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(54) Title: A METHOD FOR QUORUM QUENCHING

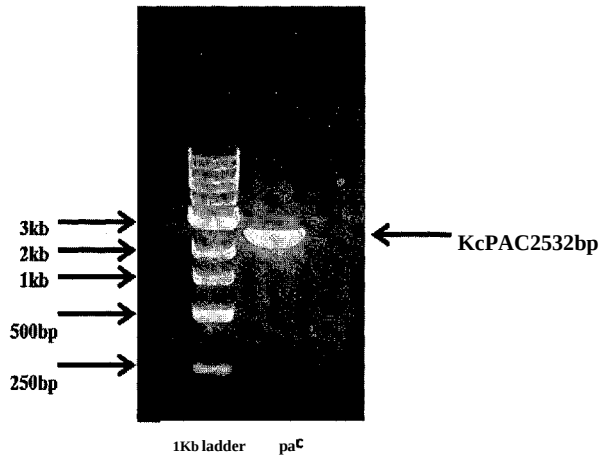

Figure 1: PCR amplification of *Kc*PAC gene of 2532bp loaded on 1.5% agarose gel.

(57) Abstract: The present invention relates to a method for quorum quenching. more particularly this invention provides a Penicillin G Acylase enzyme having quorum quenching ability. The invention further relates to the application to this ability of Penicillin G acylase to prevent formation of biofilms. The current invention provides a biomolecule having high antimicrobial activity. The present invention also provides a kit for the for the quorum quenching.

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A METHOD FOR QUORUM QUENCHING

FIELD OF INVENTION

The present invention relates to a method for quorum quenching. More particularly this invention provides a Penicillin G Acylase enzyme having quorum quenching ability. The invention further relates to the application to this ability of Penicillin G acylase to prevent formation of biofilms.

BACKGROUND OF INVENTION

Penicillin acylases (PAs) have been recognized a group of enzymes of tremendous industrial importance for more than 50 years now, amongst other advantages providing a green route of obtaining the essential beta-lactam (6-APA) nucleus. Penicillin G acylase produced by *Kluyvera citrophila (KcPGA)* in the recent past has received more attention due to its numerous industrial process friendly properties, namely increased resilience to harsh conditions and ease of immobilization.

Acyl homoserine lactones (AHLs), the largest and the most well characterized class of signal molecules, mediate communication amongst gram negative bacteria and a diminished accumulation of these molecules impedes intercellular communication leading to a state of communication blackout. This forms the basis of the phenomenon known as Quorum Quenching. Of the many probable ways to quench quorum sensing, enzymatic methods have received increasing amounts of attention since 2003. Two types of enzymes that have been shown to degrade acyl homoserine lactones are AHL Lactonases and AHL Acylases.

US201 101 19595 relate to use of bacterial *Tenacibaculum* for quorum quenching. The crude, cellular extract or the cultivated supematants of *Tenacibaculumhas* capable of degrading N-acyl homoserine lactones for quorum quenching for treatment of infectious bacterial illness or to inhibit formation of biofilms.

Romero, M. *et al.* (2012) (Appl. Environ.Microbiol, Vol. 78(17), pages 6345-6348) relates to quorum quenching activity from the isolates of cultivated oceanic and estuarine sea water bacteria.

Romero, M. *et al.* (2012) (Recent Patents Biotech., Vol. 6, pages 2-12) relates to a review article which focus of various patents on quorum quenching. This review article discuss various synthetic and chemical molecules used for quorum quenching as well discuss the extracts of from various bacteria for quorum quenching.

Roche, D.M. *et al.* (2004) (Microbiology, Vol. 150, pages 2023-2028) relates to a review article which discusses the function of N-acylhomoserine-lactone degrading enzymes and their role in quorum quenching.

Bokhove, M. *et al.* (2010) (PNAS, Vol. 107(2), pages 686-691) relates to role of AHL acylase PvdQ enzyme from *Pseudomonas aeruginosa.* The functional activity PvdQ enzyme has been shown to be similar to pencillin G acylase and cephalosporin acylase.

Czajkowski, R. and Jafra, S. (2009) (Acta Biochem. Pol. Vol.56, No. 1/2009, pages 1-16) is review article which discuss that two main classes of AHL inactivating enzymes have been identified such as AHL lactonases which hydrolyse the lactone rings in AHLs; and AHL acylase which liberate a free homoserine lactone and a fatty acid.

Krzeslak, J. *et al.* (2007) (In book. *Pseudomonas.* Vol.5: A model system in biology, chapter 15: Quorum Qenching acylases in *Pseudomonas aeruginosa)* is a broad and review discussion of role of Quorum Qenching acylases in *Pseudomonas aeruginosa.*

US20040 109852 relates to method of eliminating formation of biofilm using a composition comprising one or more acylases, particularly the invention also relates a composition capable of degrading lactone produced by one or more microorganisms. Thus the preventing formation or removal of biofilm.

Lin, Yi-Han, *et al.* (2003) (Mol. Microbiol., Vol.47(3), pages 849-860), relates to isolation of AHL acylases from *Ralstonia* strain XJ12B and their function as enzymes for quorum quenching.

Sio, C.F. *et al.* (2006) (Infect. Immun. Vol.74(3), pages 1673-1682), relates to N-Acyl AHL acyklases from *Pseudomonas aeruginosa* PAOl and its functional capability to remove fatty acid chain from HAL nucleus of AHL-dependent quorum-sensing signal molecules.

Suresh, C.G, (2006) (In PhD Thesis titled: *"Investigation into the structure and activity of conjugated bile salt hydrolase and related penicillin acylase"*. University of Pune relates to extensive studies carried out on two penicillin G acylases (PGAs) (penicillin amidohydrolase, EC 3.5.1.1 1) from *Kluyvera citrophila* (KcPGA) and *Alcaligence faecalis* (AfPGA). The Thesis investigates the structural studies on PGA *from A. faecalis.*

The problem of pathogens developing resistance to exiting antibiotics and other similar agents is being combated by practicing physicians and hospitals world- wide. A number of deaths are being reported due to multi drug resistant hospital infections. While the pharmaceutical world is in the quest for newer molecules, other means and methods to combat this menace should be looked into and evolved.

SUMMARY OF THE PRESENT INVENTION

Accordingly, the main embodiment of the present invention provides a method for quorum quenching using KcPGA enzyme from *Kluyvera citrophila*

Another embodiment of the present invention provides a method as described in the present invention wherein the enzyme has degrading activity against AHLs.

Another embodiment of the present invention provides a method as described in the present invention wherein the enzyme has degrading activity against medium length AHL's of C6-C8 carbons.

Another embodiment of the present invention provides a method as described in the present invention wherein the enzyme KcPGA shows preference for degrading

homoserine lactones (HSL) in the order of 3-oxo C6 HSL> C6 HSL> C7 HSL> C8 N-octanoyl-L-homoserine lactone.

Another embodiment of the present invention provides a method as described in the present invention wherein the quorum quenching is achieved against gram negative bacteria.

Another embodiment of the present invention provides a method as described in the present invention wherein enzyme is useful against biofilm.

Another embodiment of the present invention provides a use of KcPGA enzyme from *Kluyvera citrophila* in inactivating quorum sensing signals by degrading acyl homoserine lactones.

Another embodiment of the present invention provides a use of KcPGA enzyme from *Kluyvera citrophila* as described in the present invention for inactivating quorum sensing signals by degrading acyl homoserine lactones.

Another embodiment of the present invention provides a use of KcPGA enzyme from *Kluyvera citrophila* as described in the present invention wherein the enzyme KcPGA shows preference for degrading homoserine lactones (HSL) in the order of 3-oxo C6 HSL> C6 HSL> C7 HSL> C8 N-octanoyl-L-homoserine lactone.

Another embodiment of the present invention provides a use of KcPGA enzyme from *Kluyvera citrophila* as described in the present invention wherein inactivation of quorum sensing signals by degrading acyl homoserine lactones results in quorum quenching.

Another embodiment of the present invention provides a use of KcPGA enzyme from *Kluyvera citrophila* as described in the present invention having activity against biofilm.

Another embodiment of the present invention provides a KcPGA enzyme from *Kluyvera citrophila* having antimicrobial or antibacterial activity.

Another embodiment of the present invention provides a KcPGA enzyme from *Kluyvera citrophila* having antibacterial activity against gram negative bacteria.

Another embodiment of the present invention provides a composition comprising KcPGA enzyme along with a pharmaceutically acceptable excipient.

Another embodiment of the present invention provides a composition wherein the composition is combined with antimicrobial or antibacterial antibiotic.

Another embodiment of the present invention provides a composition wherein the composition is for quorum quenching.

Another embodiment of the present invention provides a composition as described in the present invention for degrading or blocking quorum sensing signals by degrading AHL's.

Another embodiment of the present invention provides a composition as described in the present invention for quorum quenching of biofilms.

Another embodiment of the present invention provides a method of treating or preventing or slowing down a process a condition in a subject by administering a composition as described in the present invention.

Another embodiment of the present invention provides a method of treating or preventing or slowing down a process a condition wherein the condition is a disease caused by microbe or bacteria.

Another embodiment of the present invention provides a method of treating or preventing or slowing down a process a condition wherein the condition is caused due to quorum sensing activity or signaling of microbe or bacteria.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: PCR amplification of *KcPAC* gene of 2532bp loaded on 1.5% agarose gel.

Figure 2: Expressed enzyme checked by 15% SDS PAGE.

Figure 3: Matrix-assisted laser desorption ionization / time-of-flight time-of-flight mass spectrometry (MALDI-TOF-TOF).

Figure 4:Degradation of C6-HSL by *K.citrophilia* PGA.

Figure 5: Quantitative estimation of violacein production by CV026 in response to C6 HSL progressively being degraded by the action of *K.citrophlia* PGA.

Figure 6: Graphs depicting optimizing the time, temperature and pH for incubation.

Figure 7: Anti bio filming activity of KcPGA, concentration of enzyme used was 15-150ug/ml and this led to approximately 10-40% reduction in biofilm formation as determined by crystal violet staining. In case of KcPGA also about 40% decrease in biofilm formation was observed with 135ug of enzyme against 24 hour old culture of *V.cholerae.*

DETAILED DESCRIPTION OF INVENTION

While the invention is susceptible to various modifications and/or alternative processes and/or compositions, specific embodiment thereof has been shown by way of example in the drawings/figures and tables and will be described in detail below. It should be understood, however that it is not intended to limit the invention to the particular processes and/or compositions disclosed, but on the contrary, the invention is to cover all modifications, equivalents, and alternative falling within the spirit and the scope of the invention as defined by the appended claims.

The graphs, tables, formulas and protocols have been represented where appropriate by conventional representations in the drawings, showing only those specific details that are pertinent to understanding the embodiments of the present invention so as not to obscure the disclosure with details that will be readily apparent to those of ordinary skill in the art having benefit of the description herein.

The following description is of exemplary embodiments only and is not intended to limit the scope, applicability or configuration of the invention in any way. Rather, the following description provides a convenient illustration for implementing exemplary embodiments of the invention. Various changes to the described embodiments may be made in the function and arrangement of the elements described without departing from the scope of the invention.

The terms "comprises", "comprising", or any other variations thereof, are intended to cover a non-exclusive inclusion, such that one or more processes or composition/s or systems or methods proceeded by "comprises... a" does not, without more constraints, preclude the existence of other processes, sub-processes, composition, sub-compositions, minor or major compositions or other elements or other structures or additional processes or compositions or additional elements or additional features or additional characteristics or additional attributes.

Definitions:

For the purposes of this invention, the following terms will have the meaning as specified therein:

The present invention provides an unanticipated finding relating to ability of *Kluyvera citrophila* PGA to act as an AHL degrading enzyme.

In one aspect the present invention provides the ability of KcPGA to cleave AHLs for the first time. The recombinant purified KcPGA in the present invention has been. shown to degrade AHL molecules with acyl side chain of 8 carbons or less with or without oxo-substituent at the third carbon position of the chain.

In one aspect of the present invention the AHL acylase activity of KcPGA was initially bioassayed using biosensor strain *Chromohacterium violaceum* tn5 mutant CV026 to provide evidence of AHL degradative capability of KcPGA. Further in another aspect the present invention provides that there was almost complete degradation of C6 HSL after incubation with KcPGA over a period of three hours (Figure 5). Cleavage of AHLs by KcPGA of the present invention was also confirmed by OPA based Fluorimetric assay using a range of substrates.

In another aspect the present invention provides the optimum conditions for KcPGA catalyzed AHL degradation and it was found that deacylation activity of KcPGA was in the temperature range of 30-50°C **(Figure 6).** The pH optima for AHL acylase activity of KcPGA was found to be 8.0. Another aspect of the present invention provides the Vmax, Km values calculated using Line-weaver-Burk. The Catalytic efficiency of KcPGA calculated for two of its best substrates, namely 3-oxo-C6-HSL, and C6-HSL, yielded K_{cat}/K_m values of 0.651 x $10^3 M^{-1}s^{-1}$ and 0.103 × 10^3 $M^{-}v$, respectively **(Table 1 and Figure 5).**

Table 1: Kinetics of AHL deacylation catalyzed by KcPGA

V_{max} (mM/h/mg of protein)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})
3-oxo-C6HSL 21.37+0.85	0.1+0.01	0.06	0.67
C6HSL 10.06+0.27	0.28+0.02	0.03	0.11

Advantages of invention:

- Novel source of AHL degradation

- Useful for treatment of multi drug resistant infections

In an aspect of the invention, the composition comprising PGacylase for activity against AHLs is combined with antibiotics, anti-bacterials or agents such like for activity against AHLs.

In another aspect, the invention provides a composition comprising PGacylase, which is useful for activity against a biofilm.

In another aspect the present invention also provides for composition or pharmaceutical composition comprising KcPGA enzyme from *Kluyvera citrophila* as described in the present invention. The composition as described herein in context of the present invention can be a composition is a pharmaceutical composition as medication or medicine for a subject or a patient.

The composition as described herein in context of the present invention can be also be an antiseptic solution. The composition as described herein in context of the present invention relates to composition as described herein in context of the present invention can also be a disinfectant solution for use in hospitals, clinics, etc. The composition as described herein in context of the present invention relates to composition as described herein in context of the present invention can also be a disinfectant cleansing solution for cleaning medical instruments or devices used by medical practioners or hospitals.

The composition as described herein in context of the present invention can be also be a liquid, solid, semi-solid or the like composition for disinfecting or cleaning biofilms caused by microorganisms.

The pharmaceutical composition of the present invention is intended for parenteral and oral administration. Preferably, the pharmaceutical composition described as herein in the present invention can be administered parenterally for example, intravenously, subcutaneously, intradermally or intramuscularly. The present invention also provides for agents which function as "pharmaceutically acceptable carrier or pharmaceutically acceptable excipient", wherein the term "pharmaceutically

acceptable excipient or pharmaceutically acceptable excipient" means a pharmaceutically acceptable excipient or carrier, solution or additive to enable the delivery, dissolution or suspension of the pharmaceutical active ingredient as herein as described in the present invention. The active ingredient as described in the context of the present invention comprises of KcPGA enzyme from *Kluyvera citrophila.* The pharmaceutical composition of the present invention may also contain pharmaceutically accepted auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like. The pharmaceutical composition of the present invention may also contain pharmaceutically acceptable carriers, for example adjuvants, etc. The pharmaceutical composition of the present invention may also comprise of biomolecule as described in the present invention conjugated with polymer or biopolymer, wherein the polymers can be common or commercially known or used polymers particularly pharmaceutical administered compositions, for e.g. PEG or its derivatives, dextran etc. The pharmaceutical compositions of this invention may also be administered in any convenient form, for example tablet, capsule, injection, granule or powder form. The pharmaceutical composition of the present invention is prepared in such a manner so that it retains its enzymatic activity to bring maximum benefit to the patient on administration.

The present invention also provides a method of treating, preventing and/or slowing the progression of quorum quenching using KcPGA enzyme from *Kluyvera citrophila* as described in the present invention. Another aspect of the present invention provides for a method of treating, preventing and/or slowing quorum quenching against gram negative bacteria using KcPGA enzyme from *Kluyvera citrophila* as described in the present invention. Another aspect of the present invention provides for a method of treating, preventing and/or slowing antibacterial activity disease causing microorganisms using KcPGA enzyme from *Kluyvera citrophila* as described in the present invention. Another aspect of the present invention provides for a method of treating, preventing and/or slowing antibacterial activity against gram negative bacteria using KcPGA enzyme from *Kluyvera citrophila* as described in the present invention.

In another aspect the present invention provides for kit, wherein the kit comprises of (a) a pharmaceutical composition or composition comprising a KcPGA enzyme from *Kluyvera citrophila* (b) along with pharmaceutically acceptable carrier and (c) instruction guidelines for using the kit.

In another aspect the present invention provides for instructions guidelines with the kit wherein the instruction guidelines are in form of an instruction manual.

One aspect of the present invention provides for use of KcPGA enzyme from *Kluyvera citrophila* as an antimicrobial agent. One aspect of the present invention provides for use of KcPGA enzyme from *Kluyvera citrophila* as an antibacterial agent. Another aspect of the present invention provides for a method of preparing a medicament using KcPGA enzyme from *Kluyvera citrophila.* Another aspect of the present invention provides for method of preparing an antimicrobial medicament using KcPGA enzyme from *Kluyvera citrophila.* Another aspect of the present invention provides for method of preparing an antibacterial medicament using KcPGA enzyme from *Kluyvera citrophila.* The medicament comprising pharmaceutical composition of KcPGA enzyme from *Kluyvera citrophila* as described in the present invention for use in antimicrobial activity. The medicament comprising pharmaceutical composition of KcPGA enzyme from *Kluyvera citrophila* as described in the present invention for use in antibacterial activity.

Accordingly, the main embodiment of the present invention provides a method for quorum quenching using KcPGA enzyme from *Kluyvera citrophila.*

Another embodiment of the present invention provides a method as described in the present invention wherein the enzyme has degrading activity against AHLs.

Another embodiment of the present invention provides a method as described in the present invention wherein the enzyme has degrading activity against medium length AHL's of C6-C8 carbons.

Another embodiment of the present invention provides a method as described in the present invention wherein the enzyme KcPGA shows preference for degrading homoserine lactones (HSL) in the order of 3-oxo C6 HSL> C6 HSL> C7 HSL> C8 N-octanoyl-L-homoserine lactone.

Another embodiment of the present invention provides a method as described in the present invention wherein the quorum quenching is achieved against gram negative bacteria.

Another embodiment of the present invention provides a method as described in the present invention wherein enzyme is useful against biofilm.

Another embodiment of the present invention provides a use of KcPGA enzyme from *Kluyvera citrophila* in inactivating quorum sensing signals by degrading acyl homoserine lactones.

Another embodiment of the present invention provides a use of KcPGA enzyme from *Kluyvera citrophila* as described in the present invention for inactivating quorum sensing signals by degrading acyl homoserine lactones.

Another embodiment of the present invention provides a use of KcPGA enzyme from *Kluyvera citrophila* as described in the present invention wherein the enzyme KcPGA shows preference for degrading homoserine lactones (HSL) in the order of 3-oxo C6 HSL> C6 HSL> C7 HSL> C8 N-octanoyl-L-homoserine lactone.

Another embodiment of the present invention provides a use of KcPGA enzyme from *Kluyvera citrophila* as described in the present invention wherein inactivation of quorum sensing signals by degrading acyl homoserine lactones results in quorum quenching.

Another embodiment of the present invention provides a use of KcPGA enzyme from *Kluyvera citrophila* as described in the present invention having activity against biofilm.

Another embodiment of the present invention provides a KcPGA enzyme from *Kluyvera citrophila* having antimicrobial or antibacterial activity.

Another embodiment of the present invention provides a KcPGA enzyme from *Kluyvera citrophila* having antibacterial activity against gram negative bacteria.

Another embodiment of the present invention provides a composition comprising KcPGA enzyme along with a pharmaceutically acceptable excipient.

Another embodiment of the present invention provides a composition wherein the composition is combined with antimicrobial or antibacterial antibiotic.

Another embodiment of the present invention provides a composition wherein the composition is for quorum quenching.

Another embodiment of the present invention provides a composition as described in the present invention for degrading or blocking quorum sensing signals by degrading AHL's.

Another embodiment of the present invention provides a composition as described in the present invention for quorum quenching of biofilms.

Another embodiment of the present invention provides a method of treating or preventing or slowing down a process a condition in a subject by administering a composition as described in the present invention.

Another embodiment of the present invention provides a method of treating or preventing or slowing down a process a condition wherein the condition is a disease caused by microbe or bacteria.

Another embodiment of the present invention provides a method of treating or preventing or slowing down a process a condition wherein the condition is caused due to quorum sensing activity or signaling of microbe or bacteria.

The invention is explained in detail with specific reference to certain preferred embodiments. It is not be construed as restricting the scope of the invention. The following description is of exemplary embodiments only and is not intended to limit the scope, applicability or configuration to the invention in any way, Rather, the following description provides a convenient illustration for implementing exemplary embodiments of the invention various changes to the described embodiments may be made in the functions and arrangement of the elements described without departing from the scope of the invention.

Examples

Example 1:

Reagents and Media:

All the media ingredients used for growing bacterial cultures were supplied by Hi-media pvt. Ltd., India. Buffers and Antibiotics used for growth of mutant strains were of high purity obtained from Sigma Aldrich. Acyl homoserine lactones (AHLs) were purchased from Cayman chemicals, USA.

Example 2:

Culture conditions used for the microorganisms: (CV026, *V.cholerae)*

Chromobacterium Violaceum (ATCC 31532), was grown at 28°C in Luria Bertani broth supplemented with 100µg/ml Ampicillin and 30µg/ml Kanamycin. Culture was preserved in the form of glycerol stock and was revived every three months. *Vibrio cholerae* (MTCC 3906) used in the anti-biofilm assay was grown by picking up a

single colony from a freshly streaked LB agar plate and was grown in Luria Bertani broth without antibiotics at 37°C for 16-18 hours. ,

Example 3:

Cloning and over expression of Enzyme *Kc*PGA

A 2562 bp gene fragment covering the region from 12 nucleotides upstream from the start codon and 12 nucleotide downstream of *pac* gene was amplified from chromosomal DNA of *K.citrophila* DMSZ 2660 (ATCC 21285) by specific primers (Barbero *et al*, 1986, Accession No- M15418) using components from KOD polymerase kit (It's a kit from Novagen. KOD is a pure recombinant high fidelity DNA polymerase from *Thermococcus kodakaraensis* (KODl) and generalized PCR conditions. Amplified PCR product with desired restriction site near the ends was further purified using PCR clean up kit. Purified PCR product and Vector pET26b (+) both were digested with Ndel and Xhol. Purified vector and insert were ligated in 3:1 molar ratio of insert to vector with T4 DNA Ligase with suitable buffer at 16°C overnight. Ligation product was then transformed into NovaBlue competent cells. Positive clones were picked up from the LB + Kanamycin agar plate and plasmids were isolated. Double digestion confirmed plasmids were then sequenced to confirm the identity of the gene with reported sequences available at NCBI GenBank database. Expression of the sequence confirmed plasmids was checked in Periplasmic fraction, the Cytoplasmic soluble fraction as well as in total cell fraction after growing cells of BL21 DE3 pLysS for 16-18h at 16 °C with shaking at 200 rpm after induction with ImM IPTG Isopropyl β-D-l-thiogalactopyranoside.

Example 4:

Purification of Enzyme *Kc*PGA

Purification of recombinant C-terminal histidine tagged and expressed KcPGA, was done by Affinity chromatography using Ni+- Sepharose beads. Clarified crude lysate were loaded onto packed column of Nickel- Sepharose beads connected to Akta Explorer which were pre-equilibrated with equilibration buffer with 20mM Imidazole. The matrix was then washed with plenty of equilibration buffer. Nonspecific and weakly bound proteins were removed by washing with excess of washing buffer with

40mM and then with 50mM Imidazole. More than 90% pure KcPGA protein was eluted with 150 mM Imidazole. Protein elution was monitored by monitoring the absorbance at 280 nm of collected fractions. All the buffers used in this method of purification contained 25 mM Tris-HCl at pH 8.0, 500mM NaCl, ImM DTT dithiotreitol and were free from chelating agent EDTA. The eluted protein was separated and analyzed by SDS polyacrylamide (SDS-PAGE) as described by Laemmli (1970) Nature 227, 680-685 (15 August 1970) | doi:10.1038/227680a0, Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. The gel was stained with Coomassie brilliant blue and protein concentration was determined by Bradford method using BSA bovine serum albumin as the standard. The fractions containing *Kc?GA* protein after Nickel purification step were pooled and concentrated with Amicon centrifugal concentrator (Millipore, USA) with cutoff range of 30kDa and passed through Gel filtration column (Sephacryl S-200) connected to Akta Explorer and fractions were eluted with 10 mM potassium phosphate buffer pH 7.5 containing 150mM NaCl. The aliquots of the fractions were checked for the presence of PGA by enzyme assay. The purity and homogeneity of positive fractions was checked using 12% (w/v) SDS-PAGE .The fractions were concentrated with centrifugal concentrator and the protein was stored at -80 °C.

Example 5:

Cloning and Over expression of *Kc*PGA in *E.coli* BL21DE3 pLysS

Isolation and cloning of *"pad"* gene from *Kluyvera citrophila* DMSZ 2660 *(KcPAC)*

A 2562 bp PCR fragment covering the region from 12 nucleotides upstream from the start codon of *K.citrophila* "pac" gene and 12 nucleotide downstream was amplified from chromosomal DNA from *Kxitrophila* DMSZ 2660 (ATCC 21285) as a template, using gene specific primers designed according to the published coding sequence (Barbero *et al,* 1986, Accession No- M15418) **Figure 1.**

SEQ ID No:l PacNdeF 5'-caagaggatcatatgaaaaatagaaatcgtatgatcgtg-3' and

SEQ ID No:2 PacXhoR 5'-gccgaactcgaggcgctgtacctgcagcactt-3'

(Eurofins MWG Operon, Ebersberg, Germany) with Ndel and Xhol restriction sites underlined and in bold.

The gene was amplified using PCR under the following conditions. Initial denaturation at 98°C for 3min. Denaturation step at 95 °C for 15sec, annealing of primers at 61 °C for 30 sec and extension at 72°C for 50sec.These steps were repeated for 30 cycles using KOD polymerase **(Figure 1).**

The vector pET-26b(+) was digested with Ndel and Xhol, dephosphorylated with Shrimp Alkaline phosphatase and purified with PCR purification kit .The PCR products were digested using same restriction enzyme and purified using gel extraction kit. Both restriction enzyme digestions were carried under 37 °C with suitable buffers. Purified vector and purified insert was ligated in 3:1 molar ratio of insert to vector with T4 DNA Ligase (NEB) with suitable buffer at 16°C overnight and transformed into NovaBlue competent cells and confirmed by restriction analysis.

Example 6:

Relative molecular mass (Mr) determination by Matrix-assisted laser desorption ionization/time-of-flight time-of-flight mass spectrometry (MALDI-TOFTOF)

The mass spectrum was recorded by using AB SCIEX TOF/TOF™ 5800 system (AB SCIEX, USA) with 1000 Hz high-repetition laser. 5µl of 100µg/ml of the purified native *Kc*PGA was mixed with equal volume of matrix solution in microcentrifuge tube. The matrix solution of 10 mg/ml sinnapic acid was prepared in 30 % acetonitrile (ACN). About 5 µl of the mixture was applied to a stainless steel sample holder and introduced into the mass spectrometer after drying. **Figure 3,** shows major peaks of α-chain and β chain at 23,588 Da and 62901 Da respectively.

After confirmation by restriction digestion, 3-4 plasmids were sequenced .Sequence analysis of clones showed the size of 2565 bp containing "pac" gene coding sequence of **(IN NCBI : 2734 bp)** 2538bp.Sequencing result showed the KcPAC ORF of 2565bp (including nucleotides for hexahistidine tag and stop codon) encoding **(IN NCBI : 858 amino acids)** 855 amino acids. BLAST analysis showed the pac gene from K. citrophila has some changes both at gene as well as amino acids level from the sequence of "pac" gene from *K.citrophila* as reported by Barbero et al. Complete nucleotide sequence of the penicillin acylase gene from *Kluyvera citrophila.*

Barbero JL, Buesa JM, Gonzalez de Buitrago G, Mendez E, Pez-Aranda A, Garcia JL. Gene 1986, 1986 (Figure 1). Some changes in the gene sequence were also reported by Rao et al Biochem J. 1994 Nov 1;303 (Pt 3):869-75. Changing the substrate specificity of penicillin G acylase from *Kluyvera citrophila* through selective pressure. Rao A, Garcia JL, Salto F, Cortes E., (1994). The active site residues identified as directly involved in catalysis (Ser290, Glu312, Ala358 and Asn530, number corresponds to be present in precursor) were found to be strictly conserved despite of the presence of changes in amino acid sequence. Expression of the sequence confirmed plasmids were checked into three expression different hosts. BL21 (DE3) pLysS cells were transformed with plasmid and the expression from the 1mM IPTG induced cultures expressed at 16°C for 16-18hrs using SDS-PAGE was checked and is as observed with reference to **Figure 2.** Expression in BL21 (DE3) pLysS cells showed the production of processed protein better than the other two hosts so it was chosen as expression host for further studies.

Example 7:

Penicillin G acylase assay

Bomstein and Evans (1965 (1965) Automated colorimetric determination of 6-aminopenicillanic acid in fermentation. Anal Chem) devised a specific assay for 6-APA, which depended on the reaction of the 6-amino group with p-dimethylaminobenzaldehyde to form a coloured Schiff base, which is estimated colorimetrically. Determination of PGA activity was carried out at 50 °C using 2% (w/v) Penicillin G. Enzyme sample was contained in a final volume of 1 ml of 50mM phosphate buffer pH 7.5 for 10 minutes. The reaction was quenched by the addition of lml of 300mM citric acid in 50mM Phosphate buffer (CPB) at pH 2.5. The 6-APA formed in the reaction mixture was estimated spectroscopically at wavelength 415 nm by the addition of 2 ml of a 0.6% (w/v) solution of p-dimethylaminobenzaldehyde in methanol according to Bomstein and Evans modified by Shewale. Appropriate dilution of the quenched reaction mixture in CPB was done to determine the absorbance in the linear range. The substrate concentration 20 mg/ml (2%) was maintained to measure the enzyme activity in all the experiments except stated otherwise. Standard graphs were drawn using pure 6APA (Sigma).

The specific activity of the *KcPGA* purified was determined using the standard curve plotted above. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmol of 6-APA per min under assay conditions.

Example 8:

AHL degradation bioassay using CV026 (Qualitative)

This assay has been designed in such a way that a zone of Violacein synthesis can be seen around the agar well containing the reaction mixture with corresponding AHL. The amount of violacein produced is directly proportional to the amount of AHL remaining in the reaction mixture after enzymatic degradation. *Chromobacterium violaceum* mutant CV026 is used as the test organism which has the ability to respond to a variety of signal molecules. (McClean et.al 1997 Quorum sensing and Chromobacterium violaceum: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology 1997) The QS signal molecule (namely C6-HSL OR C7-HSL) at a concentration of 12.5 µM was added to lml of reaction buffer (phosphate buffer pH6.0 or 5.0) containing 20-40 µg of KcPGA. The reaction mixture was kept at 35oC for a period of 3 hours. For the bioassay plate 75ul of overnight culture of CV026 was added to 10 ml of Luria bertani soft agar and mixed well. The soft agar was then overlaid onto basal LA plate, and a 4mm diameter well was dug in the centre of the plate using a sterile cork borer after the overlay is set. To the agar well 50 ul of the reaction mixture containing AHL and KcPGA was added at 0 time and after 3 hours of reaction, and the plates were incubated in upright position at 30oC for 24 hours. The diameter of zone of Violacein synthesis was then measured and compared with appropriate controls in which enzyme was absent. The biosensor strains used CV026 responds to presence of C6 HSL with violacein production and zone diameter of pigment production is proportional of the amount of AHL in the aliquoted reaction mixture. It was observed that after 3 hour incubation of AHL with .KcPGA there was some amount of AHL degradation as indicated by the decrease in the zone of Violacein production by CV026 **(Figure 4).**

Example 9:

Violacein inhibition assay (quantitative):

This assay is based on a similar principle to the one described above, that in the presence of any AHL acylase or AHL degrading enzyme quantitative decrease in the production of the purple pigment violacein can then be estimated colorimetrically.(Choo et.al 2006 Inhibition of bacterial quorum sensing by vanilla extract,LETTERS IN APPLIED MICROBIOLOGY) In the first step a reaction mixture containing 0.5mM AHL and 30ug of enzyme was incubated at 35oC for a period of 3 hours, with samples aliquoted every hour. A blank reaction was also set which was devoid of any enzyme. Then 75µ1 of overnight culture of CV026 was inoculated in 10ml LB broth in a series of tubes and 20ul of reaction mixture was added at each time interval respectively. The tubes were incubated at 30oC for 16-18 hours. The violacein produced was extracted from the culture broth by dissolution of the pigment in DMSO and separating it from the cell mass by centrifugation. The amount of AHL remaining in the reaction mixture could be correlated with decrease in the purple pigment production which could be quantitatively estimated by measuring the optical density at 570nm. Appropriate Controls were used. All experiments were done in triplicates for the sake of reproducibility. It was observed that there was almost complete degradation of C6HSL after incubation with *KcPGA* over a period of three hours and percentage decrease in Violacein production was plotted as a function of time **(Figure 5).**

Example 10:

Deacylation of N-Acyl Homoserine Lactone by *KcPGA* (HSL-OPA assay

To determine the amidohydrolase activity of KcPGA against AHLs this assay was used. Free amino acid in the form of the HSL moiety released during the deacylation reaction was estimated using o-phthaldialdehyde (OPA) in 0.1 M Na-borate. OPA stock was made by adding 4 mg of OPA dissolved in 0.1 ml of ethanol with 5 mg of dithiothreitol (DTT) dissolved in 4.9 ml of 0.1 M Na-borate buffer pH 9.0.The OPA derivatized, fluorescence active amine formed, was monitored at 340nm and O.D. was calculated. A standard plot of Pure HSL (Sigma) in the concentration range of 0.1 mM - ImM was plotted for calculating the amount of HSL released after enzymatic degradation. This assay was used for optimizing reaction conditions such as pH,

temperature and incubation time. With reference to Figure 6, the pH optima for AHL acylase activity of *KcPGA* was found to be 8.0 AND Temperature optimum was found to be 50°C. Under these optimal condition of pH and temperature the AHL deamidation reaction when continued for 3 hours gave best results as seen from **Figure** 6.

Example 11:

Anti-Biofilm activity of KcPGA using Vibrio cholerae as test organism

Biofilm forming potential of *V.cholerae* has been recognized as an important part of the pathogenesis of this well known enteric pathogen. Biofilm formation is quorum sensing mediated phenotype so any enzyme that can degrade quorum sensing signal molecules may have the potential to inhibit biofilm formed by *Vibrio* Sp. In this assay 5ul of overnight culture of *V.cholerae* was added to 200ul of culture media with or without appropriate concentration of .KcPGA in a 96 well plate. The plate was then incubated at 37°C for 16-18 hours with gentle shaking. After incubation all the spent media from the wells along with planktonic cells was discarded and the biofilms were gently washed twice with deionized water. The biofilm left in the wells was then allowed to air dry. It was then stained with 0.1% crystal violet for 10 mins. After staining excess of stain was discarded and the biofilm washed with deionized water twice. After air drying the stained biofilms 200ul of 95% ethanol was added to the wells to dissolve all the dye absorbed in the biofilm. The content from each well was then transferred to a freshly labeled well respectively. Optical density was measured at 595nm using a Microtitre plate reader. The intensity of purple color is directly proportional to the amount of biofilm formed in each well and test O.D is compared to that of control well to determine percentage inhibition of biofilm formation. KcPGA was indeed capable of reducing the extent of biofilm formed by the test organism however the concentration of enzyme capable of mediating this effect was very high as seen from **Figure** 7.

Example 12:

Comparative experimental data to establish that claimed KcPGA is better in quorum quenching substantially in comparison with EcPGA.

Enzyme used - 20 µg

Concentration of substrates used - 0.5 mM

Incubation temperature: 40°C

Enzyme used	**Substrate**	**Time of Incubation**	**HSL released (mM)**
*Kc*PGA	C6HSL	3 hours	0.31
	C7HSL	3 hours	0.34
*Ec*PGA	C6HSL	3 hours	0.12
	C7HSL	3 hours	0.16

2179WO055, Sequence Listing-Final, **20140424_ST25**

SEQUENCE LISTING

<110> Council of Scientific And Industrial Research

<120> A Method of Quorum Quenching

<130> **2179WO055**

<150> **1232/DEL/2013**
<151> **2013-04-26**

<160> **2**

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WE CLAIM:

1. A method for quorum quenching using KcPGA enzyme from *Kluyvera citrophila.*

2. A method as claimed in claim 1, wherein the enzyme has degrading activity, against AHLs.

3. A method as claimed in claims 1-2, wherein the enzyme has degrading activity against medium length AHL's of C6-C8 carbons.

4. A method as claimed in claims 1-3, wherein the enzyme KcPGA shows preference for degrading homoserine lactones (HSL) in the order of 3-oxo C6 HSL> C6 HSL> C7 HSL> C8 N-octanoyl-L-homoserine lactone.

5. A method as claimed in claims 1-4, wherein the quorum quenching is achieved against gram negative bacteria.

6. A method as claimed in claims 1-5, wherein enzyme is useful against biofilm.

7. Use of KcPGA enzyme from *Kluyvera citrophila* in inactivating quorum sensing signals by degrading acyl homoserine lactones.

8. Use of KcPGA enzyme as claimed in claim 7, wherein the enzyme has degrading activity against medium length AHL's of C6-C8 carbons.

9. Use of KcPGA enzyme as claimed in claims 7-8, wherein the enzyme KcPGA shows preference for degrading homoserine lactones (HSL) in the order of 3-oxo C6 HSL> C6 HSL> C7 HSL> C8 N-octanoyl-L-homoserine lactone.

10. Use of KcPGA enzyme as claimed in claims 7-9, wherein inactivation of quorum sensing signals by degrading acyl homoserine lactones results in quorum quenching.

11. Use of KcPGA enzyme as claimed in claims 7-9, having activity against biofilm.

12. A KcPGA enzyme from *Kluyvera citrophila* having antimicrobial or antibacterial activity.

13. The KcPGA enzyme as claimed in claim 12, having antibacterial activity against gram negative bacteria.

14. A composition comprising KcPGA enzyme along with a pharmaceutically -acceptable excipient.

15. The composition as claimed in claim 14, wherein the composition is combined with antimicrobial or antibacterial antibiotic.

16. A composition as claimed in claims 14-15, for quorum quenching.

17. A composition as claimed in claims 14-15, for degrading or blocking quorum sensing signals by degrading AHL's.

18. A composition as claimed in claims 14-15, for quorum quenching of biofilms.

19. A method of treating or preventing or slowing down a process a condition in a subject by administering a composition as claimed in claims 14-15.

20. A method as claimed in claim 19, wherein the condition is a disease caused by microbe or bacteria.

21. A method as claimed in claim 19, wherein the condition is caused due to quorum sensing activity or signaling or microbe or bacteria.

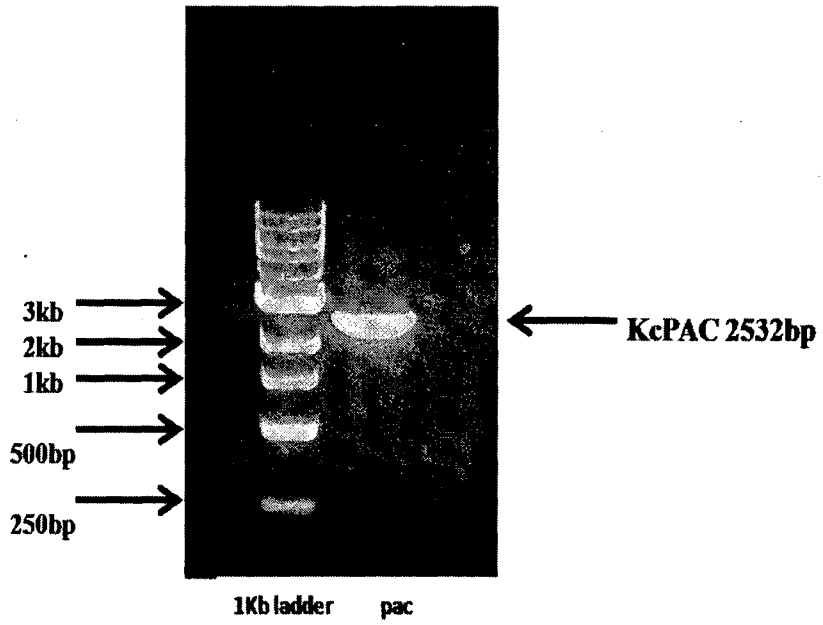
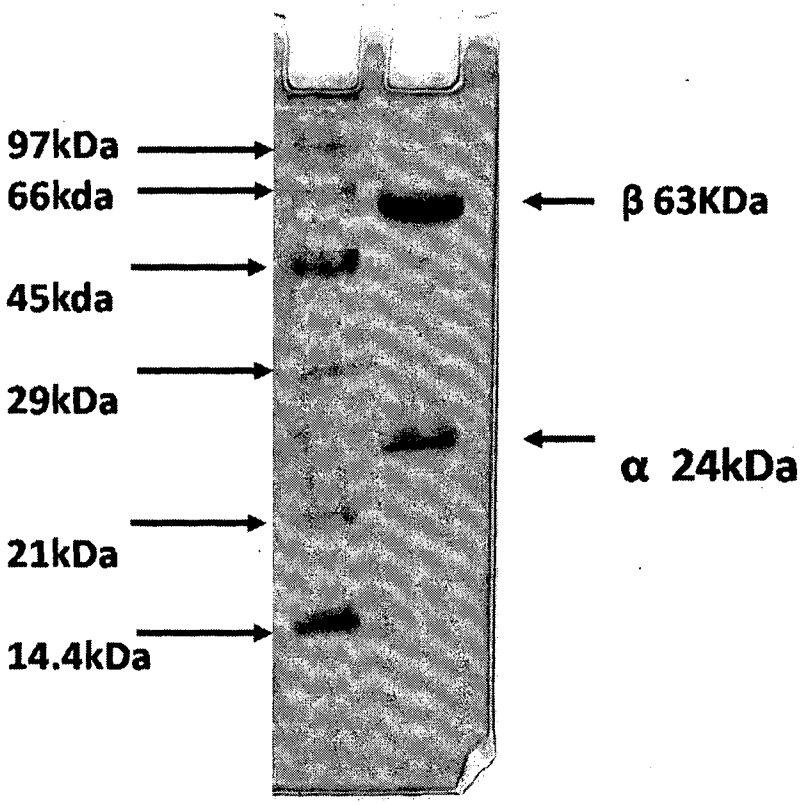


Figure 1: PCR amplification of *Kc*PAC gene of 2532bp loaded on 1.5% agarose gel.



15%SDS-PAGE

Figure 2: Expressed enzyme checked by 15% SDS PAGE.

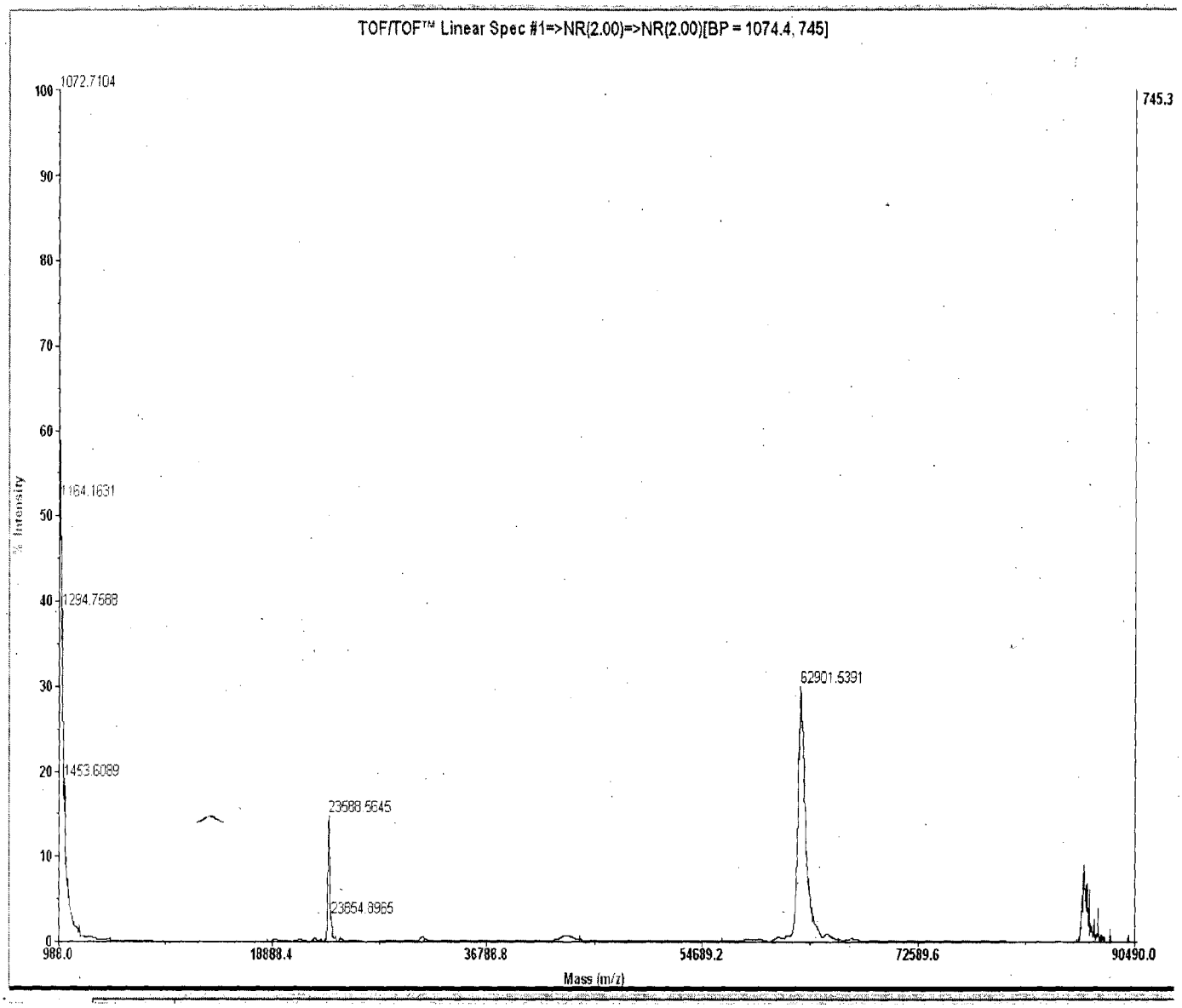


Figure 3: Matrix-assisted laser desorption ionization / time-of-flight time-of-flight mass spectrometry (MALDI-TOF-TOF).

Degradation of C6-Homoserine Lactone by *K.citrophila* PGA

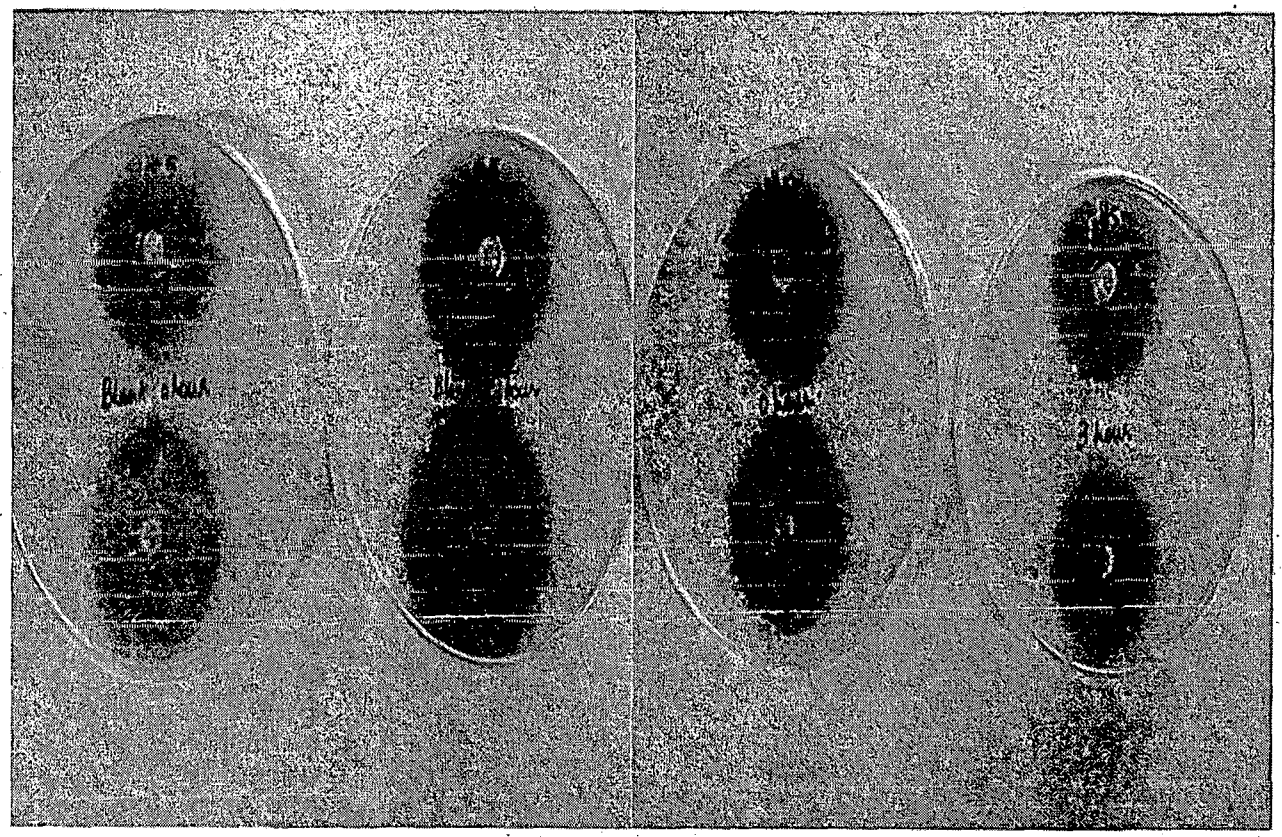


Figure 4: Degradation of C6-HSL by k.citrophilia PGA.

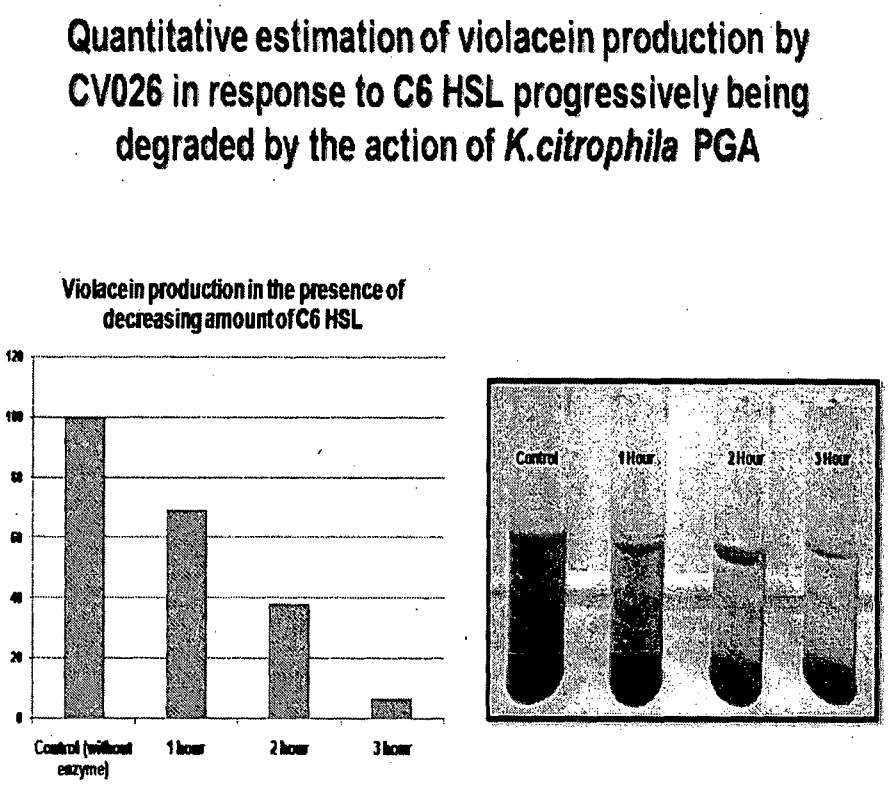


Figure 5: Quantitative estimation of violacein production by CV026 in response to C6 HSL progressively being degraded by the action of k.citrophlia PGA.

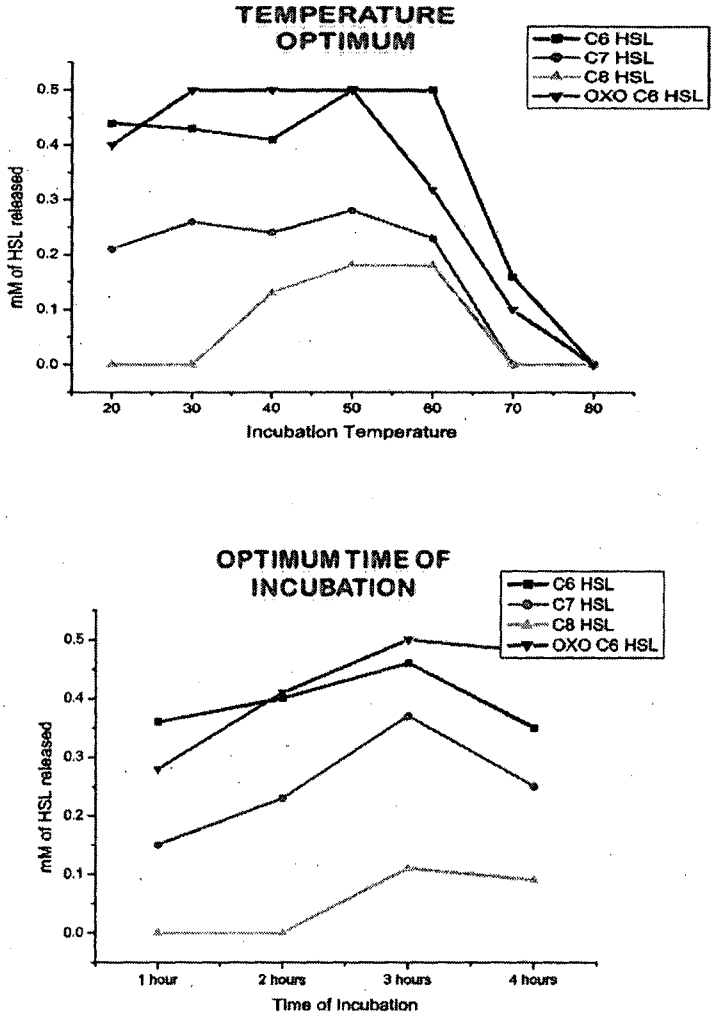


Figure 6: Graphs depicting optimizing the time, temperature and pH for incubation.

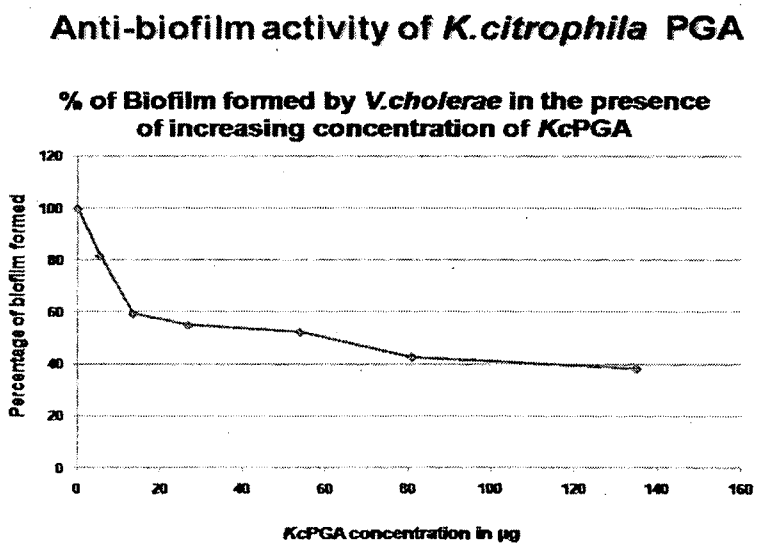


Figure 7: Anti bio filming activity of KcPGA.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2014/000265

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/50 A61P31/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	MUKHERJI RUCHI RA ET AL: "A new rol e for peni cillin acyl ases : Degradati on of acyl homoseri ne l actone quorum sensi ng si gnal s byKl uyvera ci trophi l apeni ci 11i n G acyl ase" , ENZYME AND MICROBIAL TECHNOLOGY, STONEHAM, MA, US, vol . 56, 19 December 2013 (2013-12-19) , pages 1-7 , XP028665967 , ISSN : 0141-0229 , DOI : 10. 1016/J . ENZMICTEC. 2013 . 12 .010 the whol e document -----	1-21
X	Wo 03/068951 Al (INST OF MOLECULAR AGROBIOLOGY [SG] ; ZHANG LIAN HUI [SG] ; LIN YI HAN [S) 21 August 2003 (2003-08-21) paragraphs [0027] , [0048] , [0049] ; exampl es -----	1-21
	-/- ·	

X Further documents are listed in the continuation of Box C.

X See patent family annex.

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"E" earlier application or patent but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) orwhich is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 September 2014	07/10/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fri ederi ch, Marti n

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/98214 Al (N0V0ZYMES BIOTECH INC [US]) 27 December 2001 (2001-12-27) claims ; examples ----	1-21
X	Robert Czajkowski ET AL: "Quenching of acyl-homoserine lactone-dependent quorum sensing by enzymatic disruption of signal molecules" , Acta biochimica Polonica, 1 January 2009 (2009-01-01) , page 1, XP055140727 , Poland Retrieved from the Internet: URL: http://www.ncbi .nlm.nih.gov/pubmed/19287806 the whole document ----	1-21
X	DATABASE WPI Week 198941 Thomson Scientific, London, GB; AN 1989-294792 XP002730151 , & ES 2 005 883 A (C0NSEJ0 SUPERIOR INVESTIGACION) 1 April 1989 (1989-04-01) abstract ----	12-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IN2014/000265

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		EP 1470221 A1	27-10-2004
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		US 2004109852 A1	10-06-2004
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