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Colorimetric analysis of Alkaline Phosphatase activity in S. aureus Biofilm

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TITLE:

Colorimetric Analysis of Alkaline Phosphatase Activity in *S. aureus* Biofilm

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KEYWORDS:

Biofilm, *S. aureus*, alkaline phosphatase, colorimetric, pNPP, quantitative, high throughput

SUMMARY:

In this manuscript, we established a high throughput method to quantify alkaline phosphatase activity in *S. aureus* biofilm culture from 96-well tissue culture plate

ABSTRACT:

Alkaline phosphatase (ALP) is a common enzyme expressed in both prokaryotic and eukaryotic cells. It catalyzes the hydrolysis of phosphate monoesters from many molecules at basic pH and plays an indispensable role in phosphate metabolism. In humans, eukaryotic ALP is one of the most frequently used enzymatic signals in diagnosing various diseases, such as cholestasis and rickets. In S. *aureus*, ALP is detected exclusively on the cell membrane; it is also expressed as a secretory form as well. Yet, little is known about its function in biofilm formation.

The purpose of this manuscript is to develop a quick and reliable assay to measure ALP activity in *S. aureus* biofilm that does not require protein isolation. Using p-nitrophenyl phosphate (pNPP) as a substrate, we measured ALP activity in *S. aureus* biofilm formed in 96-well tissue culture plates. Activity was based on the formation of the soluble reaction product measured by 405 nm absorbance. The high throughput nature of the 96 well tissue culture plate method provides a sensitive and reproducible method for ALP activity assays. The same experimental set up can also be extended to measure other extracellular molecular markers related to biofilm formation.

INTRODUCTION:

Alkaline phosphatase (ALP) is ubiquitously expressed in both prokaryotic and eukaryotic cells[1]. It can catalyze the hydrolysis of monophosphate from different molecules such as nucleotides, proteins, alkaloids, phosphate esters and anhydrides of phosphoric acid. In humans, eukaryotic ALP is present in many tissues including liver, bone, intestine and placenta[2]. It plays important roles in protein phosphorylation, cell growth, apoptosis, stem cell processes as well as normal skeletal mineralization. Eukaryotic ALP is also a key serum indicator for the presence of diseases

in bone, liver, and other tissues/organs when elevated[3,4].

Prokaryotic ALP has been detected in a variety of bacterial cells, including *E. coli*[5], *S. aureus*[6,7] and some common rumen bacteria in the soils[8]. Bacterial ALP activity has been used as a biosensor in the detection of pesticides, heavy metals[9] and bacterial contamination[10]. The constitutive expression of ALP has been used to identify *Staphylococci*[11] and to differentiate *Serratia* from *Enterobacter*[12]. It is further suggested that constitutive ALP production is correlated with pathogenicity in *staphylococci*[13]. Although ALP has been studied in different settings[3,4], yet little is reported for its activity and function in biofilms cultures.

A biofilm has been documented to have a differently functioning bacterial life as compared with its free-living bacterial cell counterpart[14]. In *S. aureus*, the formation of biofilm has been identified in a variety of clinical conditions and accounts for the antibiotic resistance and chronic inflammation[15,16]. Many molecules had been reported to be found in a biofilm matrix such as polysaccharides, proteins, nucleic acids, and lipids, but the mechanism of biofilm formation is not fully understood[14]. To understand the role of ALP in biofilm formation, we cultured *S. aureus* biofilms in 96 well tissue culture plates and measured the ALP activity using para-nitrophenylphosphate (pNPP).

The molecule pNPP is a ready-to-use substrate for ALP and has been widely used to measure ALP activity[6,17,18]. This colorimetric assay is based on the conversion of para-nitrophenyl phosphate (pNPP) to para-nitrophenol resulting in a colored product at 405 nm. Compared to other conventional ALP assay, such as agarose gel electrophoresis[19], wheat germ agglutinin (WGA) precipitation, and WGA-HPLC[20], this assay is highly specific, sensitive, easy to reproduce, and most importantly, allows for high throughput.

PROTOCOL:

1. Medium preparation

1.1. Prepare 1 L of Tryptic Soy Broth (TSB): Into 1 L of distilled water, add 15 g of pancreatic digest of casein, 5 g of papaic digest of soybean meal, 3 g of sodium chloride, 2.5 g of dextrose, and 2.5 g of dipotassium phosphate. Sterilize before use.

1.2. For Tryptic Soy Agar (TSA), add 15 g of agar to 1 L of TSB, autoclave. Then let it cool down to room temperature. Next, pour at a ratio of 20 mL per Petri dish (100 mm x 15 mm).

1.3. Biofilm culture medium: Add 10 g of glucose to 1 L of autoclaved TSB. Sterilize by filtration using a 0.2 µm filter.

2. *S. aureus* biofilm formation in 96 well tissue culture plate

NOTE: The *S. aureus* biofilm is cultured as previously described[6,21].

2.1. Inoculate one individual colony of *S. aureus* from TSA in 10 mL of TSB and grow overnight at 37 °C.

2.2. Dilute the overnight culture into 10 mL of TSB/glucose (10 g/L) at a 1:100 (v/v) ratio. Vortex gently to mix for a consistent concentration.

2.3. Transfer 200 μL of diluted culture into a total of 18 wells (more for experimental groups, such a non-biofilm control to suggest a relationship to ALP activity[6]) from one 96 well plate. Use triplets for each time point: 0 min, 15 min, 30 min, 45 min, 60 min, and 75 min. See step 3.2.

2.4. Incubate the 96 well plate at 37 °C for 24 h for biofilm formation[6,21].

2.5. Decant the supernatant by aspiration.

2.6. Wash each well with 1x phosphate buffered solution (PBS, pH 7.4), centrifuge for 5 min at 15,000 x *g* and decant the supernatant by aspiration.

3. Measurement of ALP activity in *S. aureus* biofilm

NOTE: ALP activity was measured as described[6,18].

3.1. Add 75 μL of buffered ALP substrate consisting of commercially available pNPP to each well from step 1.6 and begin recording the time. Record each time point in triplicate.

3.2. After incubation for a varied time: 0 min (control), 15 min, 30 min, 45 min, 60 min, and 75 min respectively, add 75 μL of 5 M NaOH to stop the reaction.

3.3. Centrifuge the plate for 5 min at 15,000 x *g*.

3.4. Transfer 100 μL of supernatant into a new 96 well plate for colorimetric measurement.

3.5. Measure the absorbance of each well at 405 nm using a 96 well plate reader. Use the 0 min time point wells to control for 405 nm background noise.

3.6. Repeat the entire experiment in three individual 96 well plates.

REPRESENTATIVE RESULTS:

Figure 1 shows a representative result of ALP activity from biofilm cultures of *S. aureus* in 96 well tissue culture plates. 75 μL of commercially available pNPP solution was added to each well and incubated at room temperature. After incubation for different times (0 min, 15 min, 30 min, 45 min, 60 min, and 75 min, respectively) 75 μL of 5 M NaOH was added to stop the reaction. The product was then measured in a 96 well plate reader at 405 nm. Each time point was repeated in triplets from a single 96 well plate, and the entire experiment was repeated in 3 individual 96

well plates.

FIGURE AND TABLE LEGENDS:

Figure 1: *S. aureus* biofilm cultures were incubated with pNPP substrate for different time (0 min, 15 min, 30 min, 45 min, 60 min and 75 min) and their ALP activity was measured at 405 nm. Error bars represent standard deviation.

DISCUSSION:

In our assay, we used pNPP as the ALP substrate. This is a working solution designed for ELISA and no dilution is needed. After hydrolysis by ALP, a yellow product develops and can be measured at 405 nm. At the end of the enzymatic reaction, we briefly centrifuged the 96 well plate and transferred the supernatant to a fresh 96 well plate to measure absorbance using the plate reader. We found that this extra centrifugation step is critical, since it increases the consistency of absorbance at 405 nm, probably by eliminating any possible suspended cells incurred during enzymatic reaction.

For biofilm formation, we use 200 μL of a 1:100 (v/v) dilution for overnight culture as reported[6,21]. This is the optimum dilution for *S. aureus* to form biofilm compared to other dilutions (data not shown). Since the purpose of this paper is to measure ALP activity in biofilm, it was crucial that we maintained optimum biofilm culture conditions. This was further emphasized by the choice in using glucose at 10 g/L for optimum biofilm growth[6]. The dilution factor and glucose concentration need to be optimized if different bacterial cells are used for biofilm formation.

During incubation of the biofilm culture with pNPP substrate, it will take time for the colored product to develop. We measured ALP activity after 15- and 30-min time points which is when the visible yellow color is observed. With the current concentration, we noticed an increased ALP activity for up to 75 min as indicated in **Figure 1**. After 75 min, no increase of the ALP activity was observed.

Finally, since ALP in *S. aureus* is an exclusively membrane bound protein[7], its activity can be tested without cell lysis. There are benefits to an enzyme activity assay that does not require whole cell destruction. Notably, the protein isolation process can sometimes yield a lower active enzyme quantity[22]. However, this protocol cannot be used if ALP is not in its membrane bound form.

As previously reported[6], ALP activity is elevated in biofilm culture as compared to its platonic counterpart. The experimental settings used in the current study can be extended to investigate factors/compounds that affect ALP activity in *S. aureus* biofilm. The findings from these studies will provide additional insight into how to control bacterial biofilm formation.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

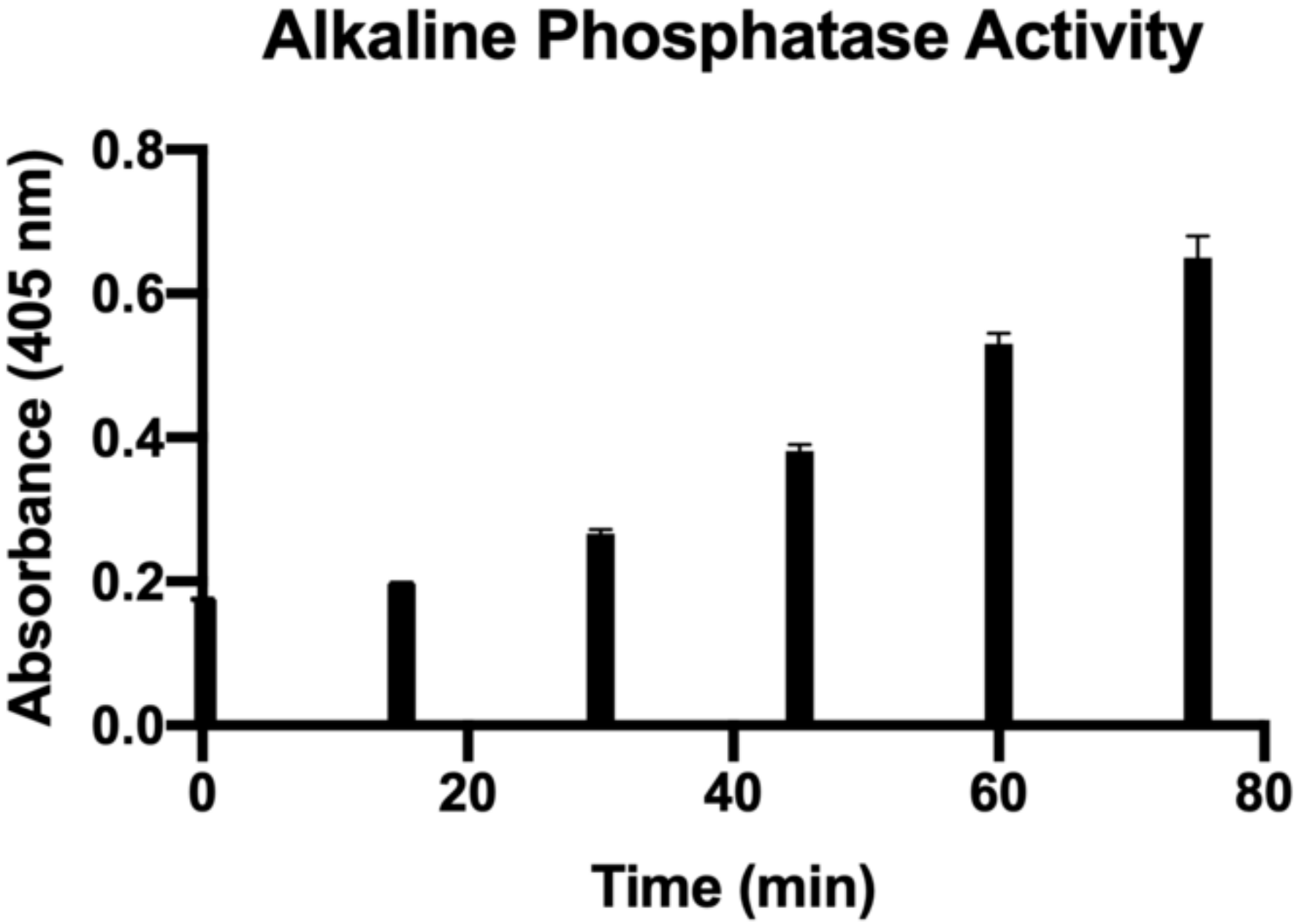

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Agar	VWR	9002-18-0	
Eppendorf Centrifuge	Thomas Scientific	5810	
Gluose	VWR	50-99-7	
NaOH pellets	VWR	SS0550-500GR	
Para-nitrophenylphosphate (pNPP)	Sigma	P7998-100ML	Typical concentrations of pNPP liquid substrates, often used in enzyme-linked immunesorbent assays (ELISA), range between 10 to 50 mM. Similar to most ready-to-use pNPP liquid substrates like the one used here, the exact pNPP concentration is not disclosed due to its proprietary nature.
10X PBS, pH7.4. 173 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 2 mM KH2PO4	Sigma	P3288-1VL	
Plate Reader	Biotek	ELx808	
S. aureus	ATCC	ATCC25923	
Tryptic Soy Agar 15g / L TSB	VWR	9002-18-0	

Tryptic Soy Broth: g/L Pancreatic Digest of Casein..... 15.0 Papaic Digest of Soyben Meal.....5.00 Sodium chloride..... 3.00 Dextrose..... 2.50 Dipotassium phosphate.....2.50	VWR	90006-098	
96 well tissue culture plates	BD	6902D09	U shaped bottom

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Line 41: Please include some sort of introduction to the biofilm formation in *S. aureus* as well.

We added some to the introduction in reference to biofilm (Line 64) and revised the paragraph as well.

Line 75: Please include composition of both liquid and agar medium. Do you sterilize the medium before use? If yes how. Please include these details in the step as well.

We add a new section of the protocol: Medium preparation (Line 82 to line 92). All compositions are included.

Line 78: What volume?

See revision, Line 97.

Line 81: Please clarify are you plating in triplicate in one 96 well or using 3 96 well per time point.

See revision, Line 103-Line 104

Line 84: Does this lead to biofilm formation? Please provide reference if any?

Yes, it does. To clarify, we cited our previous publication and another reference as well (Line 107).

Also, do you perform the centrifugation step here?

No, we didn't. Since *S. aureus* forms biofilm at the bottom and sides of the plate and we aimed to measure ALP activity in biofilm, thus we needed to remove the suspended cells by aspiration.

Line 86: How easy or difficult it is to decant by aspiration?

It is fairly easy, although it is quite tedious. If you have a multi-channel pipet it takes less than 10 pipet pumps.

You mention that ALP is secretory protein, then why do you decant the supernatant? One would assume that the protein would be found in the supernatant.

Good point, in the current revision, we further emphasized that (see Line 34-Line 35, and Line 167) "its activity has been detected exclusively on the cell membrane". This evidence was provided by Okabayashi, et al (ref # 7, pg 290).

Line 88: How do you avoid the cell lysis?

We convert G force to the respective rpm (8000 rpm), cells have been viable after this speed and

we have not seen issues in ALP activity. Cell lysis should not pose a problem with this rotation or force.

Line 91: This assay is showing the measurement with different time point of incubation with pNPP. So basically, the time it takes with NPP to develop color.

However, this does not show how ALP measurement is related to biofilm formation. Need some sort of result to show the same. Maybe different time point of biofilm and then the assay to go in alignment with the manuscript.

Data suggesting a relationship between ALP activity and biofilm has been shown in our previous study. We added a note and a reference to previous data.

Line 110: There should be a result figure to show that indeed you are checking the activity in biofilms

Then the figure presented here which suggest different incubation time with the substrate. Also needed is some sort of control.

Then one more which suggest that you are able to differentiate ALP production in biofilms generated at different time by same species or either by different species.

In our previous publication, we already established that ALP activity is elevated in biofilm compared to its suspension part, thus, ALP activity might be a molecular marker for biofilm formation or manipulation. Whether this elevated activity caused the biofilm formation or not needs further investigation.

As a follow up for this project (also as editor suggested), we will be studying the time course of ALP activity corresponding to the extent of biofilm formation. This is an ongoing project, we are not ready to publish the data yet.

Since this manuscript is to measure ALP activity in biofilm, our current data should be sufficient at this point.

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To avoid copy right issue, we produced a new figure based on our result, this figure has not been published.

Line 123:

As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We made some minor revisions in the "Discussion". For critical step, see Line 152. For modifications, see Line 159 to Line 161. For limitations, see Line 172. For future applications, see Line 175 to Line 177.