Biocatalysis and Agricultural Biotechnology 17 (2019) 605–612

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Characterization of partially purified alkaline protease secreted by halophilic bacterium *Citricoccus* sp. isolated from agricultural soil of northern India

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ARTICLE INFO

Keywords:
16S rRNA gene
Citricoccus
Protease
Detergent compatibility

ABSTRACT

The present study reports the isolation of protease producing bacteria Bact2 from agricultural soil of Regional Centre of Soil Salinity Research Institute, Lucknow, India. It was identified as *Citricoccus* sp. (KC522120.1) by 16S rRNA gene sequencing. The optimum pH and temperature for maximum enzyme production was observed to be at 10 and 40 °C respectively. The enzyme protease was partially purified and recovery of enzyme was 77.16%. A 1.66 fold increase in enzyme activity was also observed in comparison to original. After purification, the partially purified enzyme (PPE) was characterized. The optimum pH and temperature of partially purified enzyme (PPE) activity was observed to be at 10 and 40 °C respectively. The activity of PPE was enhanced in presence of Ca^{2+} and Mg^{2+} while it was inhibited by Cu^{++} and Na^{+}. The PPE was completely inhibited by Phenyl methyl sulphonyl fluoride (PMSF) indicating that it belongs to serine protease group. Finally, enzyme was found to be compatible with detergents and effective in removing stains of tea and ink indicating it's application in detergent industry. Besides, to our knowledge till date production of alkaline protease from *Citricoccus* sp. has been reported first time in the present manuscript.

1. Introduction

The chemical biocatalysts used in industrial processes could be replaced by enzymes to increase efficiency and ensuring ecological and economic sustainability of the process. Proteases are the most dominant group of enzyme constituting sixty percent of the entire enzyme industry (Sharma et al., 2017) and hydrolyze peptide bond between protein with paramount application in industrial as well as pharmaceutical sector (Lakshmi et al., 2018). They have wide applications across various industries such as detergent, food, bakery, leather, infant formulas etc. due to their attractive features like ease in production, thermo tolerance and ability to perform at varied pH range (Genckal and Tari, 2006). The commercial proteases available in market are mostly extracted from *Bacillus* strains due to their stability at high temperature and pH (Ibrahim et al., 2015; Al-Hakim et al., 2018).

A large percentage of these enzymes are used widely in many industries especially in detergent industry which constitute single largest market for enzyme at twenty to thirty percent of total sales (Haddar et al., 2009). Enzymes used in detergent should be able to resist harsh situations like surfactants, variation in pH and temperature (Verma et al., 2011a, 2011b; Waghmare et al., 2015). A large number of microorganisms have been reported to produce alkaline proteases under various physiochemical and nutritional conditions e.g *Bacillus, Micrococcus, Pseudomonas and Streptomyces* etc in last twenty years (Hashem et al., 2015; Khajuria et al., 2015). Still, there is always a need for novel microbe producing proteases with characteristics matching the need of industries. The proteases produced by microbes present in natural environment are usually unstable under extreme conditions, despite several attempts of physicochemical treatments, protein engineering and gene-shuffling methods. In this context, main focus has been given to extremophiles known for producing enzymes steady at extreme physicochemical conditions leading to denaturation of enzymes produced by their natural counterparts. Actually, limited reports are available describing the alkaline proteases isolated from halophilic and halotolerant microorganisms (Thebti et al., 2016; Ahmed et al., 2016) and only few of them have been reported further for their potential use in detergent industry. From last two decades, alkaline protease was purified and characterized by several microorganisms *viz., Bacillus* (Ibrahim et al., 2015), *Micrococcus, Staphylococcus* (Kumari, 2014), Streptomyces (Al-Askar et al., 2015). Therefore, there is a continuous need to search for

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https://doi.org/10.1016/j.bcab.2019.01.020
Received 16 October 2018; Received in revised form 21 December 2018; Accepted 11 January 2019
Available online 14 January 2019
1878-8181/

novel and better strains of microorganisms that produce alkaline protease in higher titers with better characteristics. Although the genus *Citricoccus* was reported for the first time by Alterburger et al. (2002) *viz., Citrococcus murelis* followed by another species of *Citricoccus alkalitolerant* by Li et al. (2005). Both species were found positive for lipase and glucosidase activities, there is no report available to our knowledge describing protease production any *Citricoccus* sp. Hence, we launched a screening programme for halotolerant bacteria isolated from saline agricultural soil for production of extracellular proteases. The bacterium Bact2 showing highest production of proteases was selected for further studies. The present work describes the identification of the isolate and characterization of the interesting biochemical properties of its alkaline protease making it a very potential candidate for it's application in detergent industry. To our knowledge, this is the first report stating the production of alkaline proteases from *Citricoccus* species.

2. Material and methods

2.1. Collection of soil sample

The soil sample was collected from agricultural field located near Regional Centre of Soil Salinity Research Institute, Lucknow (U.P.), India situated at 120 m above mean sea level and extended from 26° 47 ft 45 in. to 26° 46 ft 13 in latitude and 18° 46 ft 7 in to 80° 46 ft 32 in longitude. The soil samples were collected and transported immediately to the laboratory for further processing.

2.2. Isolation and screening of bacteria producing alkaline protease

The bacterial isolates were isolated by serially diluting the sample on alkaline agar media containing 1% glucose, 0.5% peptone, 0.5% yeast extract, 0.1%KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 1% Na_2CO_3 and 1.5% agar. The plates were incubated at 30 °C for 24–48 h. The production of protease enzyme was further screened on skimmed milk agar media composed of 10% skimmed milk, 1% yeast extract, 1.5% Na_2CO_3 and 2% agar and incubated at 30 °C for 24–72 h (Ibrahim et al., 2007). Strains revealing zone of hydrolysis around their colonies were considered as protease producers. The selected isolates were cultivated in 100 ml sterile alkaline broth medium and incubated at 30 °C on rotary shaker for 24–72 h under 200 rpm and finally culture was centrifuged at 10,000*g*. The enzyme activity was monitored in cell free culture supernatant using 1% casein as a substrate in 50 mM Sodium phosphate buffer (pH 8.0), 1 M Glycine-NaOH buffer (pH 10.0) separately. The activity of enzyme was monitored at regular intervals (24 h) upto 7 days. Since highest enzyme production was observed in 50 mM Sodium phosphate buffer (pH 8.0) hence same buffer was used for further experimentation. The bacterium Bact2 indicating highest alkaline protease production was then selected for further analysis.

2.3. Effect of salt on bacterial growth

The isolated bacterial strain Bact2 was evaluated by observing the growth on alkaline agar medium amended with various concentration of NaCl (2%, 5%, 7%, 9%, 11%). The control plates were also maintained at 0.05% NaCl. The plates were incubated at 30 °C for 24–48 h and growth on NaCl amended plates were compared with control plates.

2.4. Identification and biochemical characterization of bacterial isolate Bact2

The morphological characterization of bacterial isolate was determined by Bergey's manual of bacteriology (Sneath et al., 1986). Utilization of carbon source, hydrolysis of starch, gelatin and casein were determined according to Kasmpfer et al. (1991). The test for oxidase, catalase, nitrate reduction, indole production, methyl red, Voges-proskauer and urease activity was performed as described by Smibert and Krieg (1994). The molecular identification was based on 16S rRNA gene analysis. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers 5-GAGAGTT TGATCCTGGCTGGCTCAG-3 and 5-AAGGAGGTGATCCAGCCGCA-3 undergoing 35 cycles of the following steps: 94 °C for 30 s denaturation, 52 °C for 30s primer annealing and 72 °C for 1.5 min extension (Cheng et al., 2010). The obtained sequence was subjected to BLAST analysis against 16 S rRNA gene database in NCBI (National Centre for Biotechnology Information) and also reconfirmed by subjecting the deduced sequence to ribosomal database project (RDP) release 10 tool. Further we selected different Citricoccus strains from NCBI and constructed a phylogeny on basis of the 16S rRNA gene using MEGA 7 software.

2.5. Protease assay

The assay of enzyme was performed by using substrate casein 1% (Merck, Darmstadt, Germany) in 50 mM sodium phosphate buffer (pH 8.0) as modified method of Tsuchida et al. (1986). Enzyme solution was diluted in 50 mM Sodium phosphate buffer and equal volume of casein and enzyme solution (0.5 ml each) was mixed and incubated at 45 °C for 15 min consequently the reaction was accomplished by addition of 0.5 ml of trichloroacetic acid (5%). After that, the mixture was kept at room temperature and centrifuged at 10,000 rpm for 15 min at 4 °C. Then addition of 5 ml of 0.4 M Na_2CO_3 and 1 ml of (1:1) fold diluted folin ciocalteau reagent. The absorbance of reaction mixture was calculated in spectrophotometer at 660 nm. One unit of protease was calculated as amount of protease utilized in liberating 1 µg of tyrosine per minute under experimental condition.

2.6. Protein quantification

The amount of protein was quantified by using two different methods first by analyzing the absorbance at 280 nm in nanodrop spectrophotometer and second by Lowry method (Lowry et al., 1951). Bovine serum albumin was used as a standard.

2.7. Scanning electron microscopy

The isolated bacterial strain were prepared by fixing it with primary fixative (2.5% glutaradehyde/Karnovsky's for 2–6 h at 4 °C) followed by washing with 0.1 M phosphate buffer for 3 times each for 15 min at 4 °C. The post fixation was done by 1% osmium tetroxide for 2 h at 4 °C, followed by dehydration by using acetone gradient (30–95%) in dryer. After dehydration, the Bact2 was loaded into a Critical point drying (CPD) apparatus and dried with liquid carbon dioxide as transition fluid. The final coating was done in sputter coating apparatus under a low pressure argon atmosphere with a thin film of platinum (10–20 nm thick) (Richard and Wilson, 1993). The coated Bact2 strain was then observed under scanning electron microscope (Jeol 6490 LB, Japan).

2.8. Optimization of culture conditions for maximum protease production

2.8.1. Influence of pH and temperature

The production of protease was optimized by incubating the bacterial culture at temperature range 20–70 °C and pH 6–12 for 72 h. The O.D of bacterial culture was centrifuged at 10,000 rpm for 10 min and supernatant was used for enzyme assay.

2.8.2. Influence of carbon source

Bacterial culture was grown for 72 h at 40 °C with different carbon source in alkaline broth to examine the effect of source of carbon on protease production. Mono*saccharides* (Glucose and galactose), di*saccharides* (maltose, lactose and sucrose) and poly*saccharides* (starch) all were used separately as carbon source (1% w/v). The bacterial culture was centrifuged at 10,000 rpm for 10 min and supernatant was used for enzyme assay.

2.8.3. Influence of nitrogen source

The different nitrogen sources like glycine, casein, beef extract, yeast extract, peptone and tryptone (1% w/v) were used to estimate protease production. The bacterial culture was centrifuged at 10,000 rpm for 10 min and supernatant was used for enzyme assay.

2.8.4. Partial purification of protease enzyme

For the Partial purification, Bact2 isolate was cultured in alkaline broth medium (Horikoshii, 1990) and incubated at optimum temperature for 24–72 h. Further bacterial cell was removed by centrifugation in SS34 tube at 10,000 rpm for 10 min. Obtained supernatant was used as crude enzyme and precipitated by ammonium sulphate (80%). The partially purified enzyme was subjected to DEAE-cellulose column using ion exchange chromatography to obtain purified enzyme. After electrophoresis gel was stained with Coomassie Brilliant blue and visualized under light. The molecular weight of partially purified enzyme was determined by SDS-PAGE (Upadhyay and Chandrashekar, 2012). The partial purified enzyme was used for further physiological characterization of enzyme.

2.9. Effect of physiochemical/nutritional factors on protease activity

2.9.1. Effect of pH and temperature on protease activity

The enzyme was incubated at different pH (7.0–12.0) and different temperature (20–70 °C) for 1 h and the residual activity of the enzyme was measured under standard assay condition. A blank reaction without enzyme was also performed with each reaction (Cui et al., 2015).

2.9.2. Effect of metal ions on protease activity

In order to determine the enzyme activity, the enzyme was pre-incubating at 40 °C in the presence of different metal ions (NaCl, $CuCl_2$, $MgCl_2$ and $CaCl_2$) in different composition (0.5–15 mM) for 1 h. The residual activity of the enzyme was measured under standard assay condition (Cui et al., 2015).

2.9.3. Effect of different inhibitors on protease activity

The different protease inhibitors in concentration of 5 mM and 10 mM (PMSF), divalent chelator (EDTA) were pre-incubated with enzyme for 30 min at 40 °C before adding the substrate (Table 1). The residual protease activity was measured under standard assay condition (Adinarayana et al., 2003).

2.9.4. Effect of different detergents and surfactants on protease activity

The different surfactants (SDS, Tween 20, Tritox X 100) and different detergents (Surf excel, Ariel, Tide, Rin, Wheel, and Nirma) were used for studying the compatibility of alkaline protease isolated from *Citricoccus* sp. The various detergent (7 mg/ml concentration) and surfactant solutions (5%) were dissolved in distilled water as described by Banerjee et al., 1999. The protease enzyme in detergents was inactivated by incubating the detergents solution at 65 °C for 1 h after that *Citricoccus* protease enzyme was added in detergent solution at 40 °C for 1 h (Banerjee et al., 1999; Singh et al., 2001) and residual protease activity was measured under standard assay condition. The control sample (without detergents) was taken as 100%.

2.10. Detergent compatibility assay

The efficiency of alkaline protease enzyme was estimated with different detergents and surfactants. So we optimized an experiment onto four white muslin cloths pieces which were cut into 3 × 3 square and soaked with tea ingredient (tea with milk) and ink solution (1%) and dried for 2–3 h. After that the cloth pieces incubation at 40 °C in four different conical flasks containing (A) only water as control, (B) 2% Ariel detergent powder, (C) 2% Ariel detergents having 250 ml of alkaline protease enzyme and (D) 2% Ariel detergent was having 500 ml alkaline protease. After 1 h cloth pieces were washed, dried and observed the enzyme reaction. The cloth was washed and dried for 1 h.

2.11. Statistical analysis

All data were checked under means ± SD analysis of variance and statistically significant as three replicate used. The p value refers significant differences at $p < 0.05$.

3. Results and discussion

3.1. Isolation, identification and biochemical characterization of strain Bact2

About 115 isolates were isolated and screened for alkaline protease production. Bact2 strain exhibiting highest enzyme production was selected for further studies. Morphological and biochemical characteristics of the isolate revealed that it is cocci shaped, motile Gram +ve, non-spore forming and catalase positive (Table 1). Besides, it hydrolyzed various carbohydrates and ferment several sugars. Based on Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986), Bact2 belonged to the genus *Citricoccus sp.*

The results also revealed that the size of bacterial isolate was of moderate size. The color of the colony of the bacterial isolate (Bact2) was pale yellow and shape was circular with entire margin and convex elevation (Fig. 1). The isolated bacterial strain was aerobic and Gram positive. Strain bact2 grew well in the presence of 5–7% (w/v) NaCl and moderately in 7% (w/v) NaCl. No growth was observed in the presence of 9–11% (w/v) NaCl (Alterburger et al., 2002). The pH range for growth was observed between pH 9–11. In carbon utilization, Casein, gelatin showed positive while citrate and starch was determined negative. In biochemical analysis, catalase, oxidase showed positive while indole production, methyl red, Voges-proskauer, urease activity was determined negative and nitrate was not reduced to nitrite (Table 2). The molecular identification based on 16S rRNA gene (KC522120.1 accession number) revealed 66% similarity with *Citricoccus* sp 1 having Accession number JF274865.1 (Fig. 2). The identified sequence of Bact2 was submitted to NCBI and accession number KC522120.1. The isolated bacterial strain Bact2 was identified by using 16S rRNA gene analysis. Our previous study has reported that Bact2 formed a different branch from other isolates of alkaline protease producing bacteria (Saxena et al., 2014). In contrast, we selected a phylogeny between different isolates of *Citricoccus* sp. from NCBI and it was revealed that Bact2 was 66% similar to JF274865 and denoted as monophyletic origin whereas other isolates of *Citricoccus* sp belonged to polyphyletic origin. A similar study was reported by Saggu and Mishra (2017) in the case of *Bacillus infantis* SKS1.

Table 1
Different Inhibitors/surfactants used to assess their effect on protease activity.

S.No.	Inhibitors	Inhibitor concentrations (mM)	Surfactants	Surfactant concentrations (%)
1	PMSF	5.0–10	SDS	5.0
2	EDTA	5.0–10	Triton X – 100	5.0
			Tween 20	5.0

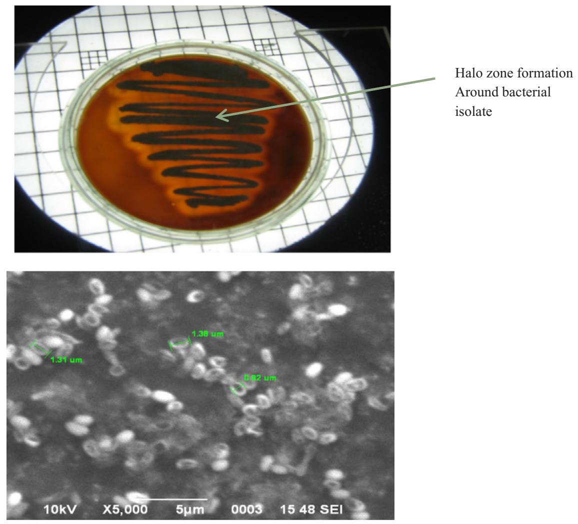

Fig. 1. Screening of Bact2 strain on skimmed milk agar plate showing translucent zone around colony and picture of Scanning electron micophotograph.

Table 2
Morphological and biochemical characterization of Bact2.

Test	Result
Gram staining	Gram positive/cocci
Endospore staining	spore forming
Motility test	(+)
Indole production	(-)
Methyl red test	(-)
Voges-proskauer test	(-)
Citrate utilization test	(-)
Starch hydrolysis	(-)
Gelatin hydrolysis	(+)
Casein hydrolysis	(+)
Urease test	(-)
Oxidase test	(+)
Catalase test	(+)
Nitrate utilization test	(-)

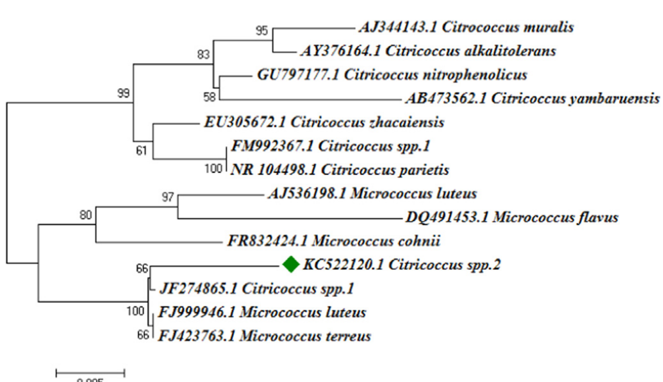

Fig. 2. Phylogenetic analysis of *Citricoccus* sp, on the basis of 16S rRNA gene analysis.

3.2. Effect of physiochemical / nutritional factors on protease production

3.2.1. Influence of pH and temperature on protease production

The optimum pH for protease production in Bact2 was observed to be 10 ((Fig. 3a). The results indicated that an increment in the pH above 10.0, a potential decline in protease production. The optimum temperature for protease production was 40 °C (Fig. 3b). The pH and temperature plays a significant role in production of enzymes by microorganisms. The pH for optimum enzyme production was found to be 10.

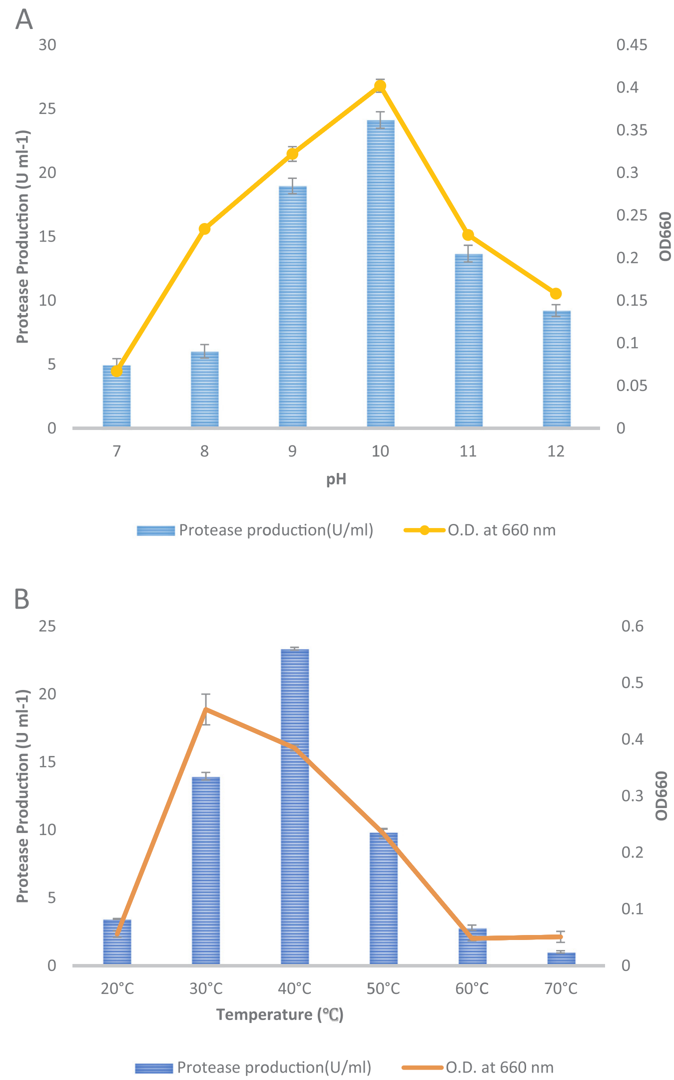

Fig. 3. a: Effect of pH on protease production by Bact2 strain. Standard error ± 2.00% $p < 0.022$. The p value refers to the comparison of protease activity at different pH. n value is an average of three independent observations. All the comparisons are statistically significant. **b:** Effect of temperature on protease production by Bact2. Standard error ± 2.50%$p < 0.025$. The p value refers to the comparisons of protease activity at different temperature. n value is an average of three independent observations and statistically significant.

This result was in conformation with Tiwari et al. (2015); Verma et al. (2011b), who have reported that pH 10 is ideal for enzyme production.

Apart from this pH between 9 and 10 indicates its alkalophilic nature. The protease production ability of the isolate was evaluated between the temperature range 20–70 °C. The maximum production of enzyme was observed at 40 °C whereas a decline in enzyme production observed at temperature beyond 60 °C indicating the thermotolerant nature of the isolate Bact2.

3.2.2. Influence of different carbon and nitrogen sources on protease production

The effect of different carbon sources such as galactose, lactose, maltose, sucrose and starch were used to replace glucose which was the original carbon source in growth media. As shown in the Fig. 4a maximum protease production (26.87 U/ml) was found with lactose followed by glucose (23.31 U/ml), maltose (21.80 U/ml), galactose (11.16 U/ml) and sucrose (5.36 U/ml). However, least enzyme production was found with starch (5.02 U/ml) (Fig. 4a). Results revealed

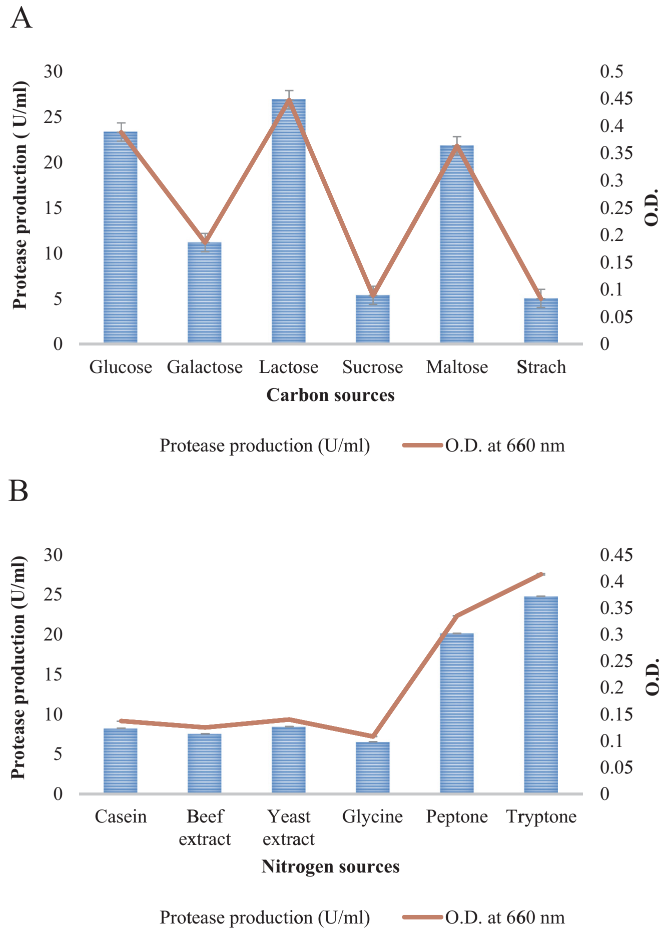

Fig. 4. a Effect of different carbon sources on protease production by Bact2 at pH 10 and temperature 40 °C. Average of three independents experiments. Standard error ± 2.20% $p < 0.002$. The p value refers to the comparison of protease at 1% glucose with other carbon sources. All the comparisons are statistically significant. **b** Effect of different nitrogen sources on protease production by Bact2 at pH 10 and temperature 40 °C. Average of the three independent experiments. Standard error ± 1.20% $p < 0.025$. The p value refers to the comparison of protease activity at 1% yeast extract with other nitrogen sources. All the comparisons are statistically significant.

that bacterial cells very efficiently produced alkaline protease when growth media was supplemented with tryptone (24.78 U/ml) as a nitrogen source in Bact2 strain followed by peptone (20.12 U/ml). Protease production was moderately inhibited in the existence of glycine (6.53 U/ml) and casein (8.24 U/ml) while it was severely declined in the presence of yeast extract (8.04 U/ml) and beef extract (7.54 U/ml, Fig. 4b). The result obtained indicated a high level of repression when inorganic nitrogen sources (ammonium salts) were used to replace the original nitrogen sources in the growth media. The present study reports that lactose and tryptone enhances enzyme production under standard conditions whereas glucose, sucrose and maltose repressed enzyme production indicating catabolite suppression of protein synthesis (Deng et al., 2010). In the previous study Ahmetoglu et al. (2015) reported that lactose is the best carbon source for protease production

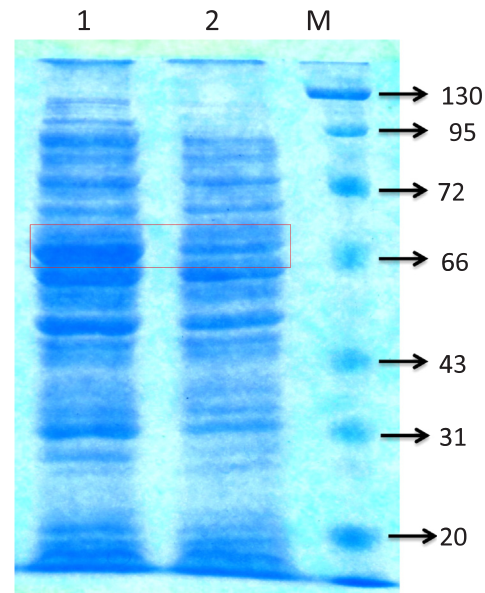

Fig. 5. Analysis of partially purified enzyme isolated from *Citricoccus* sp. on 12% Sodium Dodecyl polyacrylamide gel electrophoresis. Lane M- Protein marker, Lane 1 PPE and Lane 2 DEAE-Cellulose ion exchange chromatography.

while tryptone and peptone inhibited the enzyme production in *Bacillus* species KG5. Recently Kumar et al. (2014a, 2014b) reported that use of lactose as carbon sources gave maximum protease production comparison to others. Similarly, *Bacillus lichenformis* BBRC100053 indicated greater productivity of protease in the presence of lactose in culture media (Ghobadi et al., 2010). On Contrary combination of yeast extract and peptone was found best for protease production by *Bacillus sp* MA6 (Azad and Hoq, 2000) as organic nitrogen sources while inorganic nitrogen sources viz., ammonium chloride, ammonium sulphate suppressed the protease production (Al-Hakim et al., 2018).

3.3. Partial purification of alkaline protease

Alkaline protease produced by Bact2 strain was precipitated at 80% ammonium sulphate which was further purified by DEAE-cellulose ion exchange chromatography. The recovery of protease enzyme was found 77.16% and 1.66 fold increases in protease activity as comparison to original. The purity of enzyme increased upto 1.75 fold with 45.97% yield when enzyme was purified by ion exchange chromatography (Table 3). SDS-PAGE analysis of partially purified *Citricoccus* sp. Bact2 enzyme was run on 12% polyacrylamide (Fig. 5).

3.4. Influence of pH and temperature on protease activity

The effect of pH (7.0–12.0) on activity of the partially purified enzyme revealed that the activity of enzyme was totally dependent on the pH of the medium. A low enzyme activity was revealed at pH below 7.0 but it increased sharply as pH shifted towards the basic range. The optimum activity was observed at pH 10.0 (Fig. 6a). The pH stability of the enzyme is higher than other recently reported proteases (Annamalaia et al., 2014; Waghmare et al., 2015). The activity and stability of enzymes at higher pH signifies its importance in the laundry

Table 3
Partial purification of protease from Bact2 strain.

Purification steps	Activity (U)	Protein (mg)	Specific Activity (U/mg)	Purification Factor	% Recovery
Crude	23.21	3.17	7.32	1.00	100
Ammonium sulphate ppt.	17.91	1.47	12.18	1.66	77.16
DEAE-cellulose Ion-exchange chromatography	10.67	0.83	12.85	1.75	45.97

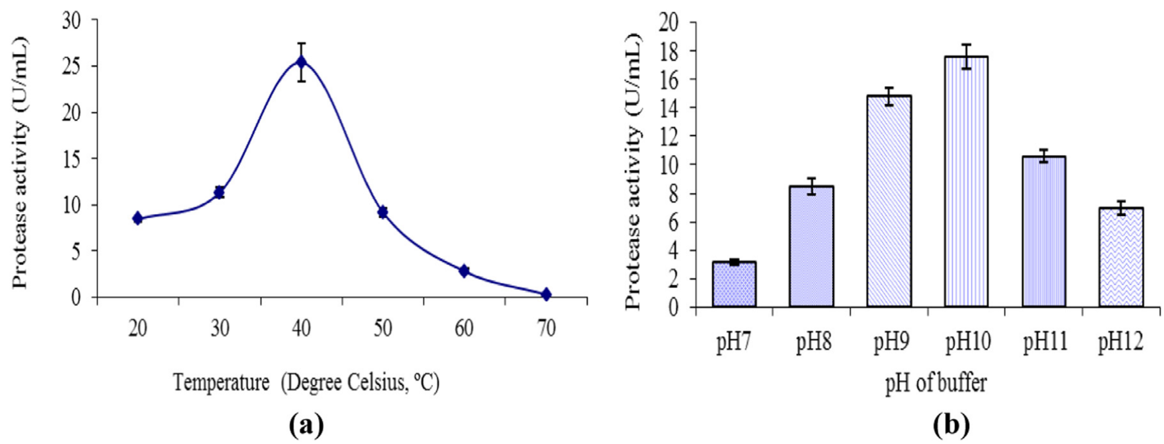

Fig. 6. Determination of optimum pH and temperature for activity of the isolated protease.

industry. A detergent compatible enzyme should tolerate alkaline conditions because pH of the detergents is generally in the range of 9–12 (Sana et al., 2006; Jain et al., 2012). The protease activity of the isolate was evaluated between the temperature range 20–70 °C. The enzyme activity was observed at 40 °C whereas a decline in enzyme activity was observed at temperature beyond 60 °C indicating the thermotolerant nature of the protease enzyme obtained from isolate Bact2 (Fig. 6b).

3.5. Influence of metals ions on protease activity

The influence of various metal ions was evaluated on the activity of partially purified enzyme. Among them cations $CaCl_2$ (Ca^{++}) increased the protease activity 35.9 ± 2.1 U/ml at 0.5 mM concentration, which was 55% higher than the control followed by $MgCl_2$ (Table 2) as also reported by Kamran et al. (2015) in the case of Ca^{++} (enhanced protease activity upto 44%). Contrary to this, when the concentration of metal ions ($CuCl_2$) increases, the protease activity was diminished while in the presence of NaCl, protease activity enhanced at 15 mM concentration (Table 4). These findings are in agreement to those reported earlier for several proteases stating the enhancement of protease activity in presence of Ca^{2+} and Na^{+} (Jain et al., 2012; Maruthiah et al., 2013). The decrease in protease activity in presence of Cu^{++} is in contrast to Maruthiah et al. (2013) reporting moderate enhancement of protease activity produced from *Bacillus subtilis* AP-MSU6.

3.6. Influence of inhibitors on protease activity

The results of Bact2 revealed that about 89% of enzyme activity was lost at 10 mM concentration of β-mercaptoethanol however 84–90% enzyme activity was reduced in presence of divalent chelators (EDTA) at 5 and 10 mM concentration (Table 5). On the other hand, the enzyme activity was completely inhibited (99%) the presence of Phenyl methyl sulphonyl fluoride (PMSF) at both 5 and 10 mM concentrations suggested that nature of enzyme as serine type (Adinarayana et al., 2003; Uyar et al., 2011) and Kamran et al. (2015) also reported that PMSF inhibited the protease activity in the presence of 5.0 mM and 10 mM concentration. The primary study of inhibition depends on the nature of the enzyme, its cofactor requirement and active reaction Centre (Sigma and Mooser, 1975). Mostly *Bacillus* strains are prominently used for the characterization of protease with their excellent stability and also diminished the enzyme activity in the presence of phenyl methyl sulphonyl fluoride (PMSF) as reported by Shaikh et al. (2018), Kamran et al. (2018). PMSF is a well-known serine protease inhibitor suggesting that protease of Bact2 belongs to serine protease.

3.7. Influence of detergents and surfactants

The activity of enzyme was significantly increased in presence of surfactants such as SDS, Tween 20, Triton X-100. The maximum protease activity (38.55 U/ml) was enhanced in presence of SDS, Tween 20, Triton X-100 (31.88 U/ml, Fig. 7a), whereas minimum activity was observed in presence of Tween 20 (19.06 U/ml). Similarly, when we compare the enzyme activity with detergent Ariel and Wheel, then enzyme activity was enhanced in case of Ariel (57.91 U/ml) than Wheel (23.80 U/ml). The evaluation of wash performance revealed that cloth piece having ink and tea stains when washed with 2% Ariel detergents having 11,540 U of alkaline protease proved to be more effective in removal of stains than cloth piece washed with only water and 2% Ariel having 5770 U of alkaline protease enzyme.

Alkaline protease enzyme have promising detergent additive characteristics and active over a broad range of temperatures which showed a current need to maintaining the fabric quality at low energy level (Mala and Srividya, 2010). Protease activity from Bact2 enzyme was treated with Ariel comparison to other detergents, retained more than 50% after 1 h incubation at 40 °C. Similar results was also performed by Singh et al. (2001), while Bhosale et al. (1995) reported that only 11% activity was revealed by Ariel detergents. Partially purified enzyme of Bact2 was found very effective in detergent formulation in ink and tea ingredient stains clothe pieces. The alkaline protease enzyme indicated a significant capability in removing process at 40 °C. Similar type of experiment was also reported by Nassar et al. (2015) who used egg yolk and blood stain in detergent formulation. Recently detergent compatibility assay with commercial detergent (Surf excel, Wheel, Ariel, Patanjali) of alkaline protease producing bacteria such as *Bacillus, Alcaligenes faecalis* and *Pseudomonas aeruginosa* was performed by Marathe et al. (2018). Here, we used tea (tea and milk mixture) stains and found that our protease was effective against that. In most of the Indian household milk is added in considerable amount while preparation of tea and protein is important component of tea therefore we used protease enzyme against tea. Pen ink stain is very common among children and office goers and some literature shows that deinking can be done with cocktail of enzymes as chemical nature of ink is neither fat, starch or protein so as our enzyme was showing some de-staining so it could be prepared as cocktail of enzymes involved in removal of stains of varying nature including ink.

4. Conclusion

The present work describes the isolation of a novel bacterium producing alkaline protease as *Citrococcus* sp (KC522120.1). This is the first report stating production of alkaline protease from halotolerant *Citricoccus* sp. The enzyme was partially purified and characterized for

Table 4
Effect of metal ions on protease activity.

S.N.	Metal Ions	Effect of metal ions on protease activity by Bact2			
		0.5 mM	1.0 mM	10 0.0 mM	15.0 mM
1	Control (without metal ion)	23.21 ± 3.10			
2	NaCl	12.3 ± 3.1	17.1 ± 3.2	26.2 ± 1.8	29.3 ± 1.4
3	$CuCl_2$	15.7 ± 4.7	14.2 ± 3.0	12.3 ± 3.1	10.5 ± 1.8
4	$CaCl_2$	35.9 ± 2.1	29.7 ± 3.2	25.8 ± 3.4	20.6 ± 3.7
5	$MgCl_2$	33.4 ± 1.7	30.9 ± 2.3	28.3 ± 1.4	21.4 ± 1.9

Table 5
Effect of inhibitors on protease activity.

Inhibitors	Concentration	Residual activity of Bact2
Control	1%	22.56 (100%)*
PMSF	5 mM	6.16 (0.27%)
	10 mM	0.89 (0.039%)
EDTA	5 mM	3.670 (16.26%)
	10 mM	2.35(10.41%)

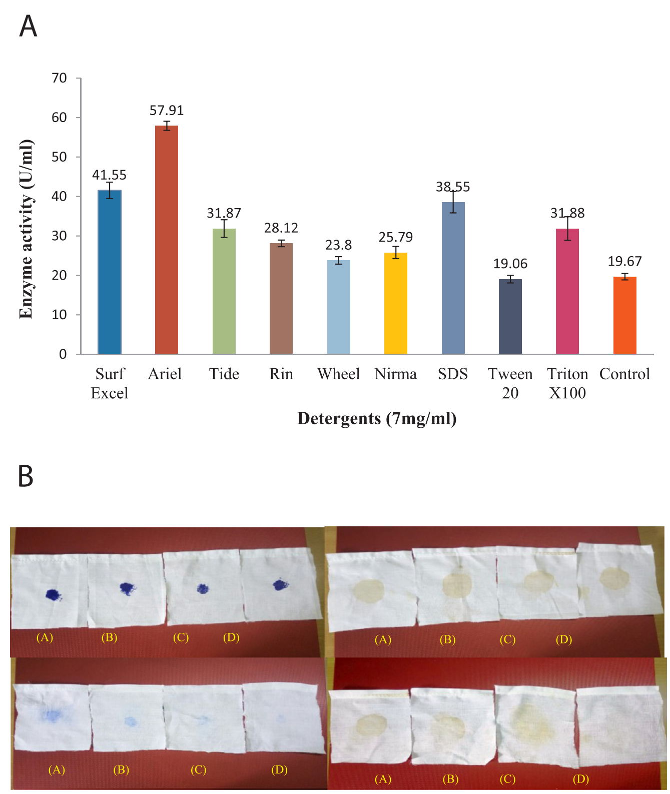

Fig. 7. a: Effect of different detergents and surfactants on protease activity. **b:** Blue ink and Tea soaked clothe pieces was washed with (A) only water, (B) Ariel detergent, (C) Ariel detergents having 250 ml of alkaline protease and (D) Ariel detergent having 500 ml alkaline protease[Cloth piece which was washed with only water (A) showed maximum color of ink and tea while cloth piece which was washed with Ariel detergent in the presence of 500 ml of enzyme (D) showed removal of stains (tea and ink)].

it's useful properties. The enzyme was active and stable at alkaline conditions (pH 10) and maximum enzyme activity was observed at temperature 40 °C. Furthermore, enzyme was stable in presence of various surfactants and metal ions. The compatibility of the enzyme was also higher with detergents and found to be very efficient in removing stains of ink and tea from clothes. These robust characters of enzymes make them an excellent candidate in detergent industries.

Acknowledgements

The first author is thankful to Department of Biotechnology, Babasaheb Bhimrao Ambedker University, Vidya Vihar, Lucknow – 226025 (U.P.), India for providing all laboratory facilities to carry out of the present work and funded by University Grant Commission (UGC) (No. F.10.-17/2007 (SA-I).

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