

Industrially important hydrolytic enzyme diversity explored in stove ash bacterial isolates

Tabbassum Kiran, Wajeeha Asad¹, Shahla Siddiqui²,
Munazza Ajaz³ and Sheikh Ajaz Rasool^{1*}

Department of Microbiology, University of Karachi, Karachi, Pakistan

Department of Statistics, University of Karachi, Karachi, Pakistan

Department of Microbiology, Federal Urdu University of Arts, Science & Technology, Karachi, Pakistan

Abstract: Extreme environments merit special attention and significance because of the possible existence of thermophilic microorganisms in such ecological niches. Keeping this in mind indigenous stove ash samples were explored for extremophilic bacteria in term of their biodiversity. Accordingly, this study reports 37 bacterial isolates from the local wood run oven (Tandoor) ash samples. All the isolated strains belong to genus *Bacillus* on the bases of morpho-cultural and biochemical considerations. The average temperature tolerance profile was >45°C thereby, indicating towards the thermophilic nature of the isolated strains. The *Bacillus* isolates were screened for 10 different hydrolytic enzymes (cellulase, xylanase, amylase, pectinase, caseinase, keratinase, lipase, esterase, dextranase and β -galactosidase) by plate screening method using the medium incorporated with specific substrate(s). It was found that keratinase was produced by all the isolates while, 36 (97.2%) isolates showed caseinase and esterase production. Amylase was produced by 35(94.6%) isolates and 34 (91.8%) isolates were able to degrade Tween-80 and xylan as substrate for lipase and xylanase respectively. The enzyme, β -galactosidase was produced by 31 (89.1%) of the isolates. Cellulase and dextranase were produced by 26 (70.2%) and 22 (59.4%) isolates respectively. None of the isolates could (under the existing conditions) produce pectin-hydrolyzing enzyme. According to the tukey's post hoc test, significant difference was found between the mean enzyme index of all the (screened) enzymes. Thus, the isolated bacterial strains with diverse hydrolytic potential may be of great value and relevance for the existing (national) industrial setups.

Keywords: Extracellular, thermophiles, hydrolytic enzyme profile, stove ash samples, enzyme index.

INTRODUCTION

For biotechnological products and processes, the microbial diversity serves as a main and rich resource. Microbes play an indispensable role in novel metabolites production, nutrient recycling, environment detoxification and the production of industrially important enzymes (Daniel, 2005; Lorenz and Eck, 2005). Out of the diverse microbial population existing in miscellaneous natural habitats including extreme environments, bacteria enjoy the status of being the most abundant (Lyngwi and Joshi, 2014). Exotic environmental sources are the ideal habitats for the isolation of bacteria with an increased sustainability at temperature extremes. These sources may include terrestrial hot springs, deep sea hydrothermal vents and other extreme ecological niches including volcanic cavities, tectonically active faults as well as decaying matter such as the droppings and deep organic landfills (Asad *et al.*, 2014). Pertaining to their spore forming ability, existence of the genus *Bacillus* spp. as natural residents of the harsh environment in these extreme conditions is quite understandable. Based on their versatility in sustaining the extreme physical conditions, the members of the genus *Bacillus* are considered as ideal bio-factories for producing the industrially important

hydrolytic extreme-enzymes with marked thermostability (Wishwanatha *et al.*, 2010). About 50% of the global enzyme market is based on the enzymes from *Bacillus* spp. (Schallmey *et al.*, 2004). A number of the ecological niche derived materials have remained untapped and hence need to be exploited. One of the sources includes the local oven wood based ash. A limited work has previously been done on the wood-based ash bacterial flora (Asad *et al.*, 2011). Thus, the present study was attempted for the isolation of thermophilic bacteria from wood-based oven ash and to explore their potential as industrial catalytic input. The projected study may facilitate to investigate the extent of biodiversity in indigenous extreme habitats and their potential bacterial flora, which may produce industrially relevant enzymes.

MATERIAL AND METHODS

Isolation of bacteria

The wood based ash samples were collected from different burning ovens, (locally known as tandoors) in sterilized bottles. These samples were serially diluted (upto 10⁻⁴) and were spread using pour plate and spread plate methods on BHI agar plates. These plates were incubated at 50°C for 24 hours. Next day; the emerged colonies were purified by successive streaking on LB agar plates (Peptone 1%, yeast extract 0.5%, NaCl 1% and

*Corresponding author: e-mail: drajazrasool@gmail.com

agar 2%). Purity of the culture was checked by Gram staining (Teodoro and Martins, 2000). Glycerol stocks were prepared for culture preservation and for further study.

Screening for Caseinase enzyme

For qualitative screening of caseinase, all the strains were inoculated onto the medium containing glucose 0.1%, yeast extract 0.25%, casein 1% and agar 2%. Plates were incubated at 50°C for 24 hours. Afterwards, zone of hydrolysis was measured and enzyme index was calculated as per formulae (Perez *et al.*, 2009).

$$\text{Enzyme index} = \frac{\text{Zone of hydrolysis in mm}}{\text{Colony diameter in mm}}$$

Screening for lipase enzyme activity

Potential for lipase production of the isolated strains was evaluated by spot inoculation on the medium consisted of peptone 1%, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01%, NaCl 0.5%, tween 80 1% and agar 2%. Plates were incubated at 50°C for 24 hours. The isolates showing zone of precipitation around the growth were identified as lipase producers (Gopinath *et al.*, 2005).

Screening for esterase enzyme activity

Esterase production (by isolated strains) was monitored by inoculating all the isolates on the medium containing peptone 1%, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01%, NaCl 0.5%, tween 20 1% and agar 2% (Kumar *et al.*, 2012). Plates were incubated at 50°C for 24 hours. Zone of precipitation around the growth was considered as positive reaction. Contrasts were enhanced using Gram iodine and the enzyme index was calculated.

Screening for amylase enzyme production

For amylase screening, medium containing glucose 0.1%, yeast extract 0.25%, starch 1% and agar 2% was used. Plates were inoculated and incubated at 50°C for 24 hours. Next day, plates were flooded with 2% iodine solution for 15 min. Zone of starch hydrolysis was observed and measured (mm) around the colony (Mazzucotelli *et al.*, 2013).

Screening for cellulase enzyme

Cellulase screening was performed by inoculating the isolates on to the mineral salt medium containing 1% cellulose and 2% agar. Plates were incubated at 50°C for 24 hours and thereafter the Petriplates were stained with 0.1% Congo red for 1 hour followed by destaining with 1M NaCl (Gautam *et al.*, 2012). Clearance zone was recorded (mm) around the colony.

Screening for xylanase enzyme

Xylanase screening of the isolated strain was performed on the medium containing; yeast extract 0.1%, xylan

0.5%, $(\text{NH}_4)_2\text{SO}_4$ 0.02%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.025%, KH_2PO_4 0.06% and agar 2%. Incubation (at 50°C) was done after inoculation (of plates) for 24 hours. Plates were then stained with Congo red solution (0.5% w/v in 50% ethanol) for 15 min and then destained with 1M NaCl (Tork *et al.*, 2013). Zone of hydrolysis was measured (mm) around the colony against the red background.

Screening for pectinase enzyme

All the strains were spotted on the medium consisting of yeast extract 0.1%, pectin 0.5%, $(\text{NH}_4)_2\text{SO}_4$ 0.14%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02%, KH_2PO_4 0.2% and agar 2% for evaluating their potential for pectinase production. Plates (after inoculation) were incubated at 50°C for 24 hours. The plates were then exposed to KI solution (0.3% iodine-0.6% KI) for 5 min (Rhoban *et al.*, 2008).

Screening for β -galactosidase enzyme

All the strains were spotted on minimal salt medium supplemented with lactose 1% (Carrim *et al.*, 2006) and incubated at 50°C for 24 hours. Next day, plates were flooded with 2% iodine solution for 15 min. Enzyme index was calculated as per above referred formulae.

Screening for dextranase production

Medium containing; glucose 0.1%, yeast extract 0.25%, dextran 1% and agar 2% was used for evaluating the dextran hydrolyzing potential of the isolated strains. After inoculation, the plates were incubated at 50°C for 24 hours. Next day, plates were flooded with 2% iodine solution for 15 min (Zohra *et al.*, 2013). Zone of hydrolysis around the colony was observed and measured (mm).

Screening for Keratinase production

Bacterial strains were spotted on the medium having 0.5% keratin, 0.03% K_2HPO_4 , 0.04% KH_2PO_4 , 0.05% NaCl, 0.01% MgCl_2 and 2% agar (Sahoo *et al.*, 2012). Plates were incubated at 50°C for 48 hours. Next day plates were stained with 0.12% coomassie brilliant blue R250 (w/v) staining solution for 30 min followed by destaining with 30% methanol and 10% acetic acid.

Temperature tolerance profile

All the isolated strains *Bacillus* TLW-1 to *Bacillus* TLW-37 were tested for their potential to survive at temperature >45°C. Maximum temperature sustained was 75°C. Isolates were kept at varying temperatures within range stated above at the respective temperatures for 24 hrs.

Statistical Analysis

All the experiments were run in triplicate. Values represent the mean \pm standard deviation. Tukey's test was applied to the enzyme screening data at the 0.05 level of significance.

RESULTS

In the present study, 37 bacterial strains were isolated from wood burned ash samples (fig.1), identified and named as *Bacillus* TLW-1 to *Bacillus* TLW-37. These strains were purified and maintained on the LB agar slants and preserved in glycerol stocks. All the isolates were aerobes, Gram-positive rods and endospore formers. The optimum growth temperature was set as 50°C (fig. 2). According to the temperature tolerance profile, 83.8% isolates were classified as thermophiles because they grow at >55°C, while 16.2% isolates were categorized as hyper-thermophiles as they grow at >60°C. These strains were screened for 10 different hydrolytic enzymes (cellulase, xylanase, amylase, pectinase, caseinase, keratinase, lipase, esterase, dextranase and β -galactosidase). Screening results are presented in fig. 3-5. The isolate TLW-3 showed the highest hydrolytic potential as it can degrade all the tested substrates (except pectin) with highest enzymatic index. Some other strains i.e. TLW-2, TLW-4, TLW-5, TLW-6, TLW-7, TLW-10, TLW-11, TLW-14, TLW-26, TLW-31, TLW-34, TLW-35 and TLW-37 were also found capable of producing all of the studied enzymes (except pectinase), but with slightly lower enzyme index in comparison to the strain TLW-3.



Fig. 1: Isolation of the bacterial strains from stove ash samples

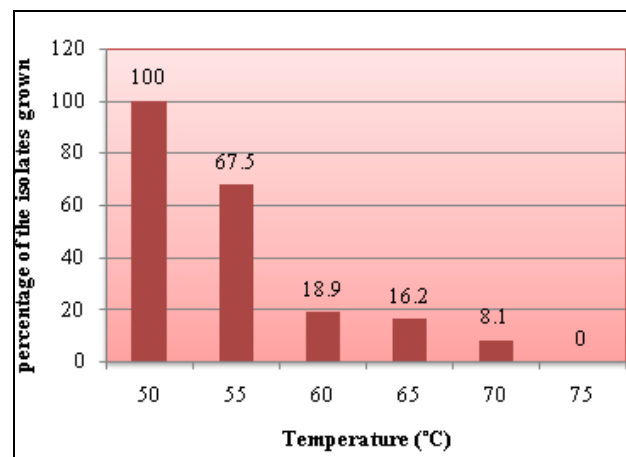
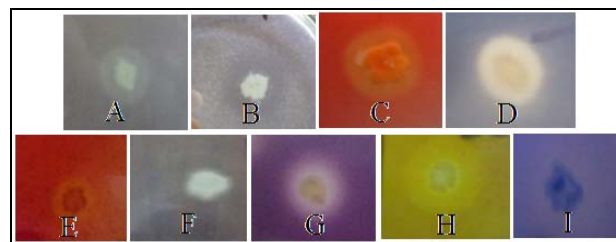


Fig. 2: Temperature tolerance profile of the *Bacillus* isolates.

Analysis of variance among different enzyme groups indicates a significant difference in the production capabilities of the isolates. Subsequent multiple comparisons by Tukey's post hoc test represents the

grouping of enzymes with respect to mean enzyme index (table 1b). The significant difference in the enzyme production ability of the isolates among the different enzyme groups lies where these groups don't share a similar letter. Results indicate that group A (caseinase enzyme) has highest mean enzyme index (2.292) when compared with the mean of other groups. Accordingly, many isolates produced a consortia of the tested enzymes with different enzyme index and the overall mean enzyme index of these enzymes differ significantly ($p=0.048$) at 95% confidence interval.



Key: A, β -Galactosidase; B, Caseinase; C, Cellulase; D, Amylase; E, Xylanase; F, Lipase; G, Dextranase; H, Esterase; I, Keratinase.

Fig. 3: Plate incorporated media screening of hydrolytic enzymes

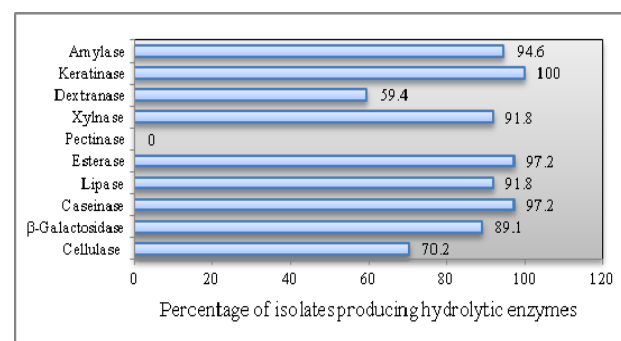


Fig. 4: Hydrolytic enzymes production profiles of the isolates

DISCUSSIONs

For a variety of industrial setups, thermophilic bacteria have turned out to be a real asset. In contrast to meso-enzymes; the applications of thermostable enzymes are not only limited to significantly reduced risk of contamination, but they also help in lowering the inlet stream viscosity and the possibility of maintaining the pH during the whole biotransformation, thus avoiding the necessary salt addition in currently operated processes (Khalil, 2011). Because of long generation time of the fungal cells and clogging of the fermentors pertaining to heavy mycelial growth, extensive research has been undertaken to explore the novel bacterial isolates that are capable of replacing fungi for the production of industrially pertinent enzymes (Ghani et al., 2013). In the

same vein, the presented work is an effort to reveal the biodiversity of the stove ash samples and screening of heterologous hydrolytic potential of the isolated thermophilic bacteria.

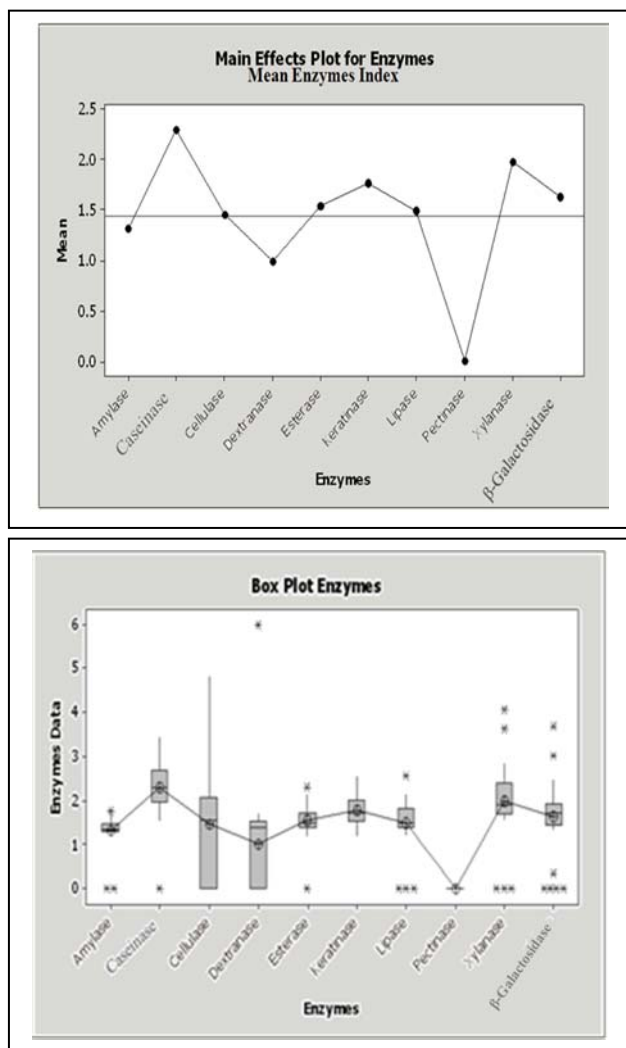


Fig. 5: Main effect and box plot of the enzymes screened

The growth conditions (*in vitro*) of the microorganisms are generally maintained according to their natural habitats (Muthulakhshmi *et al.*, 2011). Niches with high temperatures are the preferred sites for the isolation of thermophilic strains. Keeping this in view, the oven burned wood ash samples were selected for the isolation of the thermophilic bacteria. Previously, Asad *et al* (2011) reported that the bacterial strains from ash samples were able to grow at the temperatures $>65^{\circ}\text{C}$. In the current study, a total of thirty-seven bacterial strains were isolated (fig. 1). Different colonial morphologies were observed. All the isolates were found to be Gram positive and on the basis of morphological and biochemical characteristics they belong to genus *Bacillus*. *Bacillus* spp. have earlier been reported for producing different hydrolytic enzymes (Outtrup and Jorgensen, 2002; Lyngwi and Joshi, 2014).

According to Perry and Staley (1997), the organisms that can grow optimally at temperatures $>45^{\circ}\text{C}$ are thermophiles. The maximum temperature for the growth was determined by incubating them at temperature ranges from $50\text{--}75^{\circ}\text{C}$ for 24 hours (fig. 2). It was noted that all the isolates were able to grow at 50°C which is indicative of the thermophilic nature of the isolates. Some strains even could grow at 70°C , while none of them could sustain 75°C . Adiguzel *et al.* (2009) isolated bacterial strains from hot springs in Turkey which could grow at $>50^{\circ}\text{C}$.

Table 1a: One-way ANOVA: Data versus Enzymes

Source	DF	SS	MS	F-value	P-value
Enzymes	9	127.920	14.213	28.74	0.0482
Error	360	178.066	0.495		
Total	369	305.987			

Key: DF, degree of freedom; SS, sum of squares; MS, mean square

Table 1b: Tukey's Method ($\alpha = 0.05$)

Enzymes	N	Mean	Grouping
Caseinase	37	2.2942	A
Xylanase	37	1.9774	A B
Keratinase	37	1.7638	B C
β galactosidase	37	1.6300	B C
Esterase	37	1.5142	B C
Lipase	37	1.4961	B C D
Cellulase	37	1.4503	C D
Amylase	33	1.3161	C D
Dextranase	37	0.9911	D
Pectinase	37	0.0000	E

On industrial scale, simultaneous production of multipurpose enzymes by a single strain can help reducing the cost and time scale of the production setups. Traditionally, plate-screening method is globally used for selecting the potential bacterial strain (s) with versatile zymogenic ability (Ten *et al.*, 2005). The plate-screening assay revealed that almost all of the isolates were capable of producing all the enzymes tested except pectinase (fig. 3). Heteropolysaccharide nature of pectin makes it a difficult substrate for hydrolysis by a single unit of the microbial species, a complex array of pectinolytic enzymes is required that can sequentially hydrolyze pectin.

Importance of proteases has tremendously increased at industrial scale and in bio waste management. Two different proteases i.e. caseinase and keratinase were screened in this study. Fig. 4 shows the enzyme hydrolytic profile of the isolates. It was found that among all the

screened enzymes, keratinase was amply produced by all the isolates tested, followed by caseinase and esterase. Out of 37 strains, only one strain (TLW-29) could not hydrolyze casein. Keratinases can adequately degrade the poultry waste (keratin) in green manner thus, allowing the breakdown of keratin into simple peptides and amino acids to be used in animal feed and foodstuff as a supplement (Khardenavis *et al.*, 2009).

In comparison to genetic manipulation, the exploration of new industrial strains capable of producing stable amylase in bulk is feasible and cost effective. As much as 94.6% of the studied strains in the current research setup were found to be amylolytic. Narayan *et al.* (2008) reported 81.7% of the amylase producers among the bacterial strains isolated from hot springs in Fiji.

Cellulosic and hemicellulosic material is available as plentiful biomass found on earth (Sethi *et al.*, 2013) followed by starchy stuff, both serving as the significant form of renewable energy (Shaw *et al.*, 2008). The biochemically tailored cellulases that function optimally under varied conditions, including high temperatures, extreme pH conditions and in the presence of residual chemicals and inhibitors are required for the pretreatment of a variety of feedstock systems (Gao *et al.*, 2010). In the current study, 70.2% strains were able to degrade cellulose as the sole carbon source where as, the percentage of xylan degraders was found to be 91.8%. This may be due to varied nature of both the substrates in terms of structural complexity at least. Saleem *et al* (2012) reported 51% cellulase producing strains isolated from hot spring.

Lipases and esterases are extensively used in the detergent, food, pharmaceutical and cosmetic industries. When screening of these enzymes was carried out, 97.2% and 91.8% isolates showed their ability to produce these enzymes respectively. Asad *et al.* (2011) reported 66% lipase producers and 74% esterase producing bacterial strains isolated from stove ash samples.

Graphical analysis (fig 5a and b) presents the mean difference in the indices of different enzymes among all the 37 strains. The highest mean index was found for the enzyme caseinase followed by xylanase. This was further verified by including the error terms in box plot, which also showed significant difference among enzyme production capabilities of the studied bacterial strains. These results were confirmed statistically using ANOVA followed by Tukey's post hoc test as given in table (1a and b). Tukey's test highlights the significant and non-significant difference among the groups. These tables indicate that individual mean index of each enzyme is in different group thus, they differ significantly at $p=0.048$ at 95% confidence interval.

CONCLUSION

The isolated strains with diverse hydrolytic activities can be considered a potential asset after optimization studies and may result in the bulk production of these biorobotic entities.

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