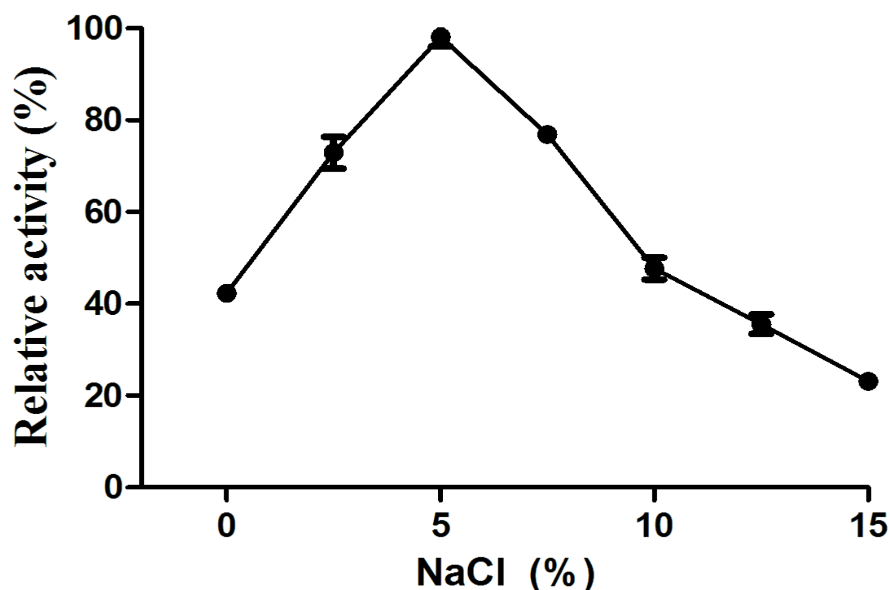

Fig. 7. Effect of NaCl on cellulase activity. Cellulase showed activity over a broad range of salt concentration ranging from 0-15% with optimum obtained at 5%. At 15% NaCl concentration the enzyme was able to retain more than 20% of activity.

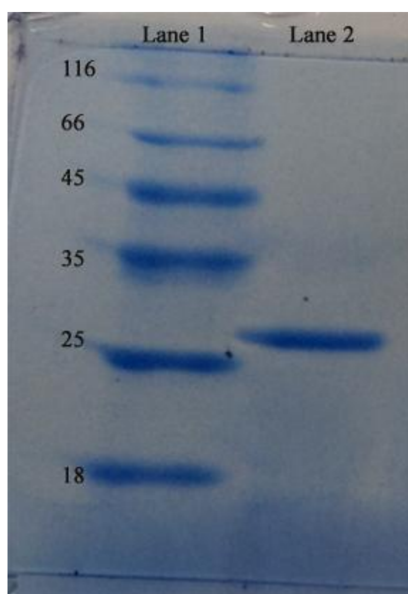

Fig. 8.SDS-PAGE (15%) analysis of purified cellulase from *B.aquimaris* VITP4 strain. Lane 1 Molecular weight markers, Lane 2: purified cellulase. Molecular mass value is approximately 26kDa.

Enzyme concentration by membrane filtration and electrophoresis

The culture supernatant was subjected to membrane filtration using 10 kDa membrane filter (Amicon) so that the volume was reduced from 50 ml to 4 ml with an increase in specific activity from 0.79 to 2.15 mg/ml. SDS-PAGE was performed to obtain the molecular weight of the VITP4 cellulase. The obtained pellet was air dried and dissolved in 50mM of Tris buffer and SDS-

PAGE was performed. A single band was observed on 15% SDS-PAGE corresponding to a molecular weight of approximately 26 kDa (Fig. 8).

Effect of additives on enzyme activity

Enzyme assay was performed in presence of metal ions (Ca^{2+}, Mg^{2+}, Hg^{2+}, K^{+}, Ni^{2+} and Fe^{2+}) and detergents (Tween 20, ß-ME, SDS and CTAB) and the results are given in Table 2. Presence of 5 mM Mg^{2+} enhanced the enzyme activity by 15% and increased to 46% at higher concentration (10mM). Additionally, Ca^{2+} enhanced the activity by 6% at 5mM and to 33% at 10mM. Presence of even Ni^{2+} and Fe^{2+} enhanced the cellulase activity. The positive effect of Mg^{2+} on cellulase activity was reported in *Bacillus* sp. MPTK 121(Mukesh *et al*., 2012) and Fe^{2+}, Mg^{2+} and Ca^{2+} caused enhancement of cellulase activity produced by *Bacillus pumilus (*Gomaa and Eman Zakaria, 2013). Zn^{2+} ions were found to inhibit the enzyme activity at higher concentration whereas Hg^{2+} ions completely inhibited the activity. The presence of SDS and Tween resulted in considerable loss of enzyme activity. However, partial activity was retained in the presence of higher concentration of SDS indicating the utility of this enzyme in industrial processes demanding the presence of the anionic detergents.

Table 2.Effect of metal ions and additives on Cellulase activity. The addition of Mg^{2+}, Ca^{2+}, Ni^{2+} and Fe^{2+}, to the supernatant before hydrolysis of cellulose resulted an increase in the amount of reducing sugar liberated. An increase in the concentration of CTAB has increased the cellulase activity, but an increase in the concentration of Tween-20, SDS and ß-ME shown inhibition on activity of cellulase.

	Relative activity (%)	
Additive (w/v)	**5 mM**	**10 mM**
Control	1.32±0.06	1.32±0.06
CaCl2	1.41±0.08	1.76±0.09
MgCl2	1.53±0.11	1.94±0.10
FeCl2	1.75±0.14	1.48±0.07
ZnCl2	0.85±0.06	0.51±0.05
NiCl2	1.47±0.09	1.69±0.09
HgCl2	0	0
AgCl2	0	0
SDS	1.13±1.05	0.95±0.07
CTAB	1.22±0.08	1.42±0.06
β -ME	1.20±0.07	1.04±0.07

Table 3.Effect of different substrates on the production of cellulose, CMCase and FPase enzymes by VITP4 strain.

Substrate	Activity (U/ml)		
	Cellulase	CMCase	FPase
Cellulose	1.34	1.27	0.39
CMC	1.31	1.58	0.24
Cellobiose	0.19	0.29	0.09

Carboxyl methyl cellulose and filter paper as substrates

With an aim to explore the substrate range for the production of cellulase, *B.aquimaris* VITP4 strain was cultured with different substrates. The cellulase, carboxyl methyl cellulase (CMCase) and filter paper cellulase (FPC) activities shown by the extracellular enzymes produced by the bacterium with the different substrates (Cellulose, CMC and Cellobiose) are given in Table no. 3. The results support the fact that type of substrate will have a profound effect on secretion of a particular set of enzymes. It has been reported that depending on the sole carbon source available and ability to utilize them, the Cellulase, CMCase and FPC activities vary from strain to strain (Antonella Amore, 2013). The results, however, indicate that the bacterium could effectively grow in the given range of substrates, indicating wider applicability.

CONCLUSION

The present study was aimed to investigate the cellulase produced by *B.aquimaris* VITP4 strain. The parameters, which resulted in the maximal production of the extracellular cellulase by the bacterium, are 2% cellulose, ammonium chloride, galactose, 5% NaCl and pH 8.0. Also the enzyme produced displayed maximal activity in the presence of Mg^{2+}, 5% NaCl, pH 8.0 and a temperature of 40 ºC. In spite of these conditions for maximal activity, the enzyme was active over a wide range of experimental conditions, indicates its broader application range. This was also supported by the fact that the bacterium could utilize cellulose, carboxy methyl cellulose or cellobiose to display the corresponding cellulase, CMCase and FPC activity. This is the first report on the cellulase production and its characterization, from an industrial perspective, by a halotolerant bacterium *B. aquimaris*.

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Conflict of interest: We have no conflict of interest with this publication.

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