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Research article

The main Aflatoxin B1 degrading enzyme in *Pseudomonas putida* is thermostable lipase

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ABSTRACT

Aflatoxin B1 is a carcinogenic and mutagenic mycotoxin mainly produced by *Aspergillus flavus* and *A. parasiticus,* and prevalent in food and feed. Microbial degradation is a promising strategy which can be performed in mild and environmental friendly condition. This work is a step towards identifying the enzyme responsible for biodegradation of AFB1 by *P. putida*. Experiments were performed with *P. putida* lysate and compared with commercial lipase to see the degradation efficiency and the temperature stability. The cell free lysate of *P. putida* efficiently degraded AFB1 in a range of temperature from 20 to 90 °C. The lysate is thermostable and could retain its activity on pre-incubation up to 90 °C. Highest rate of degradation was observed at 70 °C. These observations show that the *P. putida* lysate is not only stable at higher temperatures but its enzymatic activity increases after incubation. Similarly, the commercial lipase degraded AFB1 efficiently. However, both, the *P. putida* lysate and lipase ceased degradation in presence of a lipase inhibitor, $HgCl_2$. The Hill function accurately predicted enzyme activity at various times and temperatures. Like lipase, the lysate also hydrolyses the p-nitrophenyl palmitate to p-nitrophenol. Kinetic parameters such as V_{max}, K_m and n values are good measures to characterize the lysate response with respect to changing paranitro phenyl palmitate levels. The substrate specificity test of lipase showed linear correlation between the absorbance at 410 nm vs amount of product paranitro phenol. The value of Km, Vmax and n are 0.62 mM, 355.7 μmol min^{-1} and 1.29, respectively. The lipase gene presence in *P. putida* was confirmed using PCR technique. These observations indicate that the main enzyme responsible for AFB1 degradation by *P. putida* is lipase. Thus, lipase as a multifunctional biocatalyst provides a promising future for a variety of industries and may also help to ensure the food safety by degrading the mycotoxins.

1. Introduction

Aflatoxins are well known common contaminants of staple crops. It is class one carcinogen with well established hepatotoxicity, immunotoxicity and teratogenicity. Decontamination of aflatoxins from food and feed is a challenge for scientists and technologists to improve the food safety. These are very stable organic molecules even at high temperature. Therefore bioremediation using natural enzymes may prove an effective way to degrade aflatoxins from food in mild conditions.

Aflatoxins are produced by field fungi like *Aspergillus flavus* and *A. parasiticus* (Miazzo et al., 2000; Oueslati et al., 2012). AFB1 consists of a tetra hydro-cyclopentanone ring with oxygen functionality at position 1, 4 and 11 (Stern et al., 2001). A large number of chemicals like bases, acids and oxidizing agents can react with aflatoxins and turn them into compounds that are either more or less toxic and mutagenic (Park et al., 1988; McKenzie et al., 1998). Me'ndez-Alborese et al. (2008) described that detoxification of AFB1 includes the development of a beta-keto acid structure in the presence of acidic medium, followed by lactone ring hydrolysis. After the reaction, aflatoxin D1 (AFD1) was produced by decarboxylation of the lactone ring, which is 450 times less mutagenic and has an 18-fold lower toxicity than AFB1. Samuel et al. (2014) proposed that lactone ring opened in the presence of *P. putida* resulted in the decarboxylation of AFB1 to AFD1 and AFD2. A study by Liu et al. (1998) reported that AFB1 is detoxified by opening the difuran ring by a multi-enzyme isolated from *Armillariella tabescens.* According to Motomura et al. (2003), some of the enzymes can cleave the lactone ring in resulting in loss of signatory fluorescence of AFB1. Reduction in the fluorescence spot on thin layer chromatograph (TLC), indicates the enzymatic cleavage of the lactone ring. It was reported that Lactonohydrolases, which catalyze hydrolysis (ring-opening) of lactone compounds,

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belong to the group of lipase and esterase family (Azerad, 1998; Laskin et al., 1998; Patel, 2000). There are some reports on other mycotoxin such as ochratoxin (OTA) hydrolysis. Enzymes such as amidases (EC 3.5), lipases (EC 3.1), and many commercial proteases (EC 3.4) are able to perform OTA hydrolysis (Stander et al., 2000; Abrunhosa et al., 2006). Lipase preparation from *A. niger* was shown to hydrolyze OTA. Laccase enzyme produced by *A. niger* was found to degarade 55% of AFB1 and showed significant loss of mutagenicity (Alberts et al., 2009). Laccases and manganese peroxidases from white rot fungi have been found to degrade aflatoxins resulting in a variety of metabolites (Motomura et al., 2003; Wang et al., 2011). Zeinvand et al. (2015) measured the laccase activity using the enzyme specific substrate; 2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The substrate specificity of enzyme assays showed a significant difference between laccase and lipase enzymes. Lipase nature of the enzyme was confirmed by assaying the cleavage of p-nitrophenyl palmitate (pNPP) (Stander et al., 2000). Lipase is widely known as an enzyme used for opening of lactone ring in several mycotoxins including deoxynivelanol, zearalenone, patulin (Yang et al., 2017; Li et al., 2017; Tang et al., 2018). Lipases (triacylglycerol hydrolases) are a distinctive form of enzyme class 3 esterases and have been allocated EC number 3.1.1.3 referring to their hydrolyzing specificity for carboxylic acid ester bonds (Patil et al., 2011). Lipases are found in nature profusely and have been found to be synthesized naturally by several bacteria including *Bacillus sp.*, *Acinetobacter sp.* (Iftikhar et al., 2011) and *Pseudomonas sp.* (Sharma et al., 2001). Lipase may be extracellular or intracellular in nature depending on the organism (Saxena et al., 2003). Lipases from microbial sources are of much more importance as they have reduced manufacturing costs, greater stability and accessibility than other sources (Kiran et al., 2008). Lipases are commonly produced on fatty acids, oils, glycerol or tweens (Gupta et al., 2004). It was discovered that lipid carbon sources are usually essential for achieving an elevated lipolytic enzyme output, although some scientists have achieved significant lipase yields without using fats or oils (Sharma et al., 2001). In the present study, aflatoxin was used as a substrate for the production of lipase enzyme by *P. putida*. Samuel et al. (2014) reported the AFB1 degradation by *P. putida* and the molecular analysis showed that degradation of AFB1 is due to lactone ring opening and the product AFD1 and AFD2 were identified from the culture medium. Further, it is reported that cell free lysate of *P. putida* can degrade AFB1 and degradation may be protein mediated as boiling of the lysate or the presence of SDS reduced its activity (Singh and Mehta, 2019). However, critical challenges such as identifying the enzyme and completely understanding the mechanism for AFB1 degradation by *P. putida* remains to be explored. The stability of an enzyme is the most important factor for its practical utility. Therefore, the thermostability of the *Putida* lysate was evaluated before studying the mechanistic detail and identifying the enzyme. The enzymetic activity of the lysate was confirmed on AFB1 along with pNPP a substrate known to study the lipase reaction. The kinetic study was also performed to know the effect of different concentrations of substrate over time and temperature on the lysate (enzyme) activity. Studies were performed in presence of $HgCl_2$, a lipase inhibitor. Presence of lipase gene in *P. putida* used in present study was confirmed by amplification of partial DNA with specific primer by PCR.

2. Material and methods

2.1. Microorganisms and culture conditions

AFB1 was produced and purified from the toxigenic strain *of Aspergillus flavus* (PP3) which was previously isolated and identified in our laboratory (Aiko and Mehta, 2013). *P. putida* strain (MTCC 2445) used in this study was procured from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India, and maintained on nutrient agar medium (HiMedia Laboratories Pvt. Ltd., India) at 4 °C. For experiments, *P. putida* strain (MTCC 2445) was cultured in mineral salt glucose medium (MSG) at 37 °C temperature and incubated in a shaker at 120 rev min^{-1} for different time intervals (Kulkarni and Chaudhari, 2006).

2.2. P. putida growth and degradation of aflatoxin B1 in the presence and absence of mercuric chloride

P. putida growth and AFB1 degradation was studied in presence of three different concentrations of $HgCl_2$ (6.4, 32 and 64 μg ml^{-1}). One millilitre of fresh *P. putida* culture at 0.5 OD_{600} corresponding to $1.86\times10^6 \pm 0.03$ CFU ml^{-1} was inoculated in 9 mL of MSG medium containing different concentrations of $HgCl_2$. The growth of the *P. putida* strain was monitored in terms of optical density (OD) at 600 nm by spectrophotometer for 24 h at different time intervals. A degradation study of AFB1 was carried out in MSG medium for 24 h following the method given by Samuel et al. (2014). The stock of AFB1 was prepared at a concentration of 200 μg ml^{-1} in dimethyl sulfoxide (DMSO). The final concentration of AFB1 in the medium was 0.2 μg ml^{-1}. AFB1 degradation was monitored in both presence and absence of the $HgCl_2$ using equation (1). Four experimental sets were prepared as given in Table 1. After each incubation period, the culture was centrifuged at 3000 rpm (Eppendorf centrifuge 5804 R, rotor A-4-44) for 10 min at 4 °C to separate the bacterial cells and supernatant. The supernatant was extracted with an equal volume of chloroform in a separating funnel. Extraction was repeated twice; the extracts were pooled and concentrated to a volume of 1 ml. The remaining AFB1 in the sample was analyzed qualitatively and quantitatively using TLC and UV-Vis spectrophotometer. All experiments were performed in triplicate and standard deviation was calculated.

The formula used for calculating % degradation of AFB1 in test:

$$\%\ \ D = \frac{Ac - At}{Ac} \times 100 \qquad (1)$$

where, D = degradation, A_c = amount in control (μg mL^{-1}), A_t = amount in test (μg mL^{-1}).

2.3. Cell lysate preparation and Thermal stability

Cells obtained from the fresh culture of *P. putida* are lysed in presence of protease inhibitor (PMSF). Cell lysis was performed using a sonicator at 4 °C for 4 pulse cycles with each lasting 10 min as described by Teniola et al. (2005). The lysed cells were centrifuged at 12,000 rpm for 20 min at 4 °C and supernatant was collected. This supernatant was further filtered through a Nupore 0.22-μm pore size syringe filter and stored at -80 °C for subsequent use. The thermal stability was assessed by incubating the lysate at a range of temperature between 20-100 °C for 24 h before checking the activity. The lysate was further incubated with AFB1 for 8 h to check the degradation activity. The controls were MSG medium processed in the same manner as the test. Quantification of AFB1 was carried out as described in section *2.2*.

2.4. Kinetic study using hill function

2.4.1. Para nitro phenol (pNP) standard graph

In order to determine the amount of product formed during the lipase reaction on para-nitrophenyl palmitate (pNPP), the standard

Table 1. Samples of *P. putida* were prepared in different conditions for experimental studies.

Set	Sample	Condition
I	Blank	MSG media
II	Control	MSG + AFB1
III	Test 1	MSG + Culture + AFB1
1V	Test 2	MSG + Culture + AFB1+$HgCl_2$

graph between absorbance and concentration of the product para-nitrophenol (pNP) was used. Known concentrations of pNP in the range of 0.01–0.2 mM were prepared from a stock of 100 mM pNP dissolved in milli Q water. Absorbance was measured at 410 nm using UV spectrophotometry.

2.4.2. Preparation of para-nitrophenyl palmitate (pNPP)

The hydrolysis of emulsified pNPP was assessed as described by Kordel et al. (1991). Prior to use, pNPP was equilibrated at room temperature. Solution A was prepared by dissolving 30 mM pNPP in 10 ml of 2-propanol. Solution B contained 50 mM Tris-HCI buffer (pH 8) with 0.4% Triton X-100 and 0.1% gum arabic. The solutions remained stable for about 2 weeks in a refrigerator. A fresh reaction mixture consisting of 1 part solution A and 9 parts solution B was prepared before the assay. To 900 µl of the reaction mixture, a 100 µl volume of an appropriate dilution of the lipase enzyme was added. Incubation time was optimized between 10-60 min. The product (pNP) formation was monitored by taking the absorbance at 410 nm (Gupta et al., 2002). The amount of enzyme that liberated 1 µmol of pNP from pNPP per min was defined as one unit of lipase activity.

2.4.3. The effect of substrate concentration (pNPP) on lysate activity

Different working concentrations of pNPP were used for the reaction, ranging from 0.1 to 5 mM. The reaction was performed at 37 °C in waterbath (Gupta et al., 2002). The absorbance was measured in the manner described section 2.5.2. The lineweaver-burk graph was plotted between 1/v and 1/s to calculate km (half saturation constant) and Vmax (maximum reaction velocity) value of the lysate using Michaelis-Menten (MM) Kinetics (see supplementary section A and B). Further, Hill function was used to fit the data. Three parameters Vmax, Km and n (hill coefficient) were calculated for enzymatic reaction rate vs. substrate concentration data.

$$V = V_{max}[pNPP]^n /(K^n + [pNPP]^n) \quad (2)$$

Where,

V = Enzyme activity.

V_{max} = Maximum enzyme activity.

K= Half-saturation constant

n = Hill coefficient.

2.4.4. Lysate reaction at varying time and temperature

Effect of time and temperature was studied on lysate activity using different substrate concentrations. The time and temperature interval ranged between 10-60 min and 20–100 °C, respectively. The concentration of pNPP was varied from 0.2 to 5 mM. The reaction volume was made up to 3 ml using Tris-Cl buffer (pH 7). Absorbance was measured at 410 nm. Hill equation [2] is used for fitting the enzyme activity vs substrate concentration data.

Furthermore, the thermal stability of lysate was also studied on pNPP. Pre-incubation conditions were kept the same as described in section 2.3. Reaction was performed with 30 mM pNPP as the substrate. Thermal stability of lysate was measured by monitoring absorbance at 410 nm of pNP released from the hydrolysis of pNPP (Griebeler et al., 2011).

2.5. Degradation of AFB1 by commercial lipase

Lipase enzyme (Triacylglycerol lipase from *Pseudomonas* sp) was procured from sigma-aldrich. The stock of lipase enzyme was prepared at a concentration of 20 mg ml^{-1} dissolved in 0.1 M Tris- Cl buffer (pH 7). For the experiments, five different concentrations of lipase enzyme were used in the range of 50–400 µg ml^{-1}. The final emulsion volume (0.1 M Tris-Cl, Triton X-100) was 3 ml. The final concentration of AFB1 in the medium was 0.2 µg ml^{-1}. The control tube is containing all the components except the lipase enzyme. All the tubes were incubated for different time interval (10, 30 and 60 min) at 37 °C. Chloroform extraction was performed as mentioned above. The absorbance was measured for AFB1 at 360 nm using UV-Vis spectrophotometer. The same experiment was performed in presence of lipase inhibitor, the Mercuric chloride ($HgCl_2$) at a concentration of 6.4 µg ml^{-1}. Further quantitative analysis was performed as described in section *2.2.*

2.6. Lipase gene identification

The lipase gene in *P. putida* (MTCC 2445) was identified by PCR technique using gene specific primers. The genomic DNA was isolated as described by Wilson (2001). A set of specific primers (forward 5'AAGATTGCTGCCAGGTGT3' and reverse 5'CCGGAAACACC TGATCTT3') were designed based on lipase gene sequence (NC_002947.4) from *P. putida*. PCR was performed using Taq DNA polymerase for 30 cycles. The annealing temperature kept at 53 °C. Agarose gel electrophoresis was done to check the expected band size of the amplified lipase gene.

2.7. Statistical analysis

All the experiments are performed minimum in triplicates and were analyzed by ANOVA using Microsoft Excel 2010 to know variation among and between test sets. A significant F-test at $p < 0.05$ level of probability was then reported. The results represent the average of triplicate and are expressed as mean ± standard deviation.

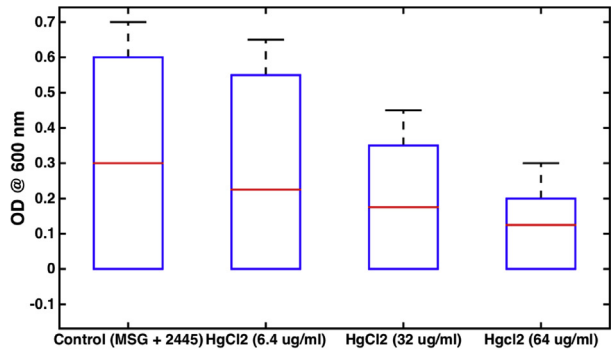
3. Results

3.1. Growth and AFB1 degradation by P. putida in the presence and absence of lipase inhibitor $HgCl_2$

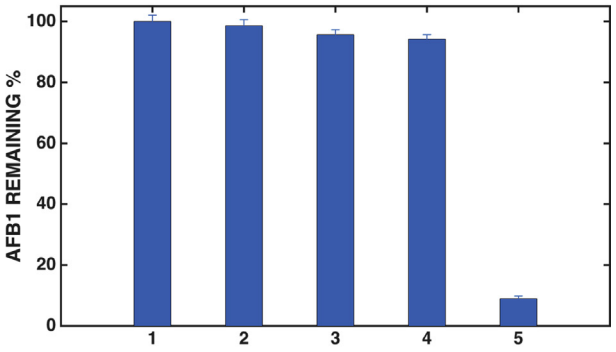
P. putida grows well in salt media (MSG) with an 8 h doubling time. The exponential growth continues till 24 h reaching the plateau (stationary phase) in 72 h (Singh and Mehta, 2019). *P. putida* is known to produce lipase enzyme which is involved in lipid degradation (Lee and Rhee 1993, 1994). To find, if the degradation of the aflatoxin by *pseudomonas* is mediated by lipase enzyme, experiments were carried out in the presence of $HgCl_2$ a well known inhibitor of lipase (Moriyama et al., 1999; Mukke and Chinte, 2012; Sabat et al., 2012). Studies were conducted in presence of 6.4, 32 and 64 µg ml^{-1} of $HgCl_2$. The concentrations of $HgCl_2$ (6.4 µg ml^{-1}) that inhibits the enzyme activity had no deleterious effect on the growth of *putida* (Figure 1a). However, 10 times higher concentrations retarded the growth by 50%. Our previous studies revealed that *P. putida* can degrade and detoxify AFB1 (Singh and Mehta, 2019). Further, studies were performed to identify the enzyme involved in the AFB1 degradation. The AFB1 degradation was calculated based on residual AFB1 content analysed by spectrophotometry. It is observed that *P. putida* degrades AFB1 very effectively. More than 85% reduction in AFB1 was observed within 24 h of incubation with *Putida* culture. Same time very insignificant AFB1 degradation was noted in presence of all the three concentrations of $HgCl_2$ studied. Less than 5% reduction in AFB1 content was measured after 24 h of incubation with 6.4 µg ml^{-1} of $HgCl_2$ (Fig. 1b, c). This experiment suggests that the reduction of AFB1 in culture medium may be due to the lipase enzyme produced by *P. putida*. In presence of inhibitor the lipase is unable to act on the AFB1, therefore AFB1 content remains same after incubation with *putida*.

3.2. Thermal stability of the lysate assayed with AFB1 degradation

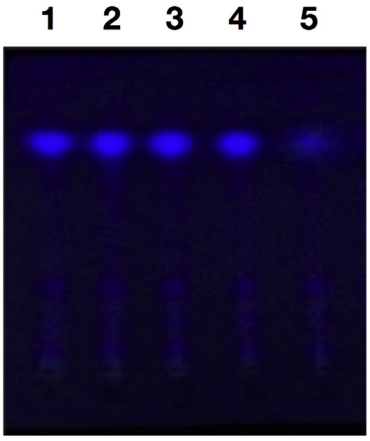
To know the thermal stability, the lysate was pre-incubated at various temperature ranging from 20 – 100 °C for 24 h before checking the activity in terms of AFB1 degradation. Lysate showed 84% AFB1 degradation after incubating at 30 °C. The AFB1 degradation capability



(a)



(b)



(c)

Figure 1. (a) Boxplots showing the growth of *P. putida* 2445 in absence and presence of $HgCl_2$ (6.4, 32, 64 μg/ml) after 24 h of incubation time. The values are means of triplicates. The central mark in red is median, edges of the boxes are the 25th and 75th percentiles, the whiskers extend to the most extreme data points. (b) Degradation of AFB1 by *P. putida* in the presence of $HgCl_2$ after 24 h of incubation- (1) AFB1 (Control) (2) AFB1+ *P. putida* + $HgCl_2$ (64 μg/ml) (3) AFB1+ *P. putida* + $HgCl_2$ (32 μg/ml) (4) AFB1+ *P. putida* + $HgCl_2$ (6.4 μg/ml) (5) AFB1+ *P. putida*. The values are the mean of triplicates. Error bars represent the standard deviations. (c) Silica gel Thin-layer chromatogram of aflatoxin B1after incubation for 24 h in the presence of $HgCl_2$- (1) Standard AFB1 (2) AFB1+ *P. putida* + $HgCl_2$ (64 μg/ml) (3) AFB1+ *P. putida* + $HgCl_2$ (32 μg/ml) (4) AFB1+ *P. putida* + $HgCl_2$ (6.4 μg/ml) (5) AFB1+ *P. putida*.

of lysate increased to 94% at 37 °C and reached 100% at 50 °C. Lysate maintained its activity up to 90 °C incubation temperature. These observations show that the lysate is not only stable at higher temperatures, but also increases its enzymatic activity after incubation at higher temperatures. At 100 °C it lost its activity and only 20% AFB1 degradation was observed (Table 2).

Table 2. Thermal stability profile of *P. putida* lysate prepared in the presence of protease inhibitor (PMSF) in terms of AFB1 degrading activity.

Temperature (° C)	AFB1 degradation (%) within 8 h
20	34.20 ± 0.01
30	84.20 ± 0.01
37	94.10 ± 0.03
50	100.00 ± 0.00
70	100.00 ± 0.00
90	90.00 ± 0.00
100	20.00 ± 0.00

Note: The experiment was performed in triplicates.

3.3. Activity check of the lysate using pNPP as substrate

Kinetic studies of lysate enzyme activity was calculated using Hill function. Hill equation was used to predict the rate of product formation in enzymatic reactions. As substrate concentrations increases enzyme activity increases as well, but in a non-linear fashion such that it eventually approaches a saturation level (V_{max}).

3.3.1. The effect of substrate concentration (pNPP) on commercial lipase and lysate activities

The enzyme activity in the cell lysate was measured on the substrate para nitro phenyl palmitate (pNPP) and compared with the commercial lipase enzyme. Lipase hydrolyzes the pNPP yielding yellow colored product para nitrophenol which can be measured in spectrophotometer at 410 nm. Absorbance vs. concentration of pNP data points was fitted using regression analysis. The linear correlation was observed between absorbance at 410 nm vs. amount of pNP. The R^2 value is ≥0.995 (supplementary section A, figure S1a, inset diagram). The absorbance of product has increased with the increasing amount of pNPP and gets saturated after 2 mM pNPP concentration as shown Figure 2. Moreover, the graph between the substrate (pNPP) concentration vs enzyme activity (μmol min^{-1}) is hyperbolic. At substrate conc 3.5 mM, lipase and lysate activities were 384.9 and 337.5 μmol/min respectively. The corresponding product concentration was calculated to be 0.56 mM using standard graph. Three parameters, namely, Vmax, Km and n are used to characterize the graphs. For instance, lipase and lysate activities vs pNPP data is fitted with corresponding values of Vmax = 405.70 μmol min^{-1}, Km = 0.62 mM, n = 1.29; Vmax = 355.7 μmol min^{-1}, km = 0.62 mM and n = 1.29, respectively. The n value being greater than one, suggests that the response is sensitive. Activities of both lipase and lysate are showing similar profiles with varying pNPP conc (commercial lipase activity being higher than lysate by a factor of 1.14). Michaelis menten equation was also used to fit the data (refer supplementary figure S1a).

3.3.2. Effect of time and temperature on activity of lysate

The graph between the substrate (pNPP) concentration (mM) vs. velocity (μmol min^{-1}) is hyperbolic for different time and temperature values. The reaction velocity increases with time. Highest velocity 596.7 μmol min^{-1} was obtained at 60 min and lowest velocity 411.1 μmol min^{-1} was noted after 10 min incubation time (Figure 3a). The Km, Vmax and n values were also calculated for data sets at different time points. Km increased with increasing pNPP concentration (Table 3). The reaction velocity has also shown a similar relationship with temperature. The highest reaction velocity 602.3 μmol min^{-1} was obtained at 70 °C followed by loss of activity at 100 °C (Figure 3b). The km, Vmax and n values were calculated as mentioned above and shown for the data sets obtained at different temperature (Table 4). Km and Vmax values

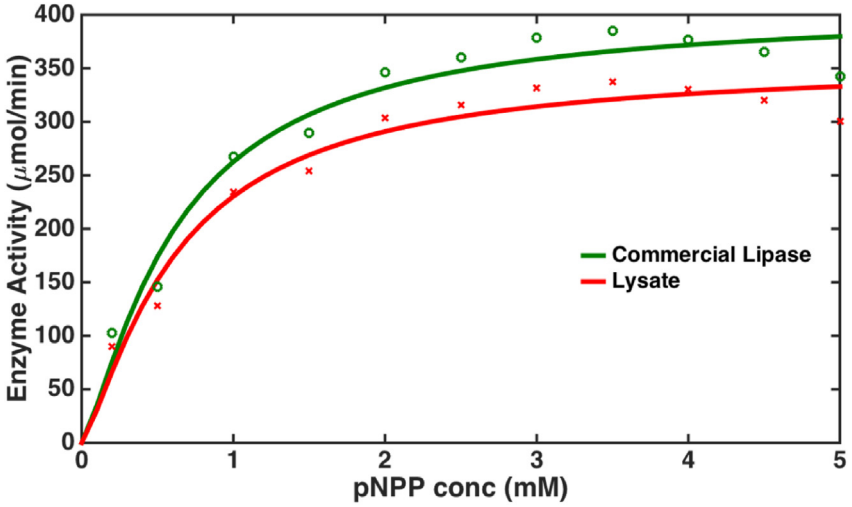


Figure 2. Lipase and lysate activity (μmol/min) vs pNPP concentration data fitted with a hill function. Corresponding parameter values for commercial lipase are; Km = 0.62, Vm = 405.70, n = 1.29, and for lysate are; Vm = 355.7 μmol/min, K = 0.62 mM and n = 1.29 respectively.

increase with rise in temperature whereas n decreases. The increase in Km values with time and temperature (up to 70 °C) shows that the enzyme activity response is getting amplified (shifting towards right). The decrease in n value indicates that the response is becoming less sensitive, whereas the increase in Vmax indicates that it is becoming saturated at higher levels.

Michaelis Menten equation was also used to characterize these graphs at different time points and temperatures, with the help of two parameters (Supplementary Figures S2 and S3) corresponding Vmax and Km values shown in supplementary Tables 1 and 2. Both Vmax and Km values increased with time from 10 to 60 min and with temperature between 20 to 70 °C.

3.4. Thermal stability of the lysate assayed on pNPP

The stability of the enzyme is the most important factor for the practical utility. Cell lysate showed excellent thermostability in the range of 20–90 °C measured in terms of hydrolysis of pNPP. The lysate not only retained its activity during pre-incubation for 24 h at these temperature but it increased. Highest activity (316.9 μmol min^{-1}) was noted in the sample incubated at 70 °C. Sample incubated at 90 °C showed slightly lower (304.7 μmol min^{-1}) activity indicating that thermal stability of lysate against pNPP increased with temperature from 30 °C to 70 °C, as determined by the amount of product formed (Table 5).

3.5. Degradation of AFB1 by commercial lipase enzyme in the absence and presence of inhibitor $HgCl_2$

To establish that the lipase from the *pseudomonas* is the key enzyme in the degradation of AFB1, studies were performed with commercially available lipase. It was observed that AFB1 degradation is time dependent as shown in (Figure 4a). Only about 10% reduction in AFB1 was noted in 10 min but more than 90% was observed in 60 min. Degradation is also dependent on the concentration of lipase enzyme in the reaction mixture. The degradation rate of 100 μg ml^{-1} was higher than that of 50 μg ml^{-1}. AFB1 degradation by lipase was studied in presence of inhibitor, $HgCl_2$ at varying enzyme concentrations and incubation times. In presence of $HgCl_2$ lipase could not degradeAFB1 and its concentration in the sample remains unchanged. Even increasing the concentration of lipase enzyme (200–400 μg ml^{-1}) did not result in a significant reduction in the amount of AFB1 (Figure 4b). These results are very well correlated with thin layer chromatogram (Figure 4c, d).

3.6. Identification of lipase gene

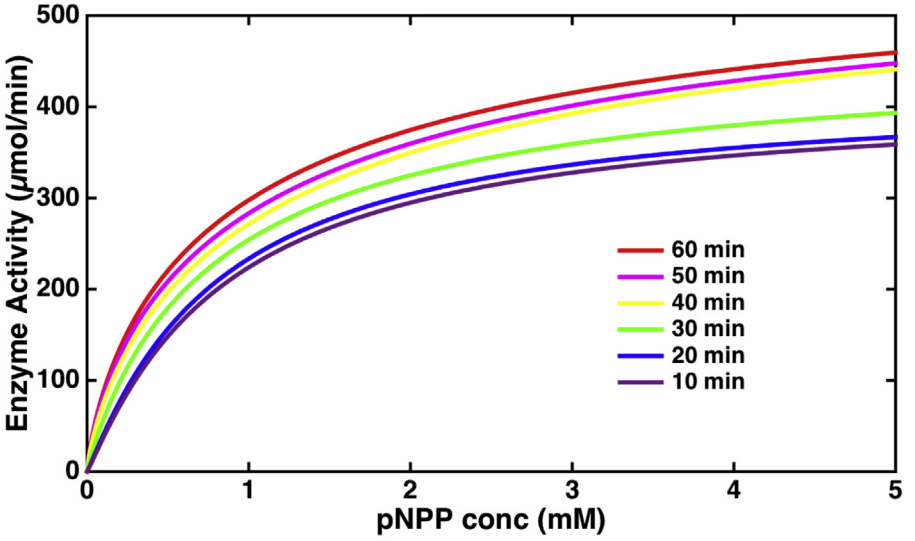
The results of PCR with lipase gene specific primers on an agarose gel showed amplification of the expected DNA size i.e. 690 bp (refer supplementary figure S4a, b), demonstrating the existence of lipase gene in *P. putida* strain (MTCC 2445).

4. Discussion

The molecular analysis of AFB1 degradation by *P. putida* showed the production of AFD1 and AFD2 due to lactone ring opening as described by Samuel et al. (2014). The *P. putida* cell lysate lost its activity on boiling as well as in presence of SDS indicating that AFB1 degradation may be protein mediated (Singh and Mehta, 2019). The quest to identify the enzyme involved led to the present study.

The cell free lysate of *P. putida* can degrade aflatoxins at high rate and in a wide range of temperature. In this study, we attempted to identify the enzyme in *P. putida* that is responsible for AFBI degradation. We discovered that *P. putida* lysate was effective at removing/degrading AFB1 in a variety of conditions. Moreover, the inhibitory effect of $HgCl_2$ was also studied on the degradation of AFB1. It was seen that *P. putida* strain (2445) was able to grow in presence of $HgCl_2$ though higher concentrations retarded the growth. However, culture lysate could not degrade AFB1 in the presence of inhibitor. Borkar et al. (2009) reported that lipase enzyme activity was decreased in the presence of metal ions such as Hg^{2+}, Zn^{2+}, Ag^{2+}, $Cu^{2+}$and Fe^{2+}. Similar results were found in our study, which showed that lysate activity was reduced for AFB1 degradation in presence of $HgCl_2$. There have been numerous biochemical and genetic studies on fungal and bacterial lipases until now (Verger 1984; Kirchner et al., 1985; Macrae and Hammond, 1985). The most important species are *Rhizomucor*, *Aspergillus*, *Penicillium* and *Geotrichum* involved in lipase production (Stocklein et al., 1993; Miura and Yamane, 1997). Lipase enzyme was reported in *P. putida* (Lee and Rhee, 1993; Jung et al., 2006; Fatima et al., 2014). Lipases in natural environment may be extracellular or intracellular. Nevertheless, the majority of bacteria are found to secrete extracellular lipases (Saxena et al., 2003). We also found the presence of lipase gene in *P. Putida* with the help of PCR analysis, indicating its involvement in AFB1 degradation.

In order to validate these results, experiments were also performed with commercial lipase. It showed faster AFB1 degradation rate compared to *P. putida* lysate. More than 80% AFB1 degradation was observed in 4 h by *P. putida* (lysate), similar degradation was noted within 1 h of incubation with commercial lipase. Furthermore, the AFB1



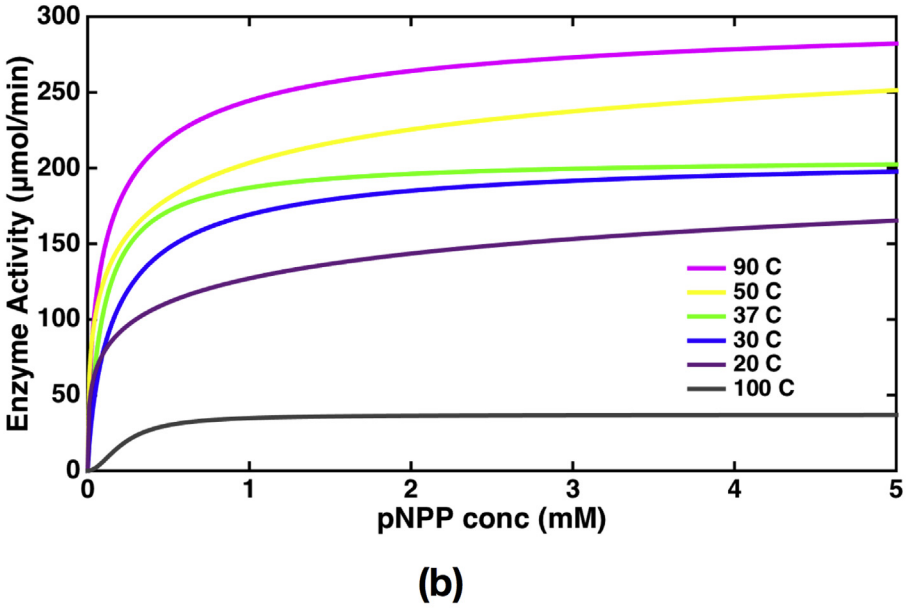


Figure 3. (a) Hill function plots representing the activity profiles of lysate at different time (10 min–60 min) with varying substrate (pNPP) concentration. Vmax increases with increase in time. (b) Hill function plots representing the activity profiles of lysate at different temperatures (20–100 °C) with varying substrate (pNPP) concentration. Vmax increases with temperature (except at 100 °C, where the value is minimum).

Table 3. Hill function parameters (km, Vmax and n) obtained for the fitted curves at different time points for lysate activity.

Time (min)	Km (mM)	Vmax (µmol/min)	n (hill coefficient)
10	0.80	411.1	1.08
20	0.82	417.8	1.08
30	0.86	465.2	0.94
40	1.13	592.6	0.76
50	1.24	594.5	0.74
60	1.32	596.7	0.70

Table 4. Hill function parameters (km, Vmax and n) obtained for the fitted curves at different temperatures for lysate activity.

Temperature (°C)	Km (mM)	Vmax (µmol/min)	n (hill coefficient)
20	1.11	434.2	0.68
30	1.58	498.9	0.65
37	1.70	574.5	0.62
50	3.45	597.4	0.55
70	4.29	602.3	0.50
90	3.62	594.3	0.52
100	0.23	37.0	1.87

Table 5. Thermal stability of lysate using pNPP substrate.

Temperature (° C)	Lysate activity (μmol/min)
20	210.5
30	293.6
37	309.0
50	310.3
70	316.9
90	304.7
100	27.7

degradation was inhibited by *P. putida* (lysate) in presence of $HgCl_2$, indicating the involvement of the lipase enzyme.

pNPP is a specific substrate used to assess lipase activity. In our study, lysate showed similar activity against para-nitrophenol palmitate (pNPP) substrate. The reaction kinetic studies with Hill function and Michaelis-Menten equation showed that km and Vmax values for lysate were comparable to those reported for lipase enzyme (Selvin et al., 2012). Lower km values indicate that the lysate has a high affinity for binding to the pNPP substrate. In a previous study, from our laboratory, the lysate activity was found to be stable in a wide range of organic solvents, as well as broad temperature and alkaline mediums (Singh and Mehta, 2019). The present study showed that *P. putida* lysate can degrade AFB1 in a wide-range of temperature. Thermal stability test by pre-incubating the lysate in temperature range (20–100 °C) showed that it remains active until 90 °C but lost it's activity at 100 °C. Presence of polyamines, hydrogen bonds, salt bridges and thermophilic amino acids leads to the higher thermal stability of lipase (Bora and Bora, 2012). *P. putida* lysate has shown wider thermal stability and activity at higher temperatures than other bacterial lipases (Kim et al., 2000; Kulkarni and Gadre, 2002). Many mechanistic studies on AFB1 degradation have reported the opening of lactone as the key step. The presence of lactone moiety is shared by several mycotoxins like Ochratoxin (OTA), Aflatoxins and Zearalenone (ZEN). Enzymes such as laccase, aflatoxin-oxidase (AFO), horseradish peroxidase, manganese peroxidase (MnP) are involved in the opening of lactone moiety present in mycotoxin. The degradation of OTA is aided by enzymes such as lipases, deoxygenases, amidases, and commercial proteases (Vanhoutte et al., 2016). Stander et al. (2000) described that crude lipase enzyme from *A. niger* was able to hydrolyze OTA. The microbial degradation of mycotoxin may involve a group of enzymes.

Lipase is widely known as an enzyme used for opening of lactone ring in several mycotoxins including deoxynivelanol, zearalenone, patulin (Yang et al., 2017; Li et al., 2017; Tang et al., 2018). Similarly, lipase derived from *Pseudomonas fluoresence* is responsible for lactone ring opening (Uyama and Kobayashi, 1993). It is already reported that opening of the lactone ring reduces the toxicity of aflatoxin (Samuel et al., 2014). The inhibition of the lysate activity in presence of $HgCl_2$, hydrolysis of pNPP by the lysate and presence of lipase gene in *P. putida* demonstrates that the active enzyme constituent in the lysate responsible for AFB1 degradation is the lipase.

The stability of an enzyme is the most important factor for its practical utility. The outstanding thermostability shown by cell lysate at higher temperatures may find application for the degradation of AFB1 during food processing.

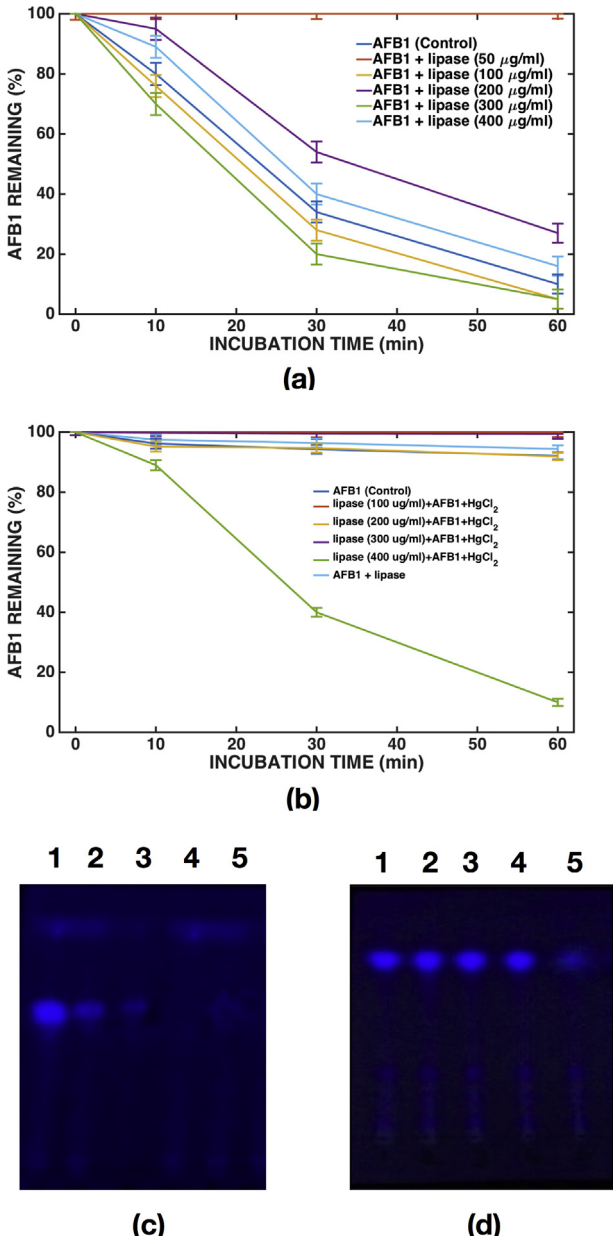


Figure 4. (a) AFB1 degradation by commercial lipase enzyme without inhibitor $HgCl_2$ during 1 h of incubation. The values are means of triplicates and the error bars represent the corresponding deviations. (b) AFB1 degradation by commercial lipase enzyme in the presence of $HgCl_2$, with 1 h of incubation. The values are means of triplicates and the error bars represent the corresponding deviations. (c) Silica gel Thin-layer chromatogram of aflatoxin B1 after incubation for 1 h in the presence of commercial lipase (1) Standard AFB1 (2) AFB1+ lipase (50 μg/ml) (3) AFB1+ lipase (100 μg/ml) (4) AFB1+ lipase (200 μg/ml) (5) AFB1+ lipase (300 μg/ml). (d) Silica gel Thin-layer chromatogram of aflatoxin B1after incubation for 1 h in the presence of Commercial lipase and $HgCl_2$ (6.4 μg/ml). (1) Standard AFB1 (2) AFB1+ Commercial lipase (100 μg/ml)+$HgCl_2$ (3) AFB1+ Commercial lipase (200 μg/ml)+$HgCl_2$ (4) AFB1+Commercial lipase (300 μg/ml)+$HgCl_2$ (5) AFB1+Commercial lipase (in sequence from left to right).

5. Conclusions

Aflatoxin B1 can be efficiently degraded by the cell free lysate of *P. putida*. The main enzyme responsible for the degradation is lipase. The cell lysate is thermostable and retains the activity even after incubation at 90 °C. The maximum degradation was observed between 50 –70 °C. The commercial lipase also showed the similar activity as *P. putida* lysate and in both cases the activity was ceased in presence of inhibitor $HgCl_2$. Biochemical process and mechanisms shall be focused sharply in future findings. The mechanistic approach involved in AFB1 degradation in presence of lipase inhibitor ($HgCl_2$) by *P. putida* lysate need to be studied. To fully comprehend the mechanism, researchers need to investigate how lipase enzymes is involved in opening the lactone ring. The microbial lipases are used in many industries like detergent, food, flavour, Pharmaceuticals, and its use can be extended to ensure the food safety from the mycotoxins.

Declarations

Author contribution statement

Prof. Alka Mehta: Conceived and design the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ms. Jyoti Singh: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

The data that has been used is confidential.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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