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A RAPID IODOMETRIC METHOD FOR QUALITATIVE DETECTION OF AHL LACTONASES

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ABSTRACT

Quenching quorum sensing by enzymatic means has been attempted by many because it is has been shown to be a powerful inhibitor of bacterial virulence without the risk of increasein resistance.AHL Lactonase is one such class of enzymes that can inhibit communication amongst gram negative bacterium by degradation of its communication signal Acyl homoserine lactone, thus not allowing successful pathogen establishment. In this brief note, a simple qualitative method for detection of AHL lactonases, in crude or partially pure enzyme preparation has been described which can be used to screen multiple samples in high throughput format.

Keywords: AHL Lactonase, Penicilloic acid, Iodine.

1. INTRODUCTION

Quorum sensing is now an accepted means by which a given bacterial population interacts socially. [1] Moreover it has also been well established at present that it is a major mechanism by which bacterial pathogens regulate their virulence gene expression. [2] Bacteria, with the help of quorum sensing signals and their cognate receptors, are able to take full advantage of the available environment and evade the deleterious host mechanisms. What one bacterium can't do, many can..! And quorum sensing makes this possible. This population density dependent phenomenon allows bacterial community to come together and function as a single entity, thus increasing the strength and the persistence of the invading pathogens. [1, 3] Quorum Sensing has been studied in both gram positive and gram negativebacterium, and the most well characterized class of signal molecules in case of gram negative bacterium is Acyl homoserine lactone. [2] Expression of a diverse range of phenotypes is mediated by QS based

regulons like, antibiotic production, pigment formation, biofilm formation ,expression of exo-enzymes and virulence factors, for example, proteases, lytic enzymes, exotoxins etc. [1,2,3] In this light, impeding quorum sensing in any way will weaken the bacterial pathogen and thus allow anti-infective therapies a chance to destroy the invader. This concept is better known as Quorum Quenching (QQ). Quorum Quenching has been predicted to be the future of antimicrobial therapy because it offers the least possible opportunity of development of resistance, by imposing no life or death selective pressure upon the pathogenic bacteria. [4] A variety of mechanisms have been proposed which can aid in quenching, quorum sensing, in gram negative systems, and the enzymatic means of QQ has been proven to be the most promising.Of the different classes of enzymes capable of quenchingAHL mediated quorum sensing, two major classes of enzymes have received significant attention, namely AHL acylase and AHL lactonase. [5]

To date AHL Lactonaseshave been identified and studied in varied organisms (Table1).The first report of an authentic AHL Lactonase was by Dong et al., wherein they identified a gene *aiiA* from *Bacillus sp*. which was subsequently shown to code for a potent AHL degrading lactonase. [6] Since then AHL lactonase producing capability has been recognized in many different genera and in fact in different species and sub-species of *Bacillus.* [7] AHL lactonase acts on the lactone ring, hydrolyzing it and inactivating the QS signal molecule (Fig.1A).Assay systems are well in place that allow detection of these AHL lactonases.Bioassay based approach utilizes a biosensor strain capable of responding to AHL signals by elaborating an easily detectable phenotype. [8] However these assays can be very time taking, too cumbersome when test sample numbers are large, and may give false positive results if appropriate controls are not available. Chemical assays used to detect AHL lactonase activity are based on the premise that hydrolysis of AHLs yields a ring-opened product along with, one proton and this production of protons can be detected by using a continuous spectrophotometric pH indicator assay. [9] However this system suffers from the requirement of very high substrate (AHL) concentration and very pure enzyme preparations. Also finding the perfect buffers and indicators pairs to match the reaction pH and indicator p*Ka* can be very tricky. In this article we propose a facile qualitative detection system, to detect AHL Lactonase activity using Penicillin G as substrate (Fig. 1B).

2. MATERIALS AND METHODS

2.1 AHL Lactonase enzyme preparation

Crude and partially purified enzyme preparations of AHL Lactonase were obtained from a novel Bacillus sp. Concentration of both crude dialyzed enzyme and Q-Sepharose partially purified enzyme was set at 1mg/ml before use in this iodometric assay.

2.2 Reagents and chemicals

Penicillin G potassium salt was procured from SIGMA-Aldrich, India. Soluble Starch and Iodine crystals of high purity were obtained from HiMedia, India.

2.3 Iodometric assay for Lactonase activity

This assay is based on the rationale that AHL lactonases possess the capability to act on intact Penicillin molecule and hydrolyzing it to Penicilloic acid. Penicllin G as such is inert to iodine in neutral aqueous solution while penicilloic acid,the inactivation product derived from Penicllin quenches 6-9 equivalents of added iodine,thus iodine (the colorimetric reagent)is not free to interact with 0.1% starch (already present in the reaction mixture) and gives no blue color. [10] However in Penicillin only control (without enzyme) since there is no formation of penicilloic acid all the added 10% iodine reagent is free to interact with the starch already present in the reactionmixture thus giving a blue color. The hydrolyzing activity of AHL lactonase on Penicillin G was estimated by incubating the reaction mixture at 40^{o}C for 15 minutes. Presence or absence of blue color, which develops as a result of interaction of free/unquenched iodine with 0.1% starch solution, was noted within 10 seconds of addition of Iodine reagent and mixing it well (Fig. 2). Appropriate controls were used to eliminate confusing observations.

3. RESULTS AND DISCUSSION

Methods, both biological and chemical, are available to detect AHL lactonase activity; however each of them suffers from their own drawbacks. The present article reports a rapid method of AHL lactonase activity detection based on a very simple principle. AHL lactonase belong to beta-lactamase superfamily.[7] Members of beta lactamase superfamily are known to act on beta-lactam ring of Penicillin and cephalosporin molecules, causing a ring opening hydrolysis reaction. The above two facts led to the musing that AHL lactonase may also possess the ability to hydrolyze penicillin molecule leading to the formation of Penicilloic acid. This forms the basis of our qualitative assay. This Penicilloic acid formed after

Penicillin inactivation could then easily be estimated using starch-iodine detector-indicator system, which is already a well-established method first reported by Cole et al. in 1973. [10]

An important point that should be noted here is that there is some amount of spontaneously degradation of Penicillin at the required incubation temperature of 40^{o}C, hence the Penicillin only control will show a slight decrease in blue color when compared with starch only control.To minimize this spontaneously degradation of the substrate, incubation time and temperature parameters must be modified in order to keep all ambiguities at bay.The assay was performed using different concentrations of partially purified AHL lactonase preparations from a novel *Bacillus sp*. and it was found that the system could detect degradation of Penicillin G with as less as 20µg of enzyme (Fig. 3). It should be also noted that Penicilloic acid formed after hydrolysis of Beta-lactum ring of Penicillin G quenches iodine very quickly hence color development should be observed within 10secs of addition of 10% iodine solution.The biggest advantage that this assay system offers is in terms of time and economy. The color development can be observed within seconds, does not require high concentration of AHLs and this assay can easily be performed in a 96 well format to screen large number of enzyme fraction within minutes.

Table1 Some AHL lactonases reported in literature

Organism	***Gene designation***	**Reference**
Bacillus sp.	*aiiA*	Dong et al. 2002
Bacillus thuringiensis	*aiiA*	Park et al. 2008
Bacillus subtilis BS-1	*aiiA*	Pan et al. 2008
Bacillus amyloliquefaciens	*aiiA*	Yin et al. 2010
Bacillus cereus	*aiiA*	Tinh et al. 2013
Bacillus weihenstephanensis	*aiiA*	Sakr et al. 2013
Arthrobacter sp.	*ahlD*	Park et al. 2003
Agrobacterium tumefaciens	*attM*	Zhang et al. 2002
Rhodococcuserythropolis	*qsdA*	Uroz et al. 2008
Pseudoalteromonasbyunsanensis	*qsdH*	Huang et al. 2012
Ochrobactrum sp.	*aidH*	Mei et al. 2010
Microbacteriumtestaceum	*aiiM*	Wang et al. 2010
Chryseobacterium sp. strain StRB126	*aidC*	Wang et al. 2012

Figure 1

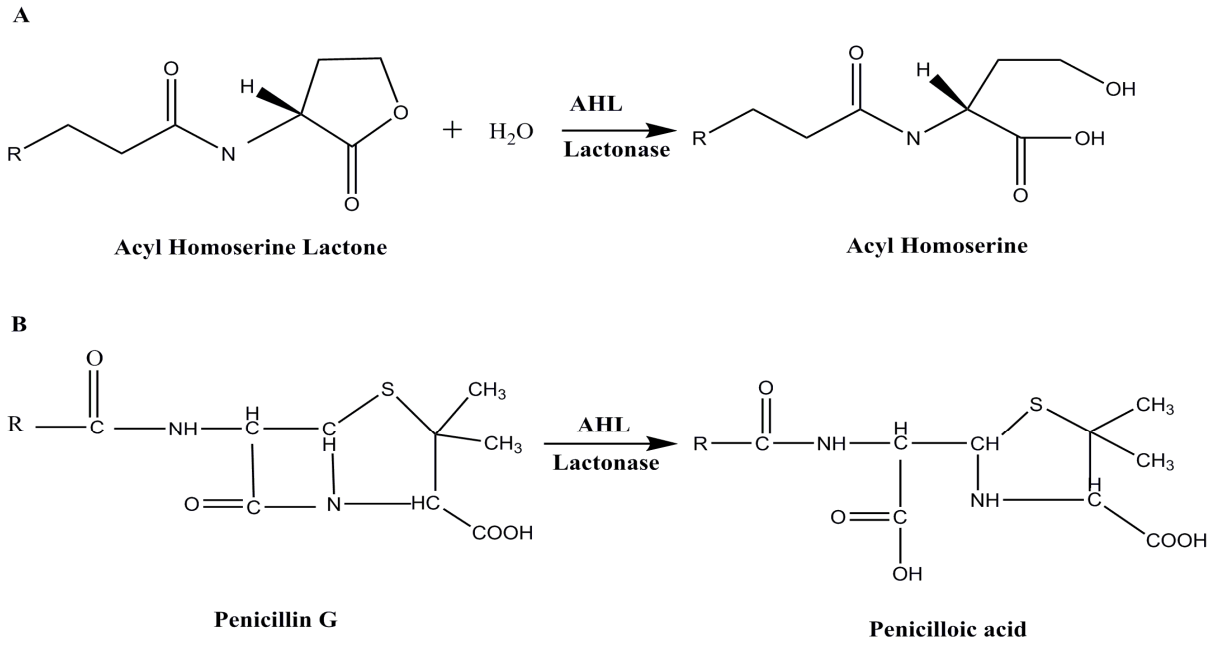


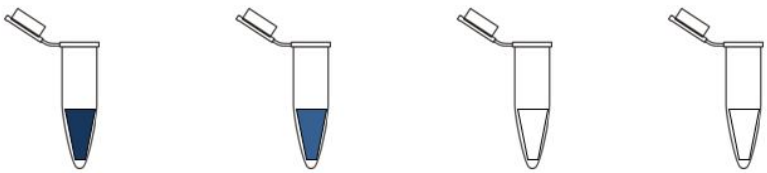
Figure 1 A Known mode of action of Lactonases on AHL molecule. BProbable mode of action of AHL Lactonase on Penicillin G

Figure 2

	Contents of the Tube (600ulofreaction Mixture)			
	1	2	3	4
Penicillin G (20mg/ml)	-	200 µl	200 µl	200 µl
Starch Indicator (0.1%)	200 µl	200 µl	200 µl	-
Lactonase enzyme preparation (1mg/ml)	-	-	200 µl	-
Milli-Q water	200 µl	200 µl	-	200 µl

Incubate at 40°C for 15 minutes & add 20µl of 10% Iodine Solution.

After addition of Iodine Solution observe blue color within 10 seconds



1 2 3 4

Figure 2 Basic iodometric assay protocol to determine hydrolyzing activity of AHL lactonase.

Figure 3

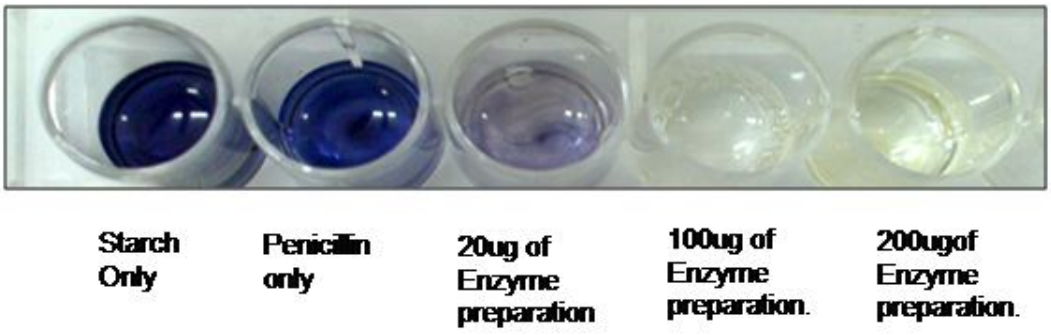


Figure 3 Photographic evidence of AHL lactonase activity on Penicillin G. With increasing concentration of enzyme preparation more amount of penicilloic acid is produced hence the decrease in blue color.

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