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Utilization of *S. aureus* strain 502A in biodegradation of insecticide acetamiprid from wetland wastewater

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ABSTRACT

In the present context of wastewater treatment, biodegradation-based techniques are well known as a low-cost and environment friendly approach, for cleaning up various agro effluents. Likewise, the efficiency of indigenous *Staphylococcus aureus* strain 502 has been investigated in the study for removal of toxic insecticide acetamiprid (ACE) from wetland wastewater. The isolation and enrichment was done in minimal salt media enhanced with 50 mg L^{-1} of ACE as sole carbon, nitrogen, and energy source, incubated at 35°C in 100 rpm for about 5 d. Central composite design (CCD) was applied to predict the optimal condition of ACE removal. The correlation of the operating variables and the response based on CCD were fitted to quadratic equation. The mathematical model fitting of growth curve of the isolated bacteria was also studied. The strain *S. aureus* 502A was observed to have maximum consumption of 61.68% of ACE in 24 h, analyzed using GC–MS. The rate kinetic analysis was studied using zero-order and three half-order kinetic models. Among the identified metabolites, Benzothiazole, 2-(2-hydroxyethylthio) with an effective peak at retention time 7.00 min was observed to be the end product of ACE biodegradation. Further, toxicological analysis on *Bacillus subtilis* exhibited no inhibition zone, suggesting the non-toxic nature of the degraded metabolite.

Keywords: Isolation; Biodegradation; Acetamiprid; Central composite design; Growth model; Rate kinetics; Quantification; Metabolite

1. Introduction

The rapidly growing industrialization along with an increasing population has resulted in the accumulation of a wide variety of toxic agrochemicals. The frequency and widespread use of man-made "xenobiotic" chemicals has led to a remarkable effort to implement new technologies to reduce or eliminate these contaminants from the environment [1]. Pesticides are being used by human beings to protect crops since ages. Since nineteenth century onwards, the worldwide production of pesticides has increased manifold because of increasing population and requirement of food grains. Excessive use of pesticides has resulted in polluting the surface and ground waters [2–4]. Agriculture, being the fundamental lynchpin of Indian economy, alarming levels of

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pesticides has been reported in air, water, soil as well as in foods and biological materials. Some of these pesticides have also been reported to be toxic, [5] mutagenic, carcinogenic, and tumorogenic [6,7]. The consumption of technical grade pesticides in India during 2000–2001 was 45,580 MT. Among the insecticides, carbamates, organochlorines, imidacloprids, and organophosphates top the list of pesticides in Indian market.

The experimental evaluation of chemical degradation in the environment is highly complicated due to multiple reasons. These include the variation of moisture, temperature, chemical, and microbiological composition of soil, ability of a chemical to volatilize and photo-degrade. The assessment test for disposability is expensive and usually time consuming [8]. By their design itself, most pesticides are highly toxic [9]. They are usually not mineralized or broken down to non-toxic products. Pesticides which drain into the water bodies through agricultural run-off and disposal of industrial wastewaters affect all aquatic fauna, especially fishes [10].

Bioremediation constitutes an attractive alternative to physicochemical methods of remediation, as it is less expensive and can selectively achieve complete destruction of organic pollutants [11]. In bioremediation, microbes that can degrade the pesticides *in situ* are used. For a successful bioremediation technique, an efficient bacterial strain that can degrade largest pollutant to minimum level is required. The survival of these indigenous bacteria under pesticide stress can provide an efficient, cheaper, and eco-friendly solution for remediation of the polluted wetland water.

Neonicotinoid insecticides are one of the most important commercial insecticides used worldwide. They are systemic broad-spectrum insecticides which exhibit a novel mode of action because they are agonists of the nicotinic acetylcholine receptor leading to paralysis and death of pest organisms [12–14]. These compounds are active against numerous sucking and biting insect pests, including aphids, whiteflies, thrips, leaf miners, beetles, and a number of coleopteran pests [15,16]. In this chemical class, acetamiprid (ACE), a chloropyridinyl neonicotinoid, was considered to be a favorable choice for controlling those pests that are severely resistant to organophosphorus, urethane, and synthetic pyrethroid pesticides; so it is regarded by EPA as an important substitute of organophosphorus pesticides [17]. Owing to its broad insecticidal spectrum and relatively low acute and chronic mammalian toxicity, ACE is used widely in crop protection [18,19]. Because of the widespread use of ACE in many areas and its potential toxicity to humans [20,21], the residues present in the environment have received considerable attention, and methods for the biotransformation of neonicotinoids are being actively researched [17].

Considering the necessity for advancement of green technology in order to protect environment, it is felt that commercial application of the insecticide-resistant bacterial strain in agriculture will greatly help to eliminate the toxic effects of frequently used chemical insecticide in the wetland areas. Likewise, in the present study, efforts were made to isolate potent bacterial strain capable of degrading ACE. Biodegradation of ACE was confirmed using GC–MS analysis. Estimation of specific growth rate is highlighted using the experimental data obtained for biomass concentration of the exponential growth phase and mathematical growth model is deduced. Emphasis is given on the effect of operating variables and their interactive effect on the overall biodegradation efficiency and optimization of the process conditions to maximize the overall removal percent of ACE insecticide from aqueous solution using central composite design (CCD). This study also involves assessment of the related product toxicity.

2. Materials and methods

2.1. Collection of agro wastewater sample

The wastewater sample was collected from Mathpukur, East Kolkata Wetlands, West Bengal, (Ramsar site No. 1208, 22°25′40′′ North, 88°22′55′′ East) India. It was stored at 0°C; the agricultural area has a history of repeated ACE application. The wastewater sample was collected from an excess running water flow channel from the agricultural land.

2.2. Chemicals and media

Analytical grade ACE (99% purity) was purchased from Sigma Aldrich; analytical-grade dichloromethane and ethyl acetate were purchased from Merck, Mumbai, India. Minimal salt media (MM) which is used as a selective media contained 1.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 1.0 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$, pH 7.0. ACE (50–300 mg L^{-1}) was added to MM as required. For MM agar preparation, 16 g L^{-1} of agar for bacteriology was added to the MM broth supplemented with ACE. Luria-Bertani (LB) media which is used as a basal media contained 10.0 g L^{-1} tryptone, 5.0 g L^{-1} yeast extract, and 10.0 g L^{-1} NaCl, pH 7.0. ACE (50 mg L^{-1}) was added as an additional component in the LB media. The strain *Bacillus subtilis* (NCIM No. 2655) was purchased from National Collection of Industrial Microorganisms (NCIM), Pune, India, to assess the toxicological

activity of ACE biodegraded metabolite. All other chemicals were obtained from Merck and Hi-Media.

2.3. Enrichment and isolation of ACE degrading bacteria

Enrichment and isolation was primarily done by inoculating 1 mL of the agro wastewater sample in 100 mL of MM media, containing 50 mg L^{-1} of ACE as the sole carbon, nitrogen, and energy source and 100 mL of the basal media (Luria-Bertani broth) with 50 mg L^{-1} of ACE. These were incubated at 40˚C on a rotary shaker at 100 rpm for about 7 d. Later 1 mL of both the enrichment cultures was transferred into 100 mL of fresh enrichment medium similar in composition as the previous media and incubated for another 7 d. Subculture slants were made on MM agar and LB agar supplemented with 50 mg L^{-1} of ACE after four rounds of transfer. After incubation at 40˚C, different colonies formed were picked up, further purified using the streak plating method, tested for their ACE-degrading ability to 50 mg L^{-1}. One potent strain, possessed the highest ACE degradation efficiency, was selected for further investigation.

2.4. Identification and characterization of isolated bacterial strain

The isolated bacterial strain was identified on the basis of morphological, 16S rRNA sequence, and biochemical properties [22] by referring the *Bergey's Manual of Determinative Bacteriology* for the tests. The cell morphology was examined by inverted microscope (Optika XDS-2) and Field Emission Scanning Electron Microscope (FESEM, JOEL JSM 6700F), using cells from an exponentially growing culture. The 16s rRNA gene sequencing was performed by BioAxis DNA Research Centre (Pvt.) Ltd. Hyderabad, India. The monoculture slants (MM agar & LB agar supplemented with 50 mg L^{-1} of ACE) were used to isolate the bacterial DNA using the standard protocol for it. Further, a pair of universal primers were made to amplify the 16S rRNA gene [17] and the sequencing was performed by ABI 3730xl Genetic Analyzer and later the 16S rRNA sequence analysis was done using BLAST and the phylogenetic tree was made using Clustal W2- Phylogeny. Also the sequences were later submitted to GenBank using the online submission tool Bankit. The nucleotide sequence of the isolated strain deposited in the GenBank database was under accession number KJ782460.

2.5. ACE biodegradation study in batch culture

The isolate growing in MM medium for about 8 h was harvested by centrifugation (10,000 rpm, 10 min) and washed twice with MM. After the cell density had been adjusted to about 1.0 × 109 (CFU mL^{-1}), an aliquot (2%, v/v) was inoculated into 200 mL of MM supplemented with 50 mg L^{-1} of ACE as the sole carbon, nitrogen, and energy source in a 500 mL Erlenmeyer flask. The cultures were incubated at 40˚C and 100 rpm on a rotary shaker. At an interval of 60 min, 2 mL of sample was taken for analysis. Bacterial growth was observed by measuring the colony-forming units (CFU mL^{-1}) and concentrations of ACE were determined by spectrophotometer following the protocol described below. Each treatment was performed in triplicates and the control sample without any inoculation and substrate were carried out under similar conditions.

Initially, 10 mL stock of different ACE dilutions was prepared and mixed with equal volume of Britton Robinson Buffer, kept for 10 min undisturbed after vigorous shaking and then optical density was measured at 269 nm (which is the λ_{max} for ACE) using the UV–vis spectrophotometer (Perkin-Elmer Lambda 25). The standard calibration curve was obtained by plotting the graph between the optical density and the different concentration of ACE. A triplicate of batch cultures were prepared by varying the different process parameters and incubated accordingly so as to determine the percentage of ACE degraded by the isolated bacteria.

The degrading efficiency of ACE was studied by varying pH of batch cultures for both the enrichment media (MM broth and LB broth, supplemented with 50 mg L^{-1} of ACE) from 2 to 10 and kept under constant process parameters (temperature 35˚C, agitation speed 100 rpm, and incubation time 28 h) followed by spectrophotometric quantification.

The degrading efficiency of ACE was studied by varying temperature of batch cultures for both the enrichment media (MM broth and LB broth, supplemented with 50 mg L^{-1} of ACE) from 10 to 80˚C and kept under constant process parameters (pH 7, agitation speed 100 rpm, and incubation time 28 h) followed by spectrophotometric quantification.

The degrading efficiency of ACE was studied by varying agitation speed of batch cultures for both the enrichment media (MM broth and LB broth, supplemented with 50 mg L^{-1} of ACE) from 0 to 120 rpm and kept under constant process parameters (pH 7, temperature 35˚C, and incubation time 28 h) followed by spectrophotometric quantification.

The degrading efficiency of ACE was studied by varying incubation time of batch cultures for both the enrichment media (MM broth and LB broth, supplemented with 50 mg L^{-1} of ACE) from 0 to 30 h, and kept under constant process parameters (temperature 35˚C, agitation speed 100 rpm, and pH 7) followed by spectrophotometric quantification.

The degrading efficiency of ACE was studied by varying initial concentration of ACE (mg L^{-1}) of batch cultures for both the enrichment media (MM broth and LB broth, supplemented with 50 mg L^{-1} of ACE) from 30 to 300 mg L^{-1} and kept under constant process parameters (temperature 35˚C, agitation speed 100 rpm, incubation time 28 h, and pH 7) followed by spectrophotometric quantification.

The results were expressed as the degrading efficiency (%*E*) of ACE by the isolated bacterial strain or the %ACE consumption, which was quantified using the following equation:

$$\%E = 100 \times (C_0 - C_f/C_0) \tag{1}$$

where C_0 is the initial ACE concentration (mg L^{-1}) and C_f is the concentration of ACE after degradation (mg L^{-1}).

2.6. Experimental design procedure

The optimum process parameters derived (initial ACE concentration, temperature, agitation speed, and pH) for both the enrichment media (MM broth and LB broth, supplemented with 50 mg L^{-1} of ACE) were given as the input in the CCD in Design Expert 8.1 Software (Stat- Ease, Inc., Minneapolis, USA), different combinations of the four parameters were obtained and then according to those batch cultures were inoculated and kept for incubation for 28 h and after treatment, the cultures are filtered and the spectrophotometric quantification was done so as to estimate the amount of ACE consumed by the bacteria. The percentage of ACE consumed was given as the response for the different combinations.

In order to examine the effects of individual parameters as well as their relative effects on the response variable, a general second-order polynomial model was selected and is deduced by equation depicted below:

$$y = b_o + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} b_{ii} X_i^2 + \sum_{i=1}^{4} b_{ii} X_i^3 \sum_{j=1}^{4} b_{ij} X_I X_j \tag{2}$$

where "*y*" is the response variable, b_o is the constant, b_i is the linear coefficient, b_{ii} the quadratic coefficient, b_{ij} the interaction coefficient, and X_i is dimensionless coded variables (X_1 depicted for initial ACE concentration, X_2 for temperature, X_3 for agitation speed, and X_4 for pH). The regression equation above was considered for optimization to maximize '*y*' using numerical optimization program of the same design software. Response surface methodology was used to maximize the percentage degradation of ACE. To ascertain the reproducibility of the data all experimental runs were conducted in triplicate.

2.7. Analysis of kinetic parameter

The ACE batch biodegradation study leads to the formation of biomass and the amount of biomass formed increases exponentially with respect to time during the log phase. Further, the increase in biomass concentration depends on the depletion of substrate concentration. As ACE biodegradation is the result of bacterial activity, the kinetics of the degradation process is related to the specific growth rate of micro-organisms, which in turn is proportional to the number of micro-organisms present at a given time in log phase. The experimental data of log phase so obtained for biomass concentration during the kinetic study were used to estimate the specific growth rate (μ) by Eq. (2).

$$\mu = \frac{1}{x}\frac{dx}{dt} \tag{3}$$

where x is the biomass concentration (mg L^{-1}) at time t (h), and dt is the change in time (h). Eq. (3) is as derived after integrating.

$$\ln x = \ln x_0 + \mu t \tag{4}$$

where x_0 is biomass concentration (mg L^{-1}) at $t = 0$. A plot of ln x against t gives a straight line with ln x_0 as its intercept and μ as the slope. Data for the other phases (log, stationary, and death) are not considered since no growth was observed. In general, the experimental values of specific growth rates so obtained were used to evaluate the growth kinetic model. Here, the Gompertz modeling of the growth curve was performed using Origin Pro 8.5.0 SR1 software (Origin Labs). The Gompertz function is based on an exponential relationship between specific growth rate and population density. Eq. (5) describes this model

$$Y = ae^{-e^{(-k(t-t_c))}} \quad (5)$$

where Y is ln(N/No), N is the population density at time t, N_o is the population density at time $t = 0$, t is the time, a is the upper asymptote, t_c is the time at which absolute growth rate is maximal, and k is the relative growth rate at t_c time.

2.8. Rate kinetic modeling

In biodegradation processes, the rate of disappearance of substrate ACE is dependent on the substrate concentration and the change of substrate concentration with time can be described by zero-order, first-order, second-order, and three half-order rate kinetics. Likewise, biomass is produced simultaneously as substrate concentration decreases [23]. In general, the first-order and second-order kinetics are not widely considered in biodegradation studies, since they do not take into account the biomass growth; therefore explanation of rate kinetics become unsatisfactory. These conceptual and mathematical difficulties led to the single deterministic model known as three half-order kinetic model [24]. It includes the term to explain biomass formation, which can be measured in terms of "p".

In the present context, the model was based on the assumption of the first-order kinetics with the introduction of an additional term that gives the detailed kinetics of biomass formation. The rate of ACE biodegradation is given by Eq. (6).

$$\frac{ds}{dt} = -k_1 S - aES \quad (6)$$

where k_1 is the proportionality constant (per unit time), E is the cell concentration, and a is proportionality constant (per unit biomass concentration per unit time). Eq. (7) is as derived after integrating and simplifying.

$$y = -k_1 - \frac{k_2 t}{2} \quad (7)$$

where $k_2 = aE/t$

$$y = \frac{1}{t}[\ln(S_0 - p + k_0 t)/S_0]$$

$$p = S_0\left(1 - e^{-k_1 t - \frac{k_2 t^2}{2}}\right) + k_0 t$$

In this equation, k_0 and S_0 are zero-order rate constant and substrate concentration at zero time, respectively. The equation contains four unknown parameters and is highly non-linear. Origin Pro 8.5.0 SR1 software (Origin Labs) was used to solve the equations. The parameters obtained by zero-order kinetics are deduced in Eqs. (8) and (9).

$$\frac{ds}{dt} = -k_0 \quad (8)$$

$$S = S_0 - k_0 t \quad (9)$$

where, k_1 and k_2 are formed by plotting y against t, which gives a straight line using the log phase data. The intercept and slope of the straight line is given by k_1 and k_2, respectively. The rate of product formation given by p is directly related to the change in biomass concentration.

2.9. Chemical analysis

The chemical analysis of ACE degradation process was performed in an attempt to predict the most probable secondary metabolite that is produced by the bacteria after degrading the pesticide. The ACE biodegradation analysis was confirmed using gas chromatography mass spectrometric technique. From the literature reviewed, analytical standard grade ACE was extracted with an equal volume of dichloromethane. The extracts were pooled, dehydrated with anhydrous Na_2SO_4, and then concentrated by rotary evaporator (Rota vapor, BUCHI). After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel using ethanol as eluent to afford the metabolite as yellow solid. The product was dissolved in a small amount (1 mL) of acetonitrile [17]. The resulting organic phases were decanted, and then dehydrated by Na_2SO_4 and were kept overnight. Later, the organic phase was concentrated by rotary evaporator (Rota vapor, BUCHI). Further, the residue was filter sterilized and analyzed in GC–MS analyzer.

A GC system (Thermo Scientific, 1300 series GC) coupled with a time of flight MS (ISQ single quadrupole MS) detector through an EI (Electron Ionization) interface was used for the present study. The column used for analysis was 30 m × 0.25 mm TG-5MS capillary column (for non polar solvents). Carrier gas used was helium (purity 99.999%) with a flow rate of 1.6 mL min^{-1}. A 1 mL aliquot of the extract was injected using the splitless mode. Temperature programming was initialized as: oven temperature held at

120°C for 1 min, ramped up at a rate of 25°C min^{-1} to 275°C and held for 3 min. The total runtime of the GC is 9.5 min. The injector temperature was set at 300°C The MS transfer line temperature was held at 250°C and ion source temperature was held at 200°C for 4 min. The mass range for detection was listed as 50–500 amu with a dwell time of 0.2 s. Solvent delay for MS is 5 min [25]. Further, sample analysis, mass measurement, and elemental compositions were performed using NIST MS library 2012 spectra.

2.10. Characterization and toxicological analysis of ACE biodegraded metabolite

To illustrate the biodegraded metabolite of ACE, the mass spectra analysis was done in (ISQ single quadrupole MS). In the present context, the toxicity potential of ACE biodegraded metabolites is tested on *B. subtilis* (NCIM No. 2655). The subculture of *B. subtilis*, grown in nutrient broth (5 g peptone; 3 g beef extract; 3 g yeast extract and 5 g NaCl in 1L distilled water at pH 7.4). The test was performed by autoclaving the Muller Hinton media, along with the petriplates at 121°C at 66.68 Pa for 20 min. About 5 mL of *B. subtilis* culture broth suspension was poured into the Muller Hinton agar media. The bacterial culture suspension along with the media was mixed well such that confluent lawn of growth would result on incubation. Small paper discs were punched using punching machine and dipped in 0.2 mL of respective pure sample of ACE, half diluted sample of ACE as well as in the biodegraded product of ACE. The small paper discs were then placed on the solidified inoculated media. Plates were then incubated for 20–24 h at 37°C prior to observation of inhibition zone.

3. Results and discussion

3.1. Strain isolation and characterization

ACE has been widely used in the control of numerous biting and sucking insect pests in the agricultural fields of East Kolkata Wetlands, West Bengal, India. ACE residues were frequently found in water and soil of that area; therefore, it is apparent that several bacteria have adapted to this ACE-contaminated environment. Using ACE as the primary source of essential components of carbon, nitrogen, and energy, several ACE degrading bacterial strains were isolated and one strain was selected for further study because of its potentially higher degrading efficiency. The selected strain was a non-spore forming, Gram positive, non-motile, cocci bacterium. Colonies grown on MSM agar were circular, irregular, and light yellow in color after 24 h incubation at 35°C.

FESEM imaging showed the magnified images of the bacterial strain. FESEM imaging was to identify the morphology, where bacterial cell length and width were 1.7 ± 0.22 μm and 0.7 ± 0.33 μm, respectively, with length/width ratio of 1:2 (Fig. 1).

Growth was observed at a variable range of temperature from 4 to 50°C (optimum at 40°C), a salinity range of 0–10% NaCl (optimum 0.7% NaCl) and a pH range of 3–9 (optimum 5). Biochemically; it showed positive results for coagulase, catalase, nitrate reduction, and carbohydrate oxidation showed fermentation

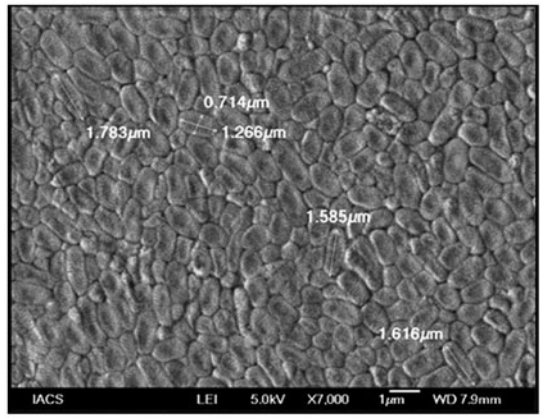
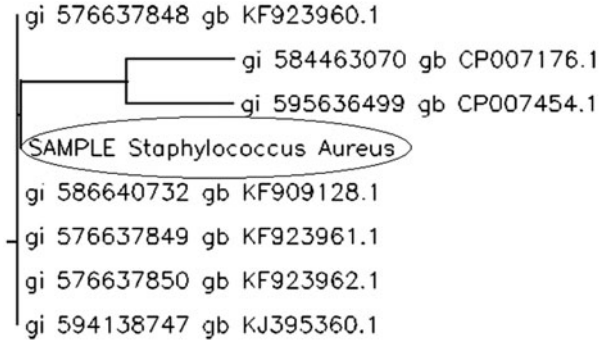


Fig. 1. FESEM micrographs of the isolated bacterial strain.



Clustal Distance Matrix

		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
gi_576637850	(1)	0.000	0.001	0.000	0.000	0.001	0.000	0.579	0.668
gi_594138747	(2)	0.001	0.000	0.001	0.001	0.001	0.000	0.579	0.667
gi_576637849	(3)	0.000	0.001	0.000	0.000	0.001	0.000	0.579	0.668
gi_576637848	(4)	0.000	0.001	0.000	0.000	0.001	0.000	0.579	0.668
gi_586640732	(5)	0.001	0.001	0.001	0.001	0.000	0.000	0.578	0.666
SAMPLE 2	(6)	0.000	0.000	0.000	0.000	0.000	0.000	0.565	0.686
gi_595636499	(7)	0.579	0.579	0.579	0.579	0.578	0.565	0.000	0.556
gi_584463070	(8)	0.668	0.667	0.668	0.668	0.666	0.686	0.556	0.000

Fig. 2. Phylogenetic tree constructed based on 16s rRNA gene sequences, also showing position of the isolate characterized in this study. The clustal distance matrix is also illustrated.

in Mannitol salt agar and phenol tolerance, and showed negative results in 70% ethanol tolerance, arginine dihydrolase, and oxidase tests.

Phylogenetic analysis of the 16S rRNA gene sequence revealed that the isolated bacterial strain grouped among *Staphylococcus* species and formed a subclade of *S. aureus* with represented identities = 100% (Fig. 2). Thus, on the basis of the results of phenotypic, genotypic, and phylogenetic properties, the isolated bacterial strain was identified as *S. aureus* strain 502A. The 16S rRNA gene sequence (481 bp) of the isolate was deposited in GenBank with the Accession No. KJ782460.

3.2. Effect of different biodegradation parameters

3.2.1. Effect of pH

In order to maintain polarity inside and outside the bacterial cell, pH of the batch affects the bacterial growth. Since ACE is the sole carbon, nitrogen source which is to be degraded, therefore, at the specific optimum pH the bacteria degrades it with maximum efficiency. For *S. aureus* optimum pH was determined at 5 (Fig. 3).

3.2.2. Effect of temperature

The ACE biodegradation process was observed to gradually increase with increasing temperature from 10˚C. As the bacterial growth is directly affected by the temperature requirements, the degradation was found to be optimum at 35˚C (Fig. 4). Here the bacteria degraded ACE with maximum efficiency. Further, with high temperature, it is probable that bacterial proteins get denatured and the metabolism gets hindered, therefore, the growth of *S. aureus* subsided along with the pesticide degradation process. For *S. aureus*, the optimum temperature for ACE degradation was determined at 35˚C.

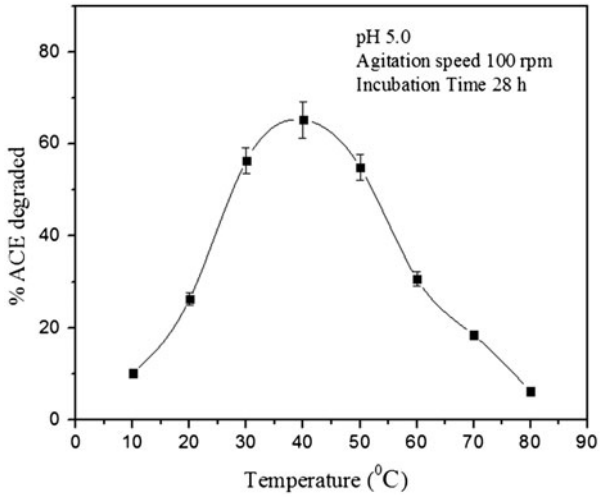


Fig. 4. Effect of temperature on % ACE biodegraded.

3.2.3. Effect of agitation speed

Aeration is one of the important factors determining the aerobic bacterial growth along with depended ACE biodegradation process. Growth of *S. aureus* was observed to increase along with the increasing ACE degradation process from minimum agitation speed of 0 to an optimum speed of 80 rpm. As the agitation

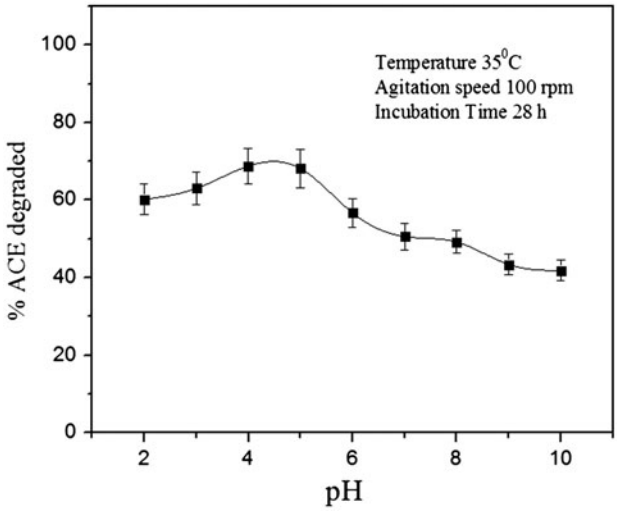


Fig. 3. Effect of pH on % ACE biodegradation.

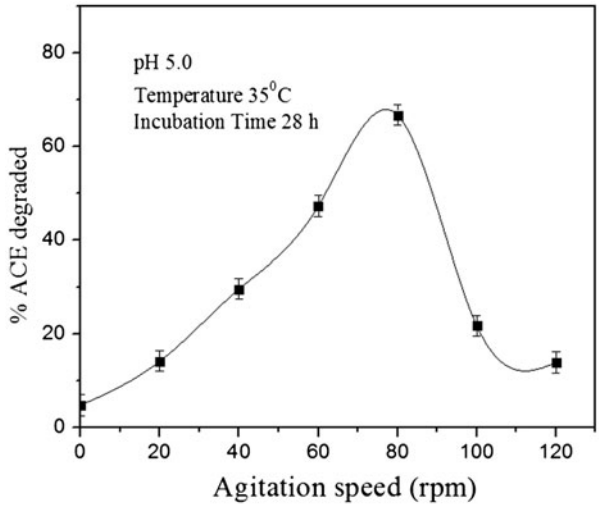


Fig. 5. Effect of agitation speed on % ACE biodegraded.

speed increased from 100 to 120 rpm (Fig. 5), growth reduced due to higher sheer stress and intercellular collision, thereby reducing the degradation of ACE.

3.2.4. Effect of incubation time

The bacterial degradation process accelerates with time. *S. aureus* was observed to have a slow exponential phase as acclimatization with media and substrate took a longer time and entered the stationary phase at about 25 h, since it reached its maximum capacity to degrade the substrate. Fig. 6 showed the percentage of ACE consumed with respect to time; *S. aureus* showed optimum incubation time at 26–28 h with a 72% ACE consumption.

3.2.5. Effect of initial ACE concentration

With the increase in concentration of ACE from 40 to 300 mg L^{-1}, the bacterial growth is reduced. *S. aureus* was unable to degrade the pesticide upon a certain level of concentration (mg L^{-1}) of ACE probably due to substrate inhibition. Therefore for *S. aureus*, the optimum initial ACE concentration was determined at 50 mg L^{-1} (Fig. 7).

3.3. Experimental design analysis

The study of CCD and ANOVA analysis using the response surface methodology with the help of Design Expert is done for the optimization of the process parameters, to illustrate the degradation of ACE by *S. aureus* strain 502A.

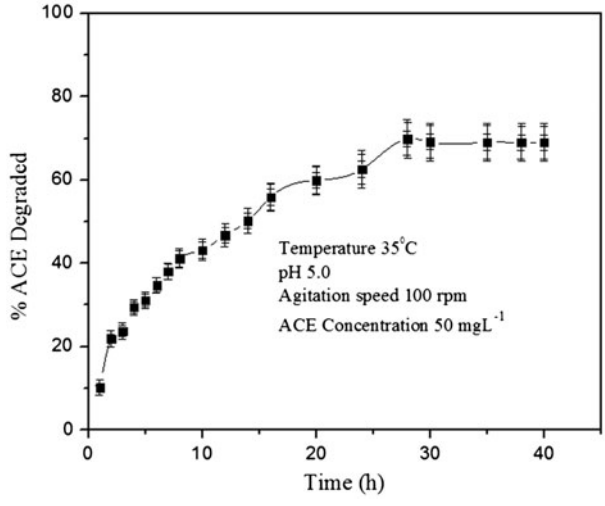


Fig. 6. Effect of incubation time on % ACE biodegraded.

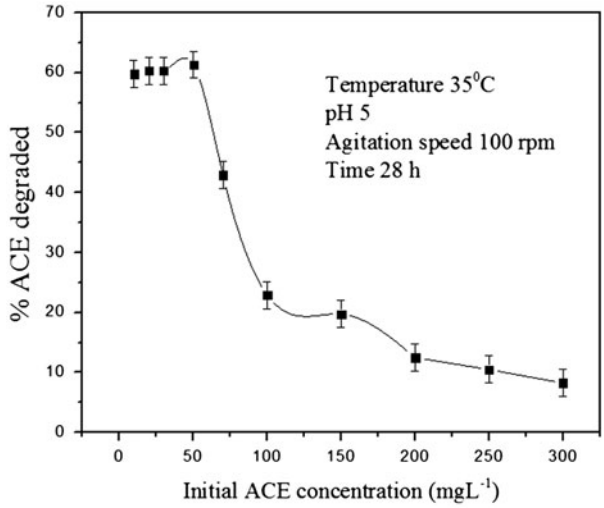


Fig. 7. Effect of initial ACE concentration on % ACE biodegraded.

The process controlling parameters such as the initial ACE concentration (c) was varied within the range of 50–90 mg L^{-1}, temperature (t) varied within the range of 30 to 50°C, agitation speed (a) varied within the range of 60–100 rpm, pH (p) varied within the range of 5.0 to 7.0. Initial ACE concentration (c), temperature (t), agitation speed (a), and pH (p), were varied as process variables according to the CCD reported in Tables 1 and 2 for optimization of the percentage ACE consumed (R) as response. CCD was created by entering factors "c", "t", "a", "p" in terms of ± 1 level to perform RSM and to predict the effect of process variables on ACE removal.

Table 1
Experimental ranges and levels of factors for response surface study

Uncoded factors	Coded factors	Units	Uncoded values	Coded values
c	X_1	mg L^{-1}	30	−1
			50	0
			70	1
t	X_2	°C	25	−1
			35	0
			45	1
a	X_3	rpm	60	−1
			80	0
			100	1
p	X_4		3	−1
			5	0
			7	1

Notes: $X_1 = (c-70)/2$; $X_2 = (t-40)/2$; $X_3 = (a-80)/2$; $X_4 = (p-6)/2$.

Table 2
Experimental design matrix

Run	Concentration (c) mg L^{-1}	Temperature (t) (°C)	Agitation speed (a) rpm	pH (p)	Response (R%)
1	60	30	90	4	63.585
2	50	35	100	5	34.107
3	40	40	90	4	42.39
4	60	40	70	4	52.919
5	50	35	80	5	68.14
6	40	40	90	6	40.78
7	30	35	80	5	50.3
8	50	35	80	5	68.14
9	70	35	80	5	39.84
10	40	40	70	4	53.64
11	50	25	80	5	65.87
12	40	30	70	6	62.076
13	60	30	70	4	60.04
14	50	35	80	5	68.14
15	50	35	60	5	58.1
16	60	30	90	6	56.01
17	60	40	70	6	57.28
18	60	40	90	6	22.2
19	40	30	70	4	40.38
20	60	40	90	4	47.43
21	50	35	80	5	68.14
22	40	30	90	6	56.28
23	60	30	70	6	63.955
24	50	35	80	3	55.281
25	50	35	80	5	68.14
26	50	35	80	5	68.14
27	40	30	90	4	51.088
28	50	35	80	7	63.046
29	50	45	80	5	59.258
30	40	40	70	6	67.86

According to statistical model fit summary reported in Table 3, a quadratic model was selected as the best-fitted model with lower standard deviation (3.25) and lowest PRESS value (915.29), higher adjusted and predicted R^2 value (0.7745) and adequate good precision in comparison to other models.

Analysis of variance (ANOVA) for quadratic model illustrated in Table 4 shows high R^2 value (0.96), and acceptable p-value (<0.0001), which proves the adequacy of the quadratic model. According to ANOVA, model insignificant terms (p-value > 0.05), are neglected and the final equation in coded form expressed as:

$$R\% = 68.14 - 0.50X_1 - 3.42X_2 - 5.27X_3 + 1.32X_4 - 3.66X_1X_2 - 0.72X_1X_3 - 4.00X_1X_4 - 4.96X_2X_3 - 1.97X_2X_4 - 4.89X_3X_4 - 5.92X_1^2 - 1.55X_2^2 - 5.66X_3^2 - 2.32X_4^2 \quad (10)$$

Table 3
Model fit summary statistics for percentage degradation of ACE

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	Press	
Linear	11.07	0.2450	0.1242	−0.0684	4337.24	
2FI	9.71	0.5583	0.3259	0.1573	3420.82	
Quadratic	*3.25*	*0.9609*	*0.9243*	*0.7745*	*915.29*	*Suggested*
Cubic	1.95	0.9934	0.9728	0.0546	3837.73	Aliased

Table 4
ANOVA results for percent degradation of ACE

Source	Sum of squares	*F* value	*p* value	
Model	3,900.54	26.30	<0.0001	Significant
A = Initial ACE concentration	6.00	0.57	0.4635	
B = Temperature	281.12	26.54	0.0001	
C = Agitation speed	665.42	62.81	<0.0001	
D = pH	41.87	3.95	0.0654	
AB	214.67	20.26	0.0004	
AC	8.34	0.79	0.3890	
AD	256.22	24.19	0.0002	
BC	394.13	37.20	<0.0001	
BD	61.96	5.85	0.0288	
CD	336.86	31.80	<0.0001	
A2	962.12	90.82	<0.0001	
B2	65.82	6.21	0.0249	
C2	880.01	83.07	<0.0001	
D2	148.17	13.99	0.0020	
R2				0.9609
C.V%				5.84

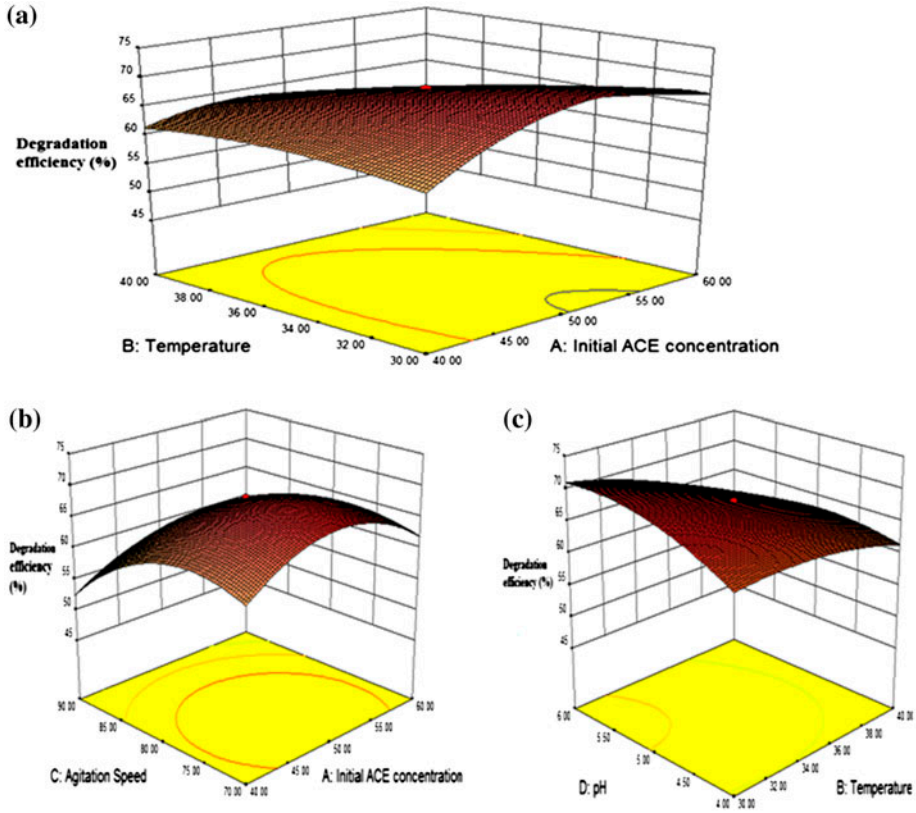


Fig. 8. Response surface 3D curves showing: (a) Dependence of % ACE degraded over initial concentration and temperature; (b) Dependence of % ACE degraded over initial concentration and agitation speed; (c) Dependence of % ACE degraded over pH and temperature.

The optimum ACE bioremediation efficiency by *S. aureus* strain 502A for different process control conditions can be predicted from the three dimensional curves Fig. 8(a)–(c). The 3D curves represent a definite number of combinations of two variables (initial ACE concentration and temperature) with the other one maintained at the middle value (Fig. 8(a)). The maximum degradation of ACE was obtained at temperature 35˚C. It is clear that below or around temperature 30 to 40˚C, the biodegradation ability is low for the isolated bacteria. Accordingly, the uptake of ACE by *S. aureus* strain 502A decreased as the temperature decreased from 40˚C, whereas the concentration increased as the temperature increased from 30˚C till it reached the optimum condition of 35˚C. Also, the maximum ACE biodegradation was obtained at 80 rpm (Fig. 8(b)). The biodegradation ability of ACE was observed to be low at agitation speed below or around 80 rpm. Therefore, uptake of ACE by *S. aureus* strain 502A decreased as the agitation speed decreased from 80 rpm, whereas the concentration increased as the agitation speed increased from 60 rpm till it reached an optimum value of 80 rpm. Likewise, it is clear that the maximum efficient biodegradation of ACE was obtained at pH 5.0 and at temperature 40˚C (Fig. 8(c)). Below or around pH 5.0 and temperature 40˚C, the biodegradation ability of *S. aureus* is low.

The optimization of the degradation parameters of ACE using CCD revealed that; all the lab scale parameters were significant in controlling the percent consumption of the pesticide. The percentage of consumption of pesticide illustrated positive linear correlation with all the four variables. Quadratic relation of all the variables showed positive effect in the consumption efficiency of ACE. Accordingly, the plot of predicted response value over the actual degradation value which proves the significance of the model for maximization of the ACE biodegradation (Fig. 9).

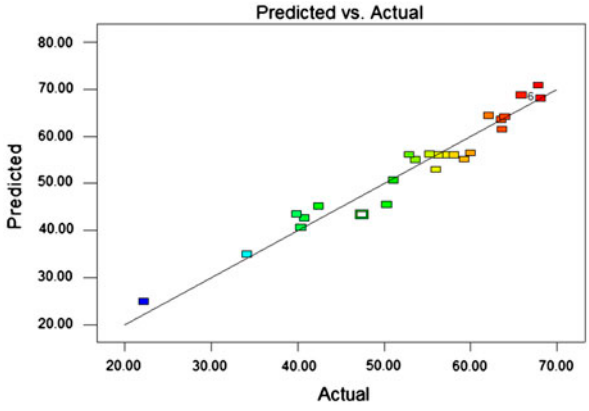


Fig. 9. Response surface plot of predicted response value over the actual value of ACE degradation.

3.4. Rate kinetics and mathematical model fitting of growth curve of S. aureus

The zero-order and three half-order kinetic constants are listed in Table 5. The regression coefficient of determination obtained for zero-order kinetics was found to be in the range of 0.93 to 0.99 for various initial ACE concentrations. The values of S_0 and k_0 were found to be in the range of 22.06–234.93 mg L^{-1} and 1.08–3.84 mg L^{-1} h^{-1}, respectively. The values of k_1 and k_2 were found to be decreasing with an increase in the initial concentration of ACE substrate. The value of regression coefficient of determination (R^2) obtained for three half-order kinetic was found to be in the range of 0.961–0.99. It indicates that the three half-order kinetic model is suitable to explain the biodegradation rate kinetic of ACE using *S. aureus* strain 502A.

From the growth curve of *S. aureus* strain 502A obtained by the experimental data, mathematical model fitting was done in Gompertz model. It was

Table 5
Parameters of zero-order and three half-order kinetic models at different initial ACE concentration

Sample number	Concentration of ACE (mg L^{-1})	Zero-order kinetics			Three half-order kinetics		
		k_0	S_0	R^2	$k_1 \times 10^4$	$k_2 \times 10^3$	R^2
1	30	1.08	22.01	0.992	3.501	4.901	0.961
2	50	3.84	66.64	0.982	−1.19	1.500	0.99
3	70	3.52	88.90	0.997	0.769	0.701	0.969
4	100	3.22	103.62	0.988	0.560	0.393	0.972
5	150	2.71	130.74	0.980	0.400	0.294	0.972
6	200	3.20	190.47	0.934	0.280	0.146	0.981
7	300	2.93	234.93	0.930	0.243	0.133	0.99

found to have adequate R^2 value, thereby suggesting the proper fit of the model in *S. aureus* in the analysis of ACE degradation study. The bacteria took longer time to acclimatize in the MM and LB nutrient medium; therefore it is probable that consumption of ACE was minimum at time interval from 0 to 5 h (Fig. 10(a)). Gradually it proceeded towards the exponential phase from 6 h and it continued till 15 h, where *S. aureus* could effectively utilize ACE as its only source of carbon, nitrogen constituents. From 16 h, there was moderate and stationary growth of the bacteria and a saturation level of consumption of ACE were observed. Growth was not accelerated and further utilization of the substrate that is the pesticide ACE was not found. The growth was in stationary phase till 32 h. The mathematical model fitted to the experimentally derived data; in accordance with the Gompertz model (Fig. 10(b)). The R^2 value was found to be 0.994 which implies adequate fitting of the model for the growth curve of *S. aureus*.

3.5. Determining biodegradation study

The endeavor of present study was to assess the utilization of biological treatment with the aid of isolated indigenous bacterial culture for ACE removal. Likewise, degradation of ACE as well as the metabolites produced during the ACE biodegradation was quantified using GC–MS (Thermo Scientific, 1300 series GC with ISQ single quadruple MS detector); since gas chromatography mass spectrometry (GC–MS) with EI are identified as techniques most often applied in multi-residue methods for pesticides at present [26]. Fundamentally, ACE insecticide belongs to the class of chloropyridyl neonicotinoid hydrocarbon derivatives; as a result, formation of several peaks in the chromatogram was observed. The mass fragmentation and m/z values as obtained were used for prediction of the proposed structures of metabolite generated after biodegradation process. The structure of purified ACE metabolite and respective mass fragmentation data was examined at a retention time (*RT*) of 9.53 min, with a molecular weight of 222 amu and m/z value of 56 (Fig. 11(a) and (b)).

The current study addresses the bioremediation approach to treat wetland wastewater, which is quite important considering the vast wetland in tropical countries and its overall impact on ecosystem. Till date, very few bacterial species have been reported for ACE degradation from wastewater. Micro-organisms that are capable to degrade ACE in soil have been identified; including the yeast *Rhodotorula* sp. which is able to hydrolyze ACE [27,28]. Also reported; 45 and 30% of ACE elimination from soil under ligninolytic and non-ligninolytic conditions, respectively, after 15 d of incubation. About 53.3% ACE was degraded by *Pseudomonas* sp. strain FH2 after incubation for 14 d in ACE-mineral medium and nearly 96.7% degraded when incubated in ACE-yeast mineral medium at 30°C for 14 d [29] Although the metabolism of ACE has been studied in honeybee, mice, spinach, and soils [30,31], no report is available in the literature regarding the metabolical reaction of ACE in effluent using a pure bacterial strain. On comparing the area of peaks representing standard sample of ACE at retention time of 9.53 with that of *S. aureus* culture sample (Fig. 12(a)); it was observed that after 24 h, 61.68% of ACE was degraded by *S. aureus,* which is significantly higher and faster as compared to the reported bacterial strains for ACE degradation,

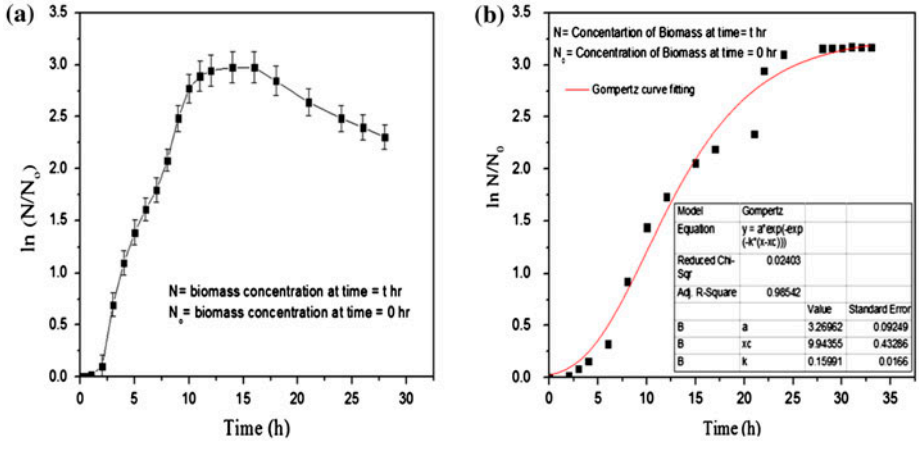


Fig. 10. Plots showing: (a) Growth curve of *S. aureus* strain 502A; (b) Gompertz growth curve modeling of *S. aureus* strain 502A.

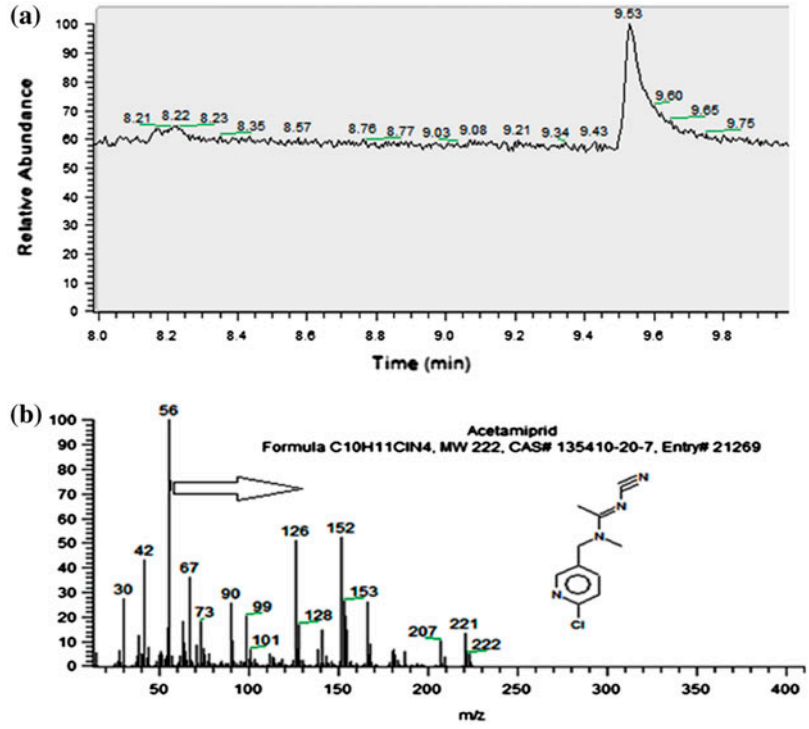


Fig. 11. GC–MS chromatograms of: (a) Quantification of standard ACE (50 mg L^{-1}); (b) Mass fragmentation of standard ACE (50 mg L^{-1}).

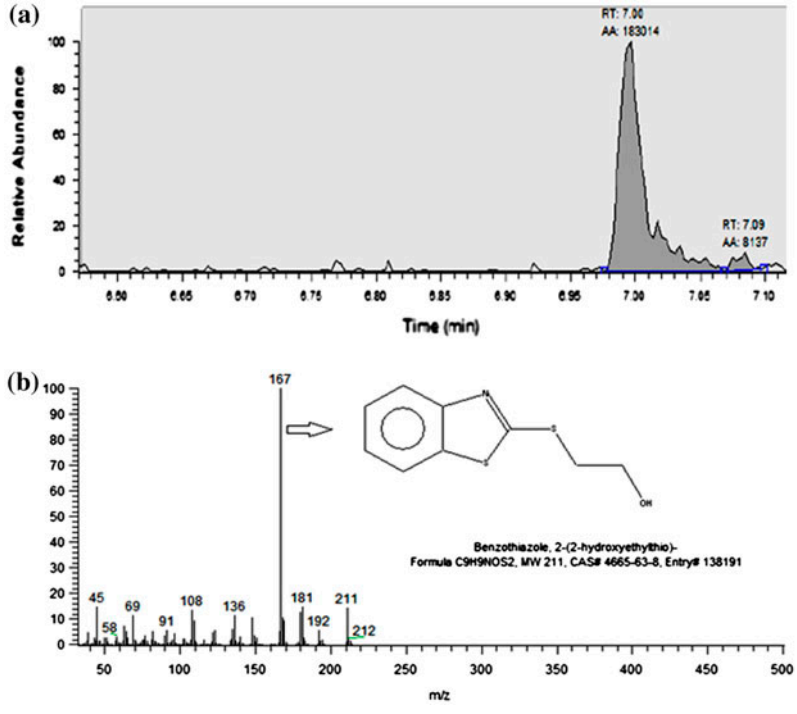


Fig. 12. GC–MS chromatograms showing: (a) Quantification of *S. aureus* strain 502A biodegraded ACE by peak area analysis; (b) Mass fragmentation of secondary metabolite of ACE having RT: 7.00 and observed to be Benzothiazole, 2-(2-hydroxyethylthio).

without any requirement of growth factors or other supplements.

Meanwhile, structure of the characteristic fragment obtained due to biodegradation of ACE and the respective mass fragmentation data showed a prominent peak at retention time (*RT*) of 7.00 min, with a molecular weight of 211 amu and m/z value of 167 (Fig. 12(b)). The compound was predicted to be Benzothiazole, 2-(2-hydroxyethylthio). From the literature reviewed, it is observed that, Benzothiazole (C_7H_5NS) is a privileged bicyclic ring system. It contains a benzene ring fused to a thiazole ring. The small and simple benzothiazole nucleus is present in compounds involved in research aimed at evaluating new products that possess interesting biological activities like cardio protective, anti-ischemic [32], analgesic, antitumour [33], antimalarial, anticonvulsant, anthelmintic, and anti-inflammatory activity [34].

The extracted sample of 24 h incubated *S. aureus* strain 502A culture in the presence of ACE substrate represented the chromatogram which effectively showed a sharp peak at a retention time of 7.00 (Fig. 13(a)), while in the absence of substrate ACE, there were no significant peak detected in the chromatogram (Fig. 13(b)), which showed *S. aureus* strain 502A is potent enough to break the toxic complex compound like ACE into a relatively simpler product.

Previously certain reported metabolic pathways of ACE biodegradation propounded that bacterium *Stenotrophomonas* sp. able to demethylate ACE in soil to form a metabolite IM 1–3 [35]. Also, N-methyl-(6-chloro-3-pyridyl) methylamine was generated in the degradation of ACE in soil by *Pigmentiphaga* sp. [17] and degradation using *Rhodococcus* sp. BCH2 with the formation of 6-chloronicotinic acid ($m/z = 157$) [36] were reviewed. Here, we report, *Staphylococcus* sp. strain 502A is promising enough to grow in MSM media with only ACE as the nutrient source and is able to remove 61.68% of the toxic compound in 24 h from the effluent; to form a non- toxic clinically significant compound Benzothiazole with a significant metabolic pathway of degradation.

Considering, all these analytical results for biodegradation of the insecticide ACE, it can be emphasized that the isolated indigenous bacteria *Staphylococcus* sp. strain 502A is potent enough to degrade a toxic insecticide like ACE (61.68%) in comparatively high concentration of 50 mg L^{-1}. The strain can be effectively utilized for further bioremediation of vast East Kolkata Wetland waters.

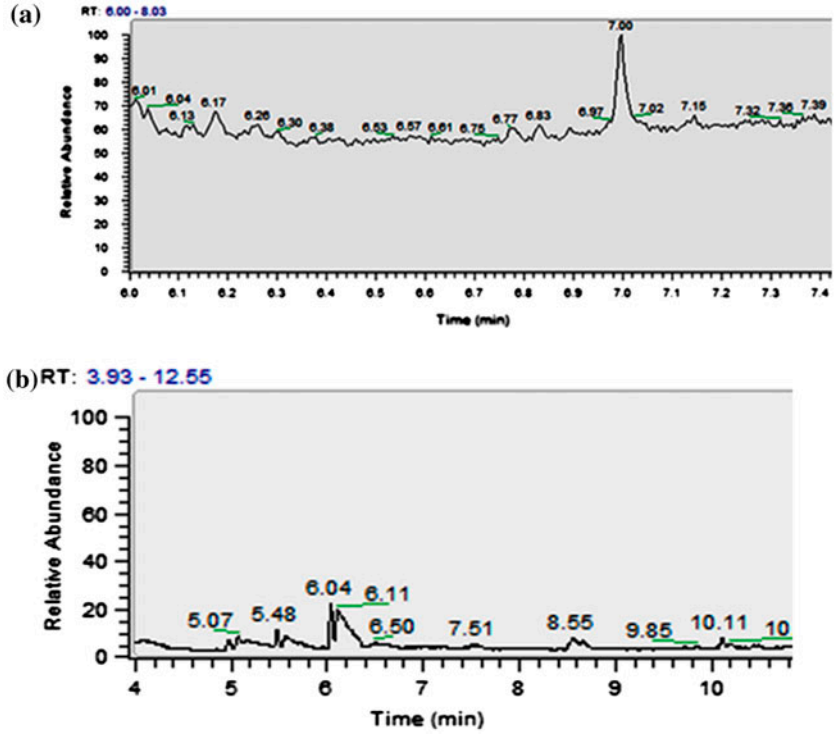


Fig. 13. GC–MS chromatograms of: (a) *S. aureus* strain 502A 24 h incubated culture in presence of ACE; (b) *S. aureus* strain 502A 24 h incubated culture in absence of ACE.

3.6. Assay of the toxicity of ACE biodegraded metabolites

The choice behind biodegradation process over other conventional wastewater treatment process lies on the ultimate fate of the substrate molecules. Safe discharge from wastewater requires meeting a standard and detail toxicological reports. In the present context, the degraded metabolite profile was investigated by mass spectroscopic analysis. Mass spectral analysis confirms the incomplete degradation, and thus imposes the investigation of the toxicological consideration of the degraded metabolites. The antimicrobial susceptibility study of the degraded metabolites was done on *B. subtilis*, known to be one of the most prevalent microorganisms in environment. The plates were observed for their respective inhibition zones. The zones of growth inhibition around each of the paper discs were measured to the nearest millimeters. The diameter of the zone is related to the susceptibility of *B. subtilis* and to the diffusion rates of pure ACE and its degraded metabolite through the agar medium [37].

The zone of inhibition around the pure sample of ACE (marked Z in Fig. 14) was found about 25 mm (diameter); the inhibition zone around the half diluted pure ACE sample (marked B in Fig. 14) was found about 7 mm (diameter) whereas no inhibition zone observed around the degraded metabolite product (marked A in Fig. 14) even at its high concentration of 50 mg L^{-1}. The degraded metabolites even at significant high concentration level (50 mg L^{-1}), as obtained from chromatogram showed no inhibition zone, which may be due to no or insignificant toxicity of the related compound (Fig. 14). From the observations, it can be foresighted that the metabolites obtained after biodegradation of ACE, upon releasing in the environment is most unlikely to cause any toxicity effect. Since, this preliminary test is performed only with microorganisms, so without pre-clinical analysis it is not justified to assay the effectiveness of the secondary metabolite righteously. The natural microflora contains *Bacillus* sp., therefore susceptibility of *B. subtilis* is tested to the degraded metabolites of ACE to predict the non-toxicity of the biodegraded metabolite on being released in the environment.

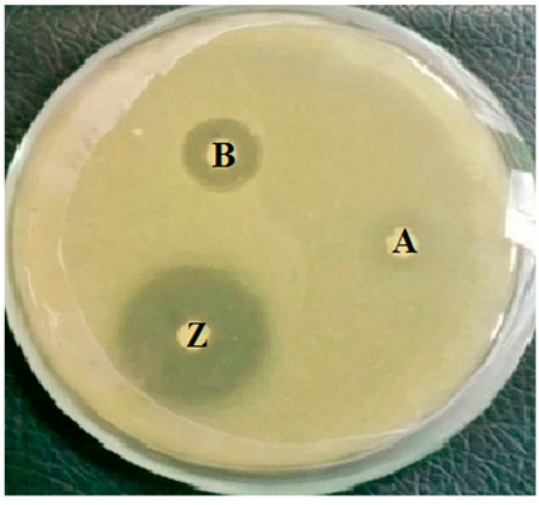


Fig. 14. Microbiological susceptibility assay of ACE.

4. Conclusion

This article illustrate about, an indigenous bacterial strain of *Staphylococcus sp.* strain 502A, isolated from agricultural wetland wastewater; studied for their potential ACE degrading capability under different optimized lab-scale conditions. The bacteria was able to degrade the toxic ACE amount present in wastewater by up to 61.68%, with the highest degrading effect observed after 24 h, at 35˚C, pH 5.0 in the presence of 50 mg $L^{-1}$of initial ACE. The end product of ACE biodegradation was observed to be Benzothiazole, 2-(2-hydroxyethylthio). Gompertz model and the lab-scale optimization analysis give several interpretations about the Sigmoidal growth pattern of *S. aureus* strain 502A. Gompertz modeling approach is observed to be ideal way to understand biomechanism of bacterial biomass growth and thigmotropism as there was 99.4% accuracy while fitting the experimental data. All the lab-scale parameters were significant in controlling the percent consumption of the insecticide ACE. The model fit summary shows the significance of the model and suggests the potency of using *S. aureus* strain 502A in effective biodegradation of the toxic ACE. The antimicrobial susceptibility test was also performed to assess the final toxicity level of the reaction products, which gave negative response suggesting a safe eco-friendly discharge to environment.

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Symbols

x — biomass concentration (mg L^{-1})
t — time (h)
t_c — absolute growth rate maximal time
k — relative growth rate
E — cell concentration (mg L^{-1})
a — proportionality constant (per unit biomass concentration per unit time)
k_0 — zero order rate constant
S — substrate concentration
S_0 — initial substrate concentration (mg L^{-1})
p — rate of Product formation (mg L^{-1})
k_1 — proportionality constant
μ — growth rate (per unit time)
dt — change in time (h^{-1})
N — population density (h)

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