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Characterization of antifungal *Paenibacillus illinoisensis* strain UKCH21 and its chitinolytic properties

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***Paenibacillus* strain, UKCH21, isolated from Uttarakhand State, north western Indian Himalayas was found to produce high levels of extracellular chitinases. The 16s rRNA gene sequence showed 100% homology with *P. illinoisensis* (Accession No. KR856190) available in the public domain and further phylogenetic study also verified the species identity. The culture supernatants have a maximum chitinase activity of 110.8 U/ml after 3 days of culturing. The isolate showed strong antifungal activity manifested in the form of progressive mycelia degradation in dual culture plates. The pathogenicity was observed as structural deformities like uneven thickening of mycelia as a result of direct degradation of chitin. The optimum pH and temperature of UKCH21 chitinases was found to be 5 and 50°C, respectively. Partial characterization of chitinase gene also confirms the family 18 status of glycosyl hydrolase with substantial variability presented here with. Above all, percent inhibition of growth and the rapid degradation of mycelia of tested plant pathogenic fungi (*Rhizoctonia solani*, *Fusarium solani* and *Sclerotium rolfsi*) in bacteria seeded medium suggest its utilization as potent antifungal biocontrol agent.**

Key words: *Paenibacillus illinoisensis*, UKCH21, chitinase, antifungal, Indian Himalayas.

INTRODUCTION

Chitin is the second most abundant polysaccharide in nature, after cellulose (Shahidi and Abuzaytoun, 2005). It is the major structural constituent of fungal mycelium providing rigidity and protective in function. Consequently, the growth and multiplication of given fungi is highly dependent on metabolism of chitin. So, any direct damage to this vital cell wall component leads to proportionate reduction in fungal development making it as an ideal target in management of plant pathogenic fungi (PPF). Chitinases (EC 3.2.1.14) are degrading enzymes belonging to family 18 glycosyl hydrolases. They cleave β 1-4 linkages between the structural residues (N-acetyl glucoseamine) of chitin (Hamid et al., 2013). So, these enzymes have ability to directly degrade

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insoluble chitin to soluble reducing sugars. Chitinases play a vital role in chitin metabolism in all the chitin containing organisms. Many studies reported presence of chitinases in non-chitinogenic organisms as well. They include a number of prokaryotic and eukaryotic organisms including higher plants (Sharma et al., 2011). The company of chitinolytic enzymes, despite lack of chitin as a structural component in these organisms, confirms some additional functions which range from nutrition to protection (Hamid et al., 2013). Amongst different chitinolytic organisms, bacteria especially *Bacillus* were found to be well known chitinase producers (Cody et al., 1990). Besides, *Bacillus* species are well known producers of antibiotics, secondary metabolites, enzymes, plant growth promoters, etc that promotes or protects plant development. The established direct toxicity and pathogenicity of chitinases and other beneficial traits associated with *Bacillus* lead to successful worldwide exploration studies of chitinolytic *Bacillus* species with antagonistic potential against a variety of PPF (Drahos and West, 2003; Gohel et al., 2006; Prasanna et al., 2013; Brzezinska et al., 2014) with potential applications in biological control programs. Keeping this in view, a study was conducted to isolate chitinolytic *Bacillus* species with potential antagonistic activity. The study led to identification of a strain designated as UKCH21, whose characterization and molecular identification of chitinases are presented.

MATERIALS AND METHODS

Isolation and identification of bacterial strain

The bacterial isolate under investigation was isolated from soil samples collected from Kalimat village of Almora district in Uttarakhand state, India at an altitude of 1276 amsl and 29° 37' N, 79° 40' E coordinates. One gram of soil sample was mixed with 10 ml of sterile distilled water and a sample of aliquot was evenly spread on to detection agar (CHDA) plate (Kamil et al., 2007), a chemical based medium containing colloidal chitin as sole carbon source. After incubation at 30°C for 3 days, bacterial colony with clear halo around (signify utilization of colloidal chitin) was picked and purified by streaking on the same media. After confirmation of purity by microscopic observation, the strain was designated as UKCH21 and stored at -80°C as 20% glycerol stock (permanent storage) and as Luria agar slant at 4°C for further study.

The bacterial strain, UKCH21 was identified using partial sequence of 16S rRNA gene. The template DNA was extracted and purified by using CTAB solution and phenol chloroform extraction procedures, respectively (Sambrook et al., 1989). The 16S rRNA fragment was amplified in a thermal cycler using previously described *Bacillus* specific primers 16S rRNA (F) '5-CAGGCCTAACACATGCAAGTC-3' and 16S rRNA (R) '5-GGGCGGTGTGTACAAGGC-3' (Yoon et al., 2001). The reaction mixture contained 100 ng of total DNA, 0.5 mM forward and reverse primers, 3 mM $MgCl_2$, 200 nM dNTPs, 5 µl of 1X Tris-HCl buffer and 2.5 U of *Taq* DNA polymerase in a final volume of 50 µl. The reaction was performed at an initial 5 min denaturation step at 94°C followed by 30 cycles of amplification consisting of 1 min denaturation at 94°C, 45 s of annealing at 45°C, 2 min of extension at 72°C, with an extra extension step of 10 min at 72°C. The amplifications were confirmed by investigating 10 µL of PCR product by electrophoresis in a 1% agarose gel. The PCR product was sequenced at Scigenome labs, Cochin, Kerala. The obtained nucleotide sequence was BLASTN searched with the whole GenBank data base and molecular evolutionary analyses were performed using the software MEGA4 (Molecular Evolutionary Genetic Analysis version 4) (Tamura et al*.,* 2007). A phylogenetic tree was constructed using standard 16S rRNA sequences of related species by neighbor-joining method using the distance matrix from the alignment.

Preparation of colloidal chitin

Colloidal chitin was prepared from commercially available chitin flakes (Himedia) according to the procedure described by Berger and Reynolds (1988). Ten grams of chitin flakes were powdered using a mortar and pestle and added slowly to 400 ml of concentrated hydrochloric acid under continuous stirring on a magnetic stirrer at 4°C. After 30 min of stirring, the mixture was incubated at 37°C for 2 h to reduce viscosity. Then, the mixture was filtered through four layer muslin cloth (to avoid any impurities) to which 4 L of ice cold distilled water was added. After thorough mixing, the solution was allowed to stand at 4°C for overnight to allow better precipitation. Colloidal chitin was collected by centrifugation at 10000 rpm for 2 min and washed thoroughly with distilled water until neutral pH was achieved. Thus, obtained colloidal chitin was made into 20% solution, autoclaved and stored at 4°C, until used.

Purification of chitinases

Chitinases from UKCH21 was obtained from cell free culture supernatants of 3 days old culture in half strength nutrient broth supplemented with 1% colloidal chitin. The isolate was inoculated to autoclaved medium (50 ml) and incubated at 30°C and 250 rpm. After three days, the fermented broth supernatants were obtained by centrifugation at 10000 rpm for 20 min followed by 0.2 µ filtration. Then, four volumes of ice cold acetone was added and allowed to stand overnight at -20°C. Thus, precipitated proteins were collected by centrifugation at 10000 rpm for 10 min and dissolved in appropriate quantity of 15 mM Tris-HCl buffer (pH 6.8) after 80% ethanol wash. The protein content of sample was measured by standard Bradford dye binding method (Bradford, 1976) and designated as partially purified chitinases (PPC).

Estimation of chitinase activity

The enzyme activity of PPC was estimated by using natural substrate, colloidal chitin at pH 5 using 50 mM acetate buffer. The reaction mixture consists of equal volumes (250 µL each) of appropriately diluted PPC and buffer containing 1% colloidal chitin. The mixture was incubated at 50°C for 30 min followed by terminating the reaction by boiling in a water bath for 10 min. The remaining colloidal chitin was precipitated by centrifugation at 10000 rpm for 5 min and supernatant was estimated for released reducing sugars by modified Schales reagent (Imoto and Yagishita, 1971). In brief, an aliquot of 450 µL of supernatant was mixed with 600 µL of Schales reagent (0.5 g potassium fericyanide in one litter of 0.5 M sodium carbonate) and boiled for 15 min in a water bath. After cooling, absorbance was measured at 420 nm and the reducing sugar was estimated from a standard curve of N-acetylglucosamine. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugar per minute.

Thermal and pH kinetics of chitinases

To study the effect of temperature and pH on chitinase activity, standard enzyme activity assays were performed at different temperatures (ranging from 30 to 80°C at intervals of 10°C) and pH (using 50 mM citrate buffer (pH 3, 4, 5 and 6), 50 mM phosphate buffer (pH 6, 7 and 8) and 50 mM borate buffer (pH 8, 9 and 10) as described. The thermal stability was assessed by incubating enzyme solutions in a microcentrifuge tube at 30 to 60°C at intervals of 10°C without substrate for 2 h followed by estimation of residual enzyme activity. The pH stability was assessed by estimating the residual activity after incubating the enzyme in different pH buffers (as described above) at 4°C overnight. All experiments were performed independently in triplicate and enzyme activities were compared with estimate percentages.

Partial characterization of *chi* gene

The purified DNA was used as template for PCR amplification of chitinase gene fragment using previously described (Williamson et al., 2000) family 18 chitinase specific primers, GA1F (5'-CGTCGACATCGACTGGGARTDBCC-3') and GA1R (5'-ACGCCGGTCCAGCCNCKNCCRTA-3'). The reaction mixture used was similar to 16S rRNA amplification modified by addition of bovine serum albumin at concentration of 3 µg/10 µL of mix. The reaction was also performed with similar conditions at an annealing temperature of 62°C. After confirming the amplification, the PCR product was purified using gel elution columns (Sigma) and sequenced from Scigenome labs, Cochin, Kerala. The obtained sequence was compared with the published sequences of chitinase gene in the GenBank databases by BLASTN nucleotide sequences aligned with the Clustal Omega (1.2.1) multiple sequence alignment (McWilliam et al., 2013). The obtained sequences were submitted to NCBI Gene Bank nucleotide sequence databases.

Estimation of antifungal activity

The antagonistic activity of Chitinolytic *Bacillus* spp. was evaluated against plant pathogenic fungi (PPF), *Sclerotium sclerotiarum, Rhizoctonia solani* and Bean *Fusarium* which were collected from local infested fields and identified according to standard protocols. Initially, the growth inhibition of PPF was evaluated in dual culture plates. A basal agar medium plate containing Luria bertain agar (LBA) and potato dextrose agar (PDA) at their half strengths was used to support the growth of both bacteria and fungi. The experimental setup consists of test bacterium streaked at the middle of plate containing solidified basal agar medium and disc inoculated PPF (0.3 mm) at both the corners of plate in parallel to streak. The dual inoculated plates were incubated at 28±2°C and the controls were devoid of bacteria. After 10 days of incubation, when the growth of PPF in control plates joined in the middle, the treatment plates were observed for growth inhibition. Further, any deformity and/or toxic effects on test mycelia were recorded using fluorescent microscope by observing contact point between test fungi and bacteria.

Further, the antagonistic activity was evaluated in bacteria seeded medium (El-Mougy et al., 2011). In brief, the test bacterium was initially grown in nutrient broth at 30°C and 250 rpm for overnight. The obtained bacterial culture was thoroughly mixed with lukewarm sterile basal medium at a rate of 10% v/v and plated in 9 mm Petri plates. After solidification, 0.5 cm diameter actively growing test fungal disc was placed at the center of plate and allowed to grow at 27±2°C. The diameter of fungal growth was measured when it reached borders in control plates with no bacteria. The *S. rolfsi* plates were further incubated to evaluate the inhibition on sclerotia formation. Triplicate plates were used to evaluate fungal growth inhibition in all the test bacteria.

RESULTS AND DISCUSSION

The present study reports a chitinolytic *Paenibacillus* strain, UKCH21 having antagonistic activity against soil inhabiting plant pathogenic fungi. Initial screening of the isolate for its chitinolytic properties in CHDA plates showed 1 cm halo around the bacterial colony of 0.4 cm after 7 days of incubation. The aerobic growth of bacterial colony was characterized by raised, creamiest white colony with smooth edges and oily appearance (Figure 1). Microscopic observation showed rod shaped vegetative cells with motility and formation of endospore confirming *Bacillus* grouping. Further species identity of UKCH21 was done using 16S rRNA gene sequencing (accession No. KX113474). The BLASTN search of the obtained 841 nucleotide sequences showed representative homology with *Paenibacillus* species with maximum homology of 100% with *P. illinoisensis* strain C1 (accession No. KR856190). The alignment and phylogenetic analysis of 16S rRNA sequences of different *Paenibacillus* species strongly suggested species status of the bacterial strain UKCH21 as *P. illinoisensis* (Figure 2). The high levels of enzyme production depicts potential degradation of chitin, in view of which, the isolate was selected to study its antagonistic potential and further identification of chitinases. Studies reported a number of *Paenibacillus* species with potential applications in biological control of variety of plant pathogenic bacteria (Budi et al., 2000; von der Weid et al., 2003; Lorentz et al., 2006; Fortes et al., 2008), fungi (Naing et al., 2015; Xu et al., 2014; Liu et al., 2008; von der Weid et al., 2003; Jung et al., 2003, 2005, 2006) and even nematodes (Jung et al., 2002). This biocidal activity of *Paenibacillus* species was supported by production of antibiotics, hydrolytic enzyme etc (Chung et al., 2000; Velázquez et al., 2004; Lorentz et al., 2006). Besides they are predominently distributed in soils and rhizosphere (Berge et al., 2002; von der Weid et al., 2002), a dynamic ecosystem. The present study also supports existence of *Paenibacillus* species in soils of Uttarakhand Himalayas as major chitinase producers.

The antifungal activity of *P. illinoisensis* strain UKCH21 in dual culture plates was manifested in the form of direct inhibition of mycelial growth upon contact with bacterial growth. Further, degradation and amputation of fungal mycelium is progressive with time. The microscopic observation of contact point between bacterial growth and test fungi showed uneven thickening of mycelia (Figure 3e) as a result of degradation and or digestion of hyphal chitin leading to loss of integrity. Senthilkumar et al. (2007) have also reported several structural deformities like hyphal lysis and bulging of the mycelium of *R. bataticola* caused by *Paenibacillus* sp. HKA-15. The bacteria seeded medium showed cent percent growth

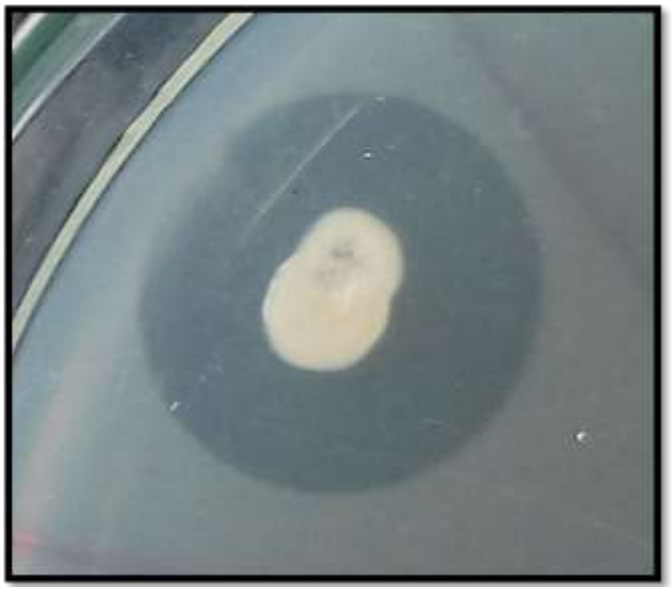

Figure 1. Chitinolytic activity of *P. illinoisensis* strain UKCH21 in CHDA plates.

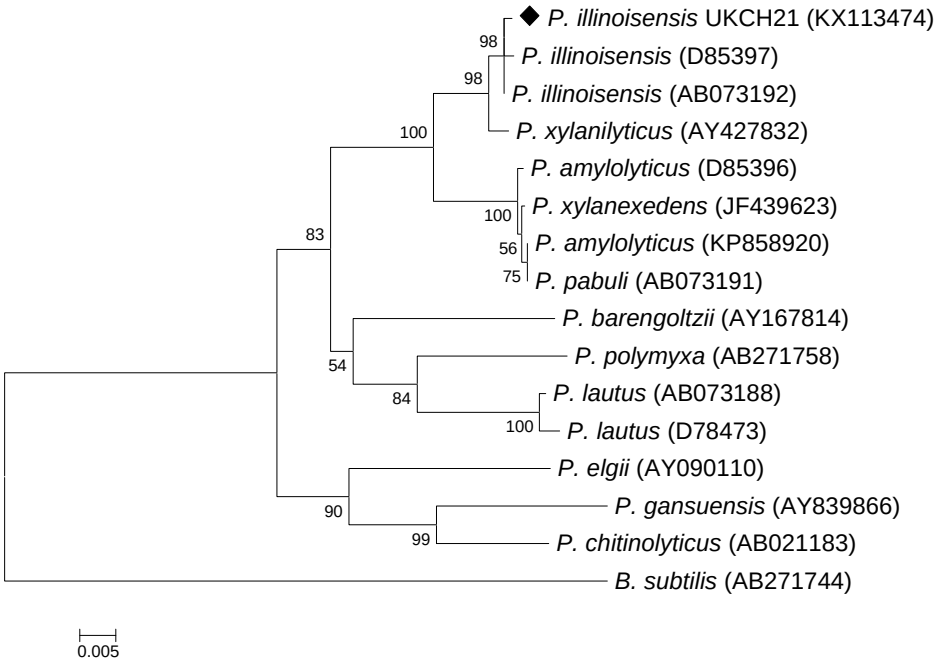

Figure 2. Phylogenetic tree constructed using 16S rRNA gene fragments by neighbor-joining method, indicating the position of the isolates UKCH21. The numbers at the nodes indicate the percentage bootstrap values for each node based on 1,000 bootstrap replicates. The tree was rooted with the 16S rRNA gene of *Bacillus subtilis* as an outgroup.

inhibition from the inoculated disc of test fungi (Figure 3). This shows that the presence of UKCH21 on given growth media completely inhibited development of tested fungi either by chitinase production or by any other secondary metabolites that act synergistically. Despite the number of *Paenibacillus* species, in particular, only minimal studies reported antagonistic potential of *P. illinoisensis*. For example *P. illinoisensis* KJA-424 against

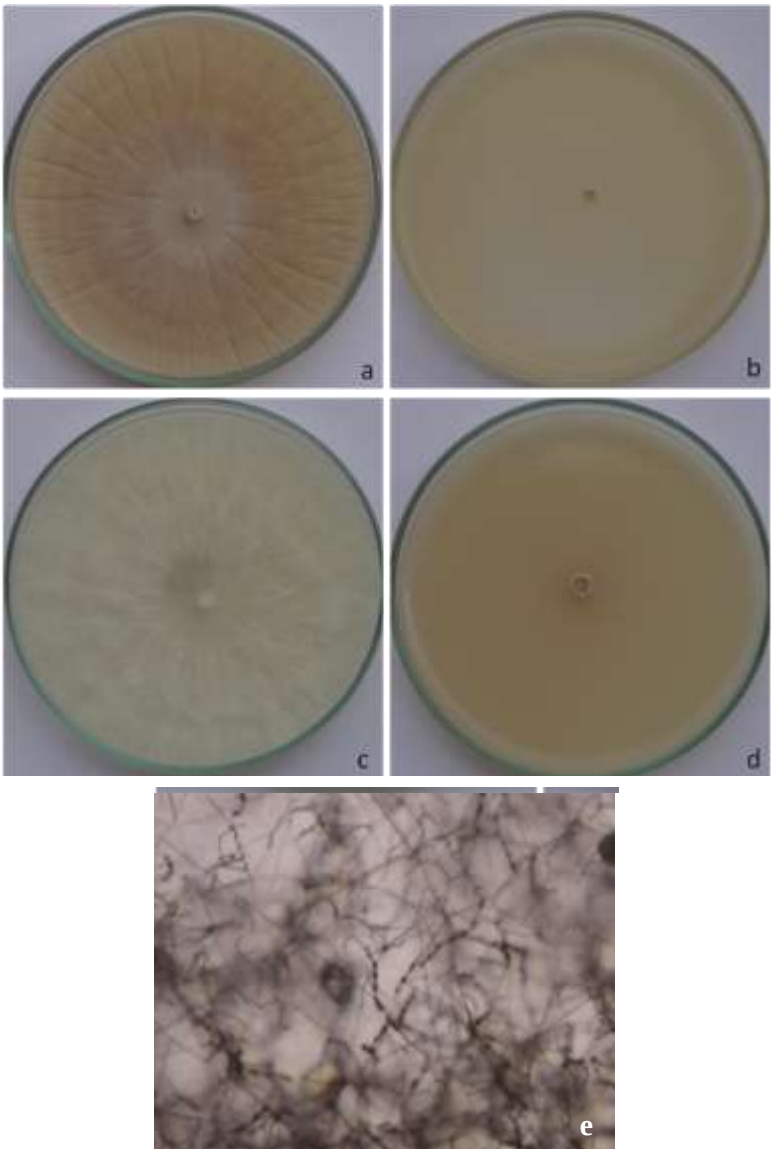

Figure 3. Antifungal activity of *P. illinoisensis* strain UKCH21 against *R. solani* and *S. rolfsi* in bacteria seeded medium. a. Control plate of *R. solani;* b. Growth inhibition of *R. solani;* c. Control plate of *S. rolfsi;* d. Growth inhibition of *S. rofsi;* e. Uneven thickening of *S. rolfsi* mycelia.

Phytophthora capsici (Jung et al., 2005; Jung et al., 2006), *Rhizoctonia solani* (Jung et al., 2003) and *Meloidogyne incognita* (Jung et al., 2002).

Partial sequencing of chitinase gene from the isolate UKCH21 resulted in 373 bp nucleotide sequence (accession no. KX446923). The nucleotide BLASTN search of obtained sequence showed maximum homology of 79% with *chiA* gene of *Paenibacillus* sp. FPU7. The sequence alignment of deduced amino acid sequence with full gene sequences from *S. marcescens* (BAA31567.1), *B. cereus* (EEK85987.1), *P. barengoltzii* (WP016312329), *P. macerans* (KFM93118.1) and *B. circulans* (AAA81528.1) showed substantial variation in amino acid composition between the species (Figure 4). Out of 121 amino acid residues, 26 amino acid were conservations (denoted by *). Especially, UKCH21 showed 17 unconserved substitutions among the tested *Paenibacillus* accessions (*P. barengoltzii* and *P. macerans*) indicating the novel characteristics of *P. illinoisensis* chitinases. However, all the sequences showed conserved characteristics of family 18 glycosyl hydrolases.

The apparent activity of any given chitinases is a function of existing temperature and pH. Studies also reported specific featured chitinases with respect to pH (Loni et al., 2014; Fu et al., 2016) and temperature

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Sma       DKVKRDRFVGSVKEFLQTWKFFDGVDIDWEFPGGKGANPNLGSPQDGETYVLLMKELRAM   345
Bce       DEKTRKVFAESTVDFLREYG-FDGVDLDWEYPGVETIPGGGSYRPEDKQNFTLLLQDVRNA   253
Pba       SQVTRETFANSAVDFLRKFN-FDGIDLDWEYPVAGGLPGNSYRPEDKQNYTKLLQEIRNK   233
Pma       DPALRENFANSAVDFLRKYQ-FDGVDLDWEYPVSGGLQGNSRRAEDKQNFTLLLQKTREK   232
Bci       TAATREVFANSAVDFLRKYN-FDGVDLDWEYPVSGGLDGNSKRPEDKQNYTLLLSKIREK   234
UKCH21    ------------------------------------------------YTLLLQKIREK   11
                                                             :. *:.. *

Sma       LDQLSVETGRKYELTSAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGAFDLKNLGHQT   405
Bce       LNKAGAEDGKQYLLTIASGASQRYADHTELKKISQILDWINIMTYDFHGGWEATSNHNAA   313
Pba       LDSAGAVDGKRYLLTIASGASPSFVSNTELGNIASIVDWINIMTYDFNGGWQQITAHNAP   293
Pma       LDAAGAKDGKRYLLTIASGASPAFAQNTELDKISDIVDWINIMTYDFNGGWQNITAHNAP   292
Bci       LDAAGAVDGKKYLLTIASGASATYAANTELAKIAAIVDWINIMTYDFNGAWQKISAHNAP   294
UKCH21    LDAAGTADNKKYFLTIASGAGPTYAANTELGNMAKYLDWINIMTYDFNGGWQTVSAHNAP   71
          *:  ..   ::* ** * .*.     ..        :* * :*:*** *.::  .  .

Sma       ALNAP-----AWKPDTAYTTVNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNGYQNNIPF   460
Bce       LYKDPNDPAA----DTKFYVDGAIDIYTNEGVPADKLVLGVPFYGRGWKSCGKENNG-QY   368
Pba       LYYDPAAGNAGVPSASVFNADAAVQGHLNAGVPANKLVLGIPFYGRGWDGVNNAGNG-QY   352
Pma       LYLDPAAVAAGVPDSESFYVEAGVRGHLNAGVPAGKLVLGVPFYGRGWTGAAAAGNG-QY   351
Bci       LNYDPAASAAGVPDANTFNVAAGAQGHLDAGVPAAKLVLGVPFYGRGWDGCAQAGNG-QY   353
UKCH21    LYTDPAAIAAGVPNADTFNVEKGVQGHINAGVPASKIVLGLAFYGRGWTG----------   121
             *          : .  .      **   *:*:*  :***** .
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Figure 4. Alignment of peptide sequence of chitinase from UKCH21 with *S. marcescens* (Sma, BAA31567.1), *B. cereus* (Bce, EEK85987.1), *P. barengoltzii* (Pba, WP016312329), *P. macerans* (Pma, KFM93118.1) and *B. circulans* (Bci, AAA81528.1). Conserved amino acids are with asterisk. Dashes indicate gaps left to improve alignment. All sequences are numbered from the Met-1 peptide.

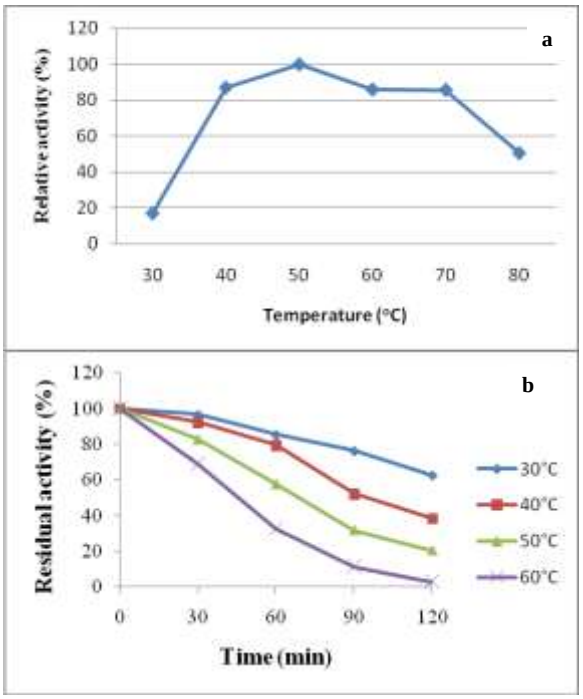

Figure 5. Effect of temperature on activity (a) and stability (b) of chitinases produced by UKCH21.

(Karthik et al., 2015) having potential in industrial applications. Besides, identification of optimum pH and temperature of any given bacterial chitinase is important to predict its activity in different environmental conditions. The culture supernatants from *P. illinoisensis* strain UKCH21 showed a maximum enzyme activity of 110.8 U/ml after 3 days of culturing with protein content of 0.69 mg/ml. Further testing of PPC from UKCH21 showed substantial enzyme activity (>80%) over a temperature range of 40 to 80°C with its peak activity at 50°C (Figure 5a). Further increase or decrease in temperature resulted in reduction in enzyme activity. However, the enzyme lacks thermal stability as revealed by the loss of more than 30% of its activity within two hours at around room temperature (30°C). At 60°C, the enzyme lost cent percent activity within 2 h of incubation is shown in Figure 5b. The enzyme showed optimum activity at pH 5 (Figure 6a). Further increase in pH showed 32% loss in activity. Interestingly, the enzyme upholds its activity between pH 6 and 9. The stability analysis showed increased enzyme activity up to pH 6 (Figure 6b).

In conclusion, the unique ecological niche presented by mountain and hill regions with associated environmental and biotic factors supports vast diversity of beneficial bacterial community with potential commercial applications.

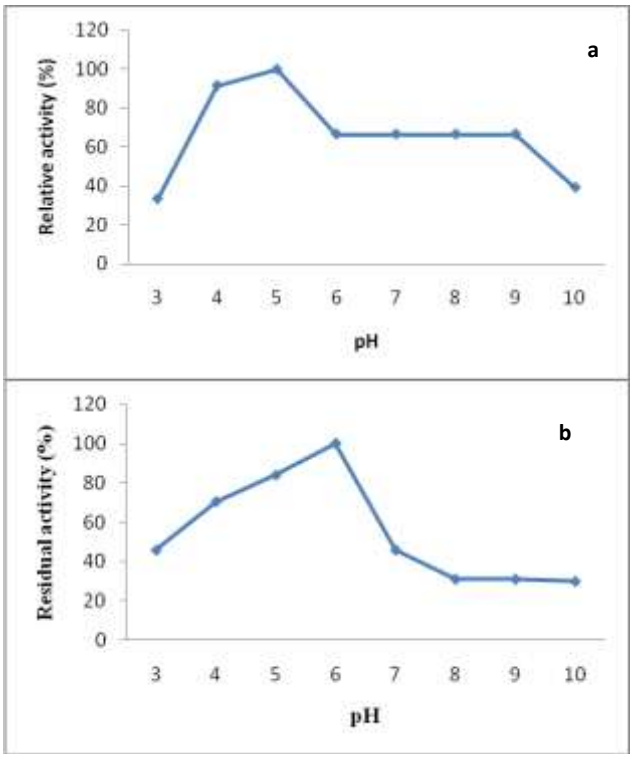

Figure 6. Effect of pH on activity (a) and stability (b) of chitnases produced by UKCH21.

To the best of the authors' knowledge, for the first time, the present study identified a strong antifungal *P. illinoisensis* strain UKCH21 from India with an extracellular chitinase production, that is, having potential applications in biological control of tested soil born plant pathogens. Keeping this in view, further studies were planned to find out the biocontrol potential of UKCH21 against other fungi and under filed conditions.

Conflict of Interests

The authors have not declared any conflict of interests.

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