International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X
www.ijpsi.org Volume 4 Issue 8 ‖ August 2015 ‖ PP.13-18

In vitro Applications of In silico Designed Biofilm Inhibitors for Staphylococcus aureus

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ABSTRACT: *In recent years anti-virulence agents have been used widely to reduce bacterial resistance and prevent damage to host cells and normal flora. Five molecules were used in this study (according to previous in silico studies) to detect their anti-biofilm activity in in vitro conditions. Three molecules gave positive results and were as follows: Acetic Acid and Acetaminophen inhibited biofilm production 100% at 5000 µg/ml concentration while Acetylsalicylic Acid inhibition was 100% at 10000 µg/ml. All the molecules at the used concentrations were found to affect biofilm production without significant change in bacterial growth. It was concluded that a structure based drug design strategy using Ligand Based Virtual Screening had a success score of about 60% and that Acetic Acid, Acetaminophen and Acetylsalicylic Acid can be used as anti-biofilm molecules. Also Non-Steroidal Anti-Inflammatory Drugs family can be a useful library for anti-biofilm future investigations.*

KEYWORDS – *Acetaminophen, Acetic Acid, Acetylsalicylic Acid, anti-biofilm, drug design, S. aureus.*

I. INTRODUCTION

A biofilm can be defined as a microbially-derived sessile community, typified by cells that are attached to a substratum, interface, or to each other, are embedded in a matrix of extracellular polymeric substance, and exhibit an altered phenotype with regard to growth, gene expression and protein production [1]. Bacterial biofilms are found in many aspects of life, including industry, nature, and in human life. In the human domain, bacterial biofilms dwell in the oral cavity as oral plaques as well as on the skin as part of protective microflora against other more aggressive pathogens [2]. In *Staphylococcus aureus*, biofilm formation is regarded as a major pathomechanism as it renders *S. aureus* highly resistant to conventional antibiotics and host defenses. This can be caused by slow diffusion of these compounds through the extracellular polymeric matrix and slow growth of the bacteria [3]. *S. aureus* biofilm mode of growth is tightly regulated by complex genetic factors. Host immune responses against persistent biofilm infections are largely ineffective and lead to chronic disease. However, current research has taken biofilm formation into account in terms of elucidating host immunity toward infection, and may lead to the development of efficacious anti-biofilm *S. aureus* therapies. Nowadays, antibiotics are the most popular form of medicine to cure *S. aureus* related diseases. In general the antibiotics also affect the beneficial bacteria/microbiota and disturb the balance state of human health and it would be a great chance for emerging of resistant organisms. The arguments are that resistance to compounds targeting the virulence factors cannot evolve and spread in the resident flora, as these bacteria lack virulence targets. It is also proposed that resistance to virulence blocking agents is likely to result in nonfunctional virulence systems, and consequently nonvirulent bacteria [4]. So if these virulence factors could be neutralized by the use of small organic molecules, virulence blockers, it is possible that the infection could be inhibited and cleared by the immune system, this would allow to design a completely novel set of antibacterial agents with a potential to act as alternatives to antibiotics [5]. In recent years *in silico* strategies have been used to predict anti-virulence agents, and the main aim of *in silico* drug design is to bring the best chemical entities to experimental testing by reducing costs and time [6].

The aim of this study was to investigate the effects of inhibitor agents for *S. aureus* biofilm formation in *in vitro* conditions which was obtained from *in silico* drug design strategies.

II. MATERIALS AND METHODS

2.1 Determination of Anti-biofilm Agents

A designed molecules in previous *in silico* study for prediction of anti-biofilm agents for *Staphylococcus aureus* were used as a starting point in this study [7]. It concluded that 29 small molecules can be used as anti-biofilm agents. Out of these molecules, only five molecules were selected to be tested *in vitro* as an anti-biofilm. These molecules were: Acetic Acid, Acetaminophen, Acetylsalicylic Acid, Ferric Ammonium Citrate, Thymol.

The selection was made on the basis of: drug likeness, low side effects in humans, market availability, low cost, ease of handling in the laboratory. The experiments were done using these molecules to investigate the biofilm production and Viable Count of the bacteria after exposure to gradient concentrations of each molecule. Biofilm monitoring only is not enough because the aim of these experiments was to disarm the virulence factor of the bacteria, not to kill them.

2.2 Strain and Growth Conditions

Staphylococcus aureus ATCC 29213 were used in this study. First, a strain was streaked and grown overnight at 37°C from a frozen stock on a tryptic soya agar plate. The plate was then kept at 4°C, never longer than two weeks. Several colonies were used to inoculate 10mL of tryptic soya broth (TSB) that was incubated at 37°C for 24 hours. From this pre-culture, 0.5mL was used to inoculate a second culture (10mL TSB) that was grown at 37°C for 24 hours. The optical density of this solution (OD_{600nm}) was used as the value at zero time. Also, the Viable Count method was done at that time by serial decimal dilution and development of bacteria on petri dishes. This is done every hour, and the plate was incubated at 37°C for 24 hours. The OD value and density of bacteria (cfu/ml) over time were used to build growth curves.

2.3 Measurement of Anti-biofilm Activity

Anti-biofilm activity of inhibitor agents was carried out by the modified method of Christensen et al. [8]. Fresh single colony of the bacterial strain was picked up and inoculated in 10mL TSB broth medium in a test tube and incubated at 37°C for 24 hours. This culture medium was used as inoculums for the biofilm assay. 10ml TSB broth tubes were inoculated with 0.5mL previously incubated bacterial culture plus different concentrations of the compounds to be tested. Then these tubes were incubated at 37°C for 24 hours. The assay of biofilm developed in medium was performed by discarding culture medium from the test tube carefully. The test tubes were rinsed (twice) with 5mL distilled water. Five millilitre (5mL) of crystal violet solution (1% w/v) was added to stain the biofilms and incubated at room temperature for 30 min. Excess stains were then washed with 5ml distilled water twice. The test tubes were then dried in air for 20 to 30 min. Five millilitre of 95% ethanol was added to the tubes and kept in room temperature for 30 min. The absorbance of the retained dye was measured by spectrophotometer at 600nm. The correlation between the OD value and biofilm production is interpreted by the standards of Stepanovic et al. [9] (Table 1). Also, the Viable Count is performed after 24 hours by serial dilution decimal and development of bacteria on petri dishes.

Table 1. Interpretation of biofilm production.

Average OD value	Biofilm production
≤ ODc	None
ODc ≤ ~ ≤ 2x ODc	Weak
2x ODc ≤ ~ ≤ 4x ODc	Moderate
> 4x ODc	Strong

ODc: Optical density cut-off value = average OD value of negative control + 3x standard deviation (SD) of negative control.

III. RESULTS AND DISCUSSIONS

3.1 Bacterial Growth Curves

Growth curves have been conducted to investigate the stage/point for biofilm production and find out the effect of quorum sensing mechanism which could lead to the formation of biofilm. Strong biofilm production strain *S. aureus* ATCC 29213 was used to estimate the growth curves of bacteria.

The correlation coefficient of OD, VC and OD slime and showed good correlation, meaning test results are accepted (Table 2). In addition, the linear regression equation between OD and VC represents a deviation of 2% ($R^2 = 0.98$) (Fig. 1C). This is because the OD values of the spectrophotometer are affected by slime production and cells that are dead and alive can affect the turbidity of the culture [10]. Experimental results show that lag phase of bacteria lasts for about 4 hours after inoculated, followed by the exponential phase of 6 to 8 hours (Fig. 1A and 1B). After this period the bacteria entered the stationary phase and an increased in biofilm formation (Fig. 1D).

The growth and survival of bacteria is dependent on the cells ability to adapt to environmental changes. *S. aureus* has evolved many mechanisms to overcome such changes, particularly in an infection. A growth curve of *S. aureus* grown under ideal conditions can be divided into three phases: lag, exponential, and stationary, as shown in Fig. 1. During exponential phase, bacterium metabolism is rapid and efficiently to ensure constant growth. As the bacteria age and stop growing (post-exponential), cellular metabolism is re-organized for long-

term survival under unfavorable conditions. In lag phase, bacteria initiate an infection, then enter exponential phase where they multiply and synthesise surface proteins and essential proteins for growth, cell division and adhesion. During post-exponential, crowding activates a quorum sensing mechanism, resulting in the production of toxins and exoproteins. This enables the bacteria to escape from the localized infection (abscess) during stationary phase and spread to new sites, where the cycle is repeated [11].

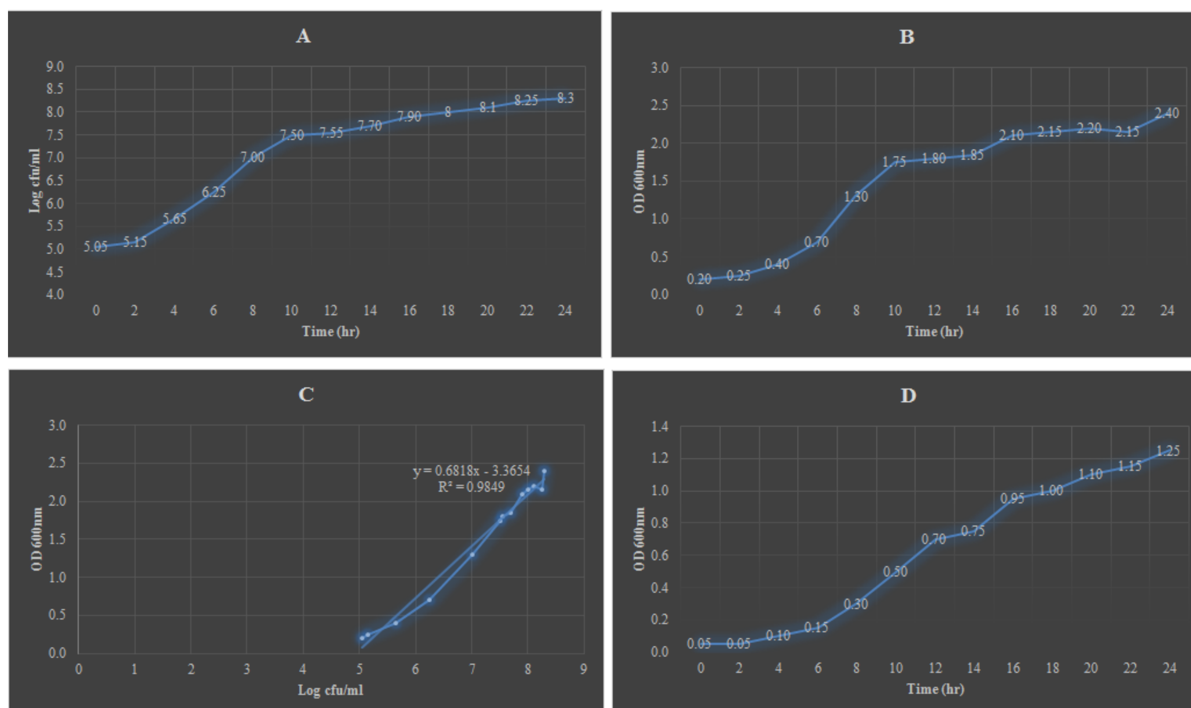

Figure 1: Growth dynamics of *S. aureus* ATCC 29213 in TSB media at 37°C/24 hours. (A) - Growth curves by using Viable Count; (B) - Growth curves by using OD; (C) - Linear regression for Viable Count and OD; (D) - Slime production.

Table 2: Correlation coefficient for Viable Count (VC), OD Curves and Slime Production of the bacteria.

Correlation Coefficient			
Viable Count	OD Curves	Slime Production	Between Slime Production and VC
0.94	0.95	0.99	0.93

Slime production begins due to quorum sensing phenomena where there is deficiency in nutrient and oxygen and an increase in crowding and waste products (i.e induction of different types of stresses). The triggering of quorum sensing systems has been shown to be responsible for a variety of physiological behavior in the bacteria including bioluminescence [12], production of antibiotics [13], release of virulence factors and biofilm formation [14]. This cooperative behavior is generally regarded to be controlled by cell density, but other circumstances, such as nutritional availability and environmental conditions, can affect quorum sensing behavior [15].

3.2 Anti-biofilm Activity Estimation

In recent years mounting problems related to antibiotic-resistant bacteria have resulted in the prediction that we are entering the post-antibiotic era. A way of preventing such a development would be to introduce novel antibacterial medicines with modes of action distinct from conventional antibiotics. Recent studies of bacterial virulence factors and toxins have resulted in increased understanding of the way in which pathogenic bacteria manipulate host cellular processes. This knowledge may now be used to develop novel antibacterial medicines that disarm pathogenic bacteria. The industrial failure to meet the public health needs of new antibacterial drugs indicates that novel approaches are needed in drug discovery. Traditional antibiotics kill or inhibit the growth of bacteria; they are either bactericidal or bacteriostatic. They have an important drawback as they not only attack bacteria causing the infection but also other bacteria. Intestinal, airway and skin bacteria build up a normal flora that is essential for our well-being when kept in balance. If the normal flora is disturbed by antibiotics, resistant bacteria are allowed to grow.

The arguments are that resistance to compounds targeting the virulence factors cannot evolve and spread in the resident flora, as these bacteria lack virulence targets. It is also proposed that resistance to virulence blocking agents is likely to result in nonfunctional virulence systems, and thus non-virulent bacteria. Further, as long as the target remains extracellular, resistance cannot emerge through the activity of bacterial efflux pumps. Finally, there will be a low selective pressure for mutations affecting the specific interaction between the drug and a virulence factor, as virulence blockers should have low effect on bacterial growth [4].

The experiments were carried out not just to estimate biofilm inhibition, but they were combined with estimation of VC. It was forwarded to use compounds that inhibit or disturb the function of sarA without affecting bacterial cell growth. The molecules selected in the study have high binding affinity to sarA protein (one of the most active proteins in *S. aureus* biofilm production process). This affinity was predicted by measuring binding free energy with the target protein using *in silico* programs [7]. This point seems quite important, as it leaves no opportunity of resistant sub-clones to dominate the population, which is the main feature of antibiotic treatment. In the latter case, a certain portion of population is resistant but is rare or at a very low level, so using compounds killing the normal (predominant) cells would offer the opportunity for such sub-population to dominate. This represents the story behind the development of resistant strains [4].

The following are schematic diagrams for biofilm production represented by spectrophotometer OD600nm readings and Viable Count (Log values) of bacterial growth versus different concentrations of the selected molecules.

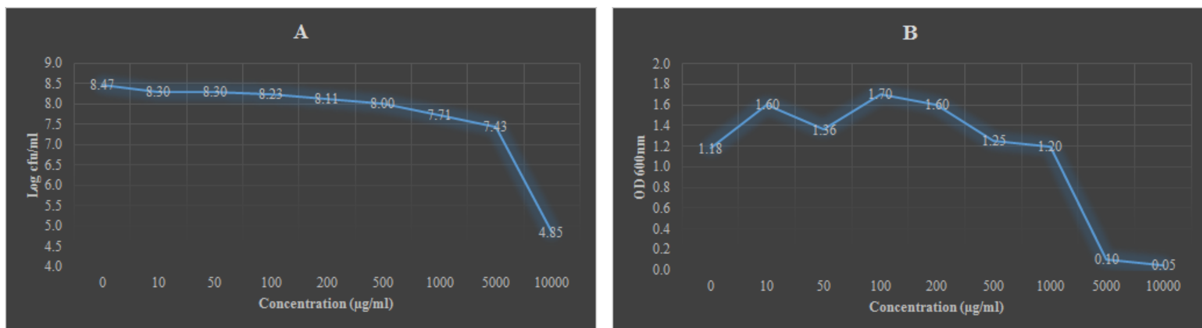

Figure 2: Effect of gradient concentrations of Acetic Acid on: (A) - bacterial growth; (B) - slime production.

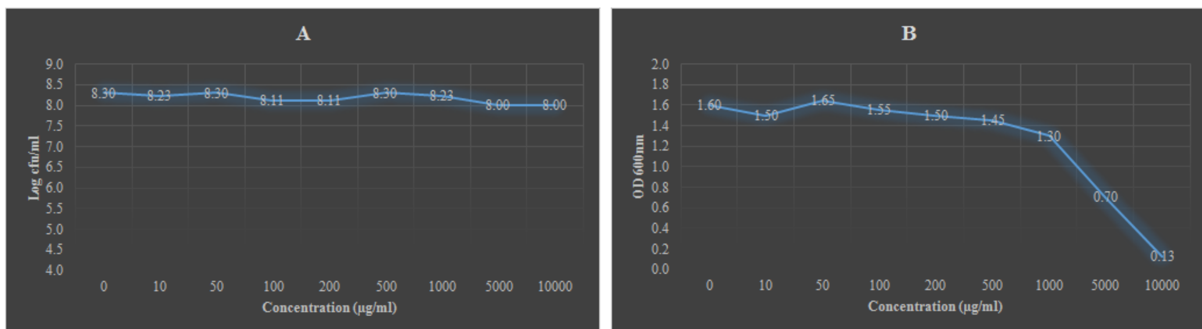

Figure 3: Effect of gradient concentrations of Acetaminophen on: (A) - bacterial growth; (B) - slime production.

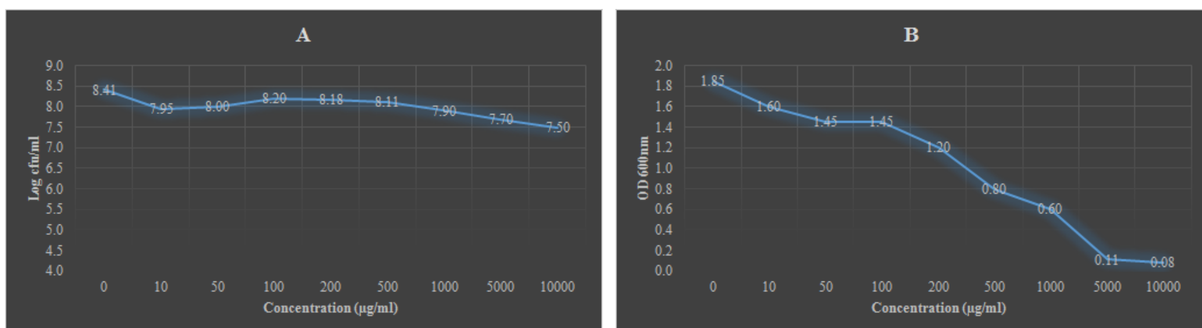

Figure 4: Effect of gradient concentrations of Acetylsalicylic Acid on: (A) - bacterial growth; (B) - slime production.

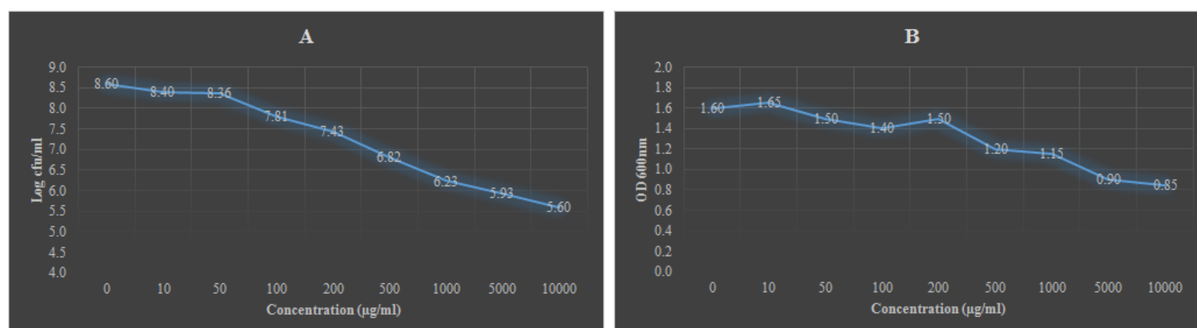

Figure 5: Effect of gradient concentrations of Ferric Ammonium Citrate on: (A) - bacterial growth; (B) - slime production.

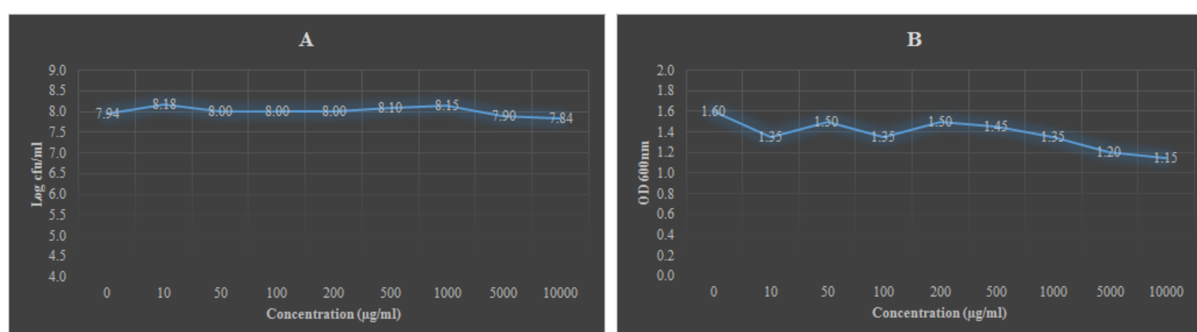

Figure 6: Effect of gradient concentrations of Thymol on: (A) - bacterial growth; (B) - slime production.

The results may lead to a conclusion that Acetic Acid, Acetylsalicylic Acid and Acetaminophen gave nearly 100% anti-biofilm activity at 5000, 10000 and 5000 µg/ml, respectively, and other molecules were poor biofilm suppressors. Aymen et al. [16] also reported in their studies on *S. epidermidis* that Acetaminophen, Acetylsalicylic Acid inhibited biofilm production 100% at 11000 and 1600µg/ml concentration, respectively, and Acetic Acid inhibition was 25% at 1000 µg/ml concentration. This leads to proposal that Non-Steroidal Anti Inflammatory Drugs (NSAID) constitute a good family for searching anti-biofilm drugs. Farber and Wolff [17] reported that Salicylic Acid inhibited adherence (55%) and biofilm production of *S. epidermidis*. A second study by Farber et al. [18] further illustrated that NSAIDs, including Sodium Salicylate, inhibit *S. epidermidis* and *P. aeruginosa* biofilm production on contact lenses, lens cases, and commonly used medical polymers in a dose-related manner. This manner has also been observed by other authors [19], [20]. Alem and Douglas [21] found in their studies on *Candida albicans* that seven of nine (NSAID) drugs tested at a concentration of 1mM inhibited biofilm formation. Acetylsalicylic Acid, Etodolac, and Diclofenac produced the greatest effects, Acetylsalicylic Acid causing up to 95% inhibition. Celecoxib, Nimesulide, Ibuprofen, and Meloxicam also inhibited biofilm formation, but to a lesser extent. Acetylsalicylic Acid was active against growing and fully mature (48-hours) biofilms; its effect was dose dependent, and it exhibited significant inhibition (20 to 80%) at pharmacological concentrations. Abd El-Aziz et al. [22] found on *P. aeruginosa* that Salicylate at a concentration of 10µg/ml reduced biofilm synthesis by 57.01% and eradicated pre-adhered biofilms by 29.19% while at 100µg/ml biofilm synthesis was reduced by 68.35% and pre-formed biofilms was disrupted by up to 62.73%. In comparison with the previous studies the results of this study show that anti-biofilm agents need higher concentrations to have effectiveness. This difference in results is due to difference in experiment conditions, and bacterial genera. The concentrations of the molecules as anti-biofilms do not meet the pharmacological limits for human use (maximum therapeutic plasma concentrations for Acetaminophen, Acetylsalicylic Acid and Acetic Acid are 150, 225 and 50µg/ml respectively) [23], [24], [25]. However *in vitro* studies and results must not be taken as final results for clinical applications because differences in environmental conditions may affect the results, where lab tools, nutrient media, temperature and solubility status differ from plasma conditions, body circulation, body temperature, body defenses. Also bacterial behavior in lab differs from that in human body, and drug molecule characteristics in lab differ from those in human body where they may undergo degradation, conformational changes, re-arrangement, plasma protein binding. Therefore, all *in vitro* experiments are considered as a beginning step and must be completed with *in vivo* studies. In this study three of five molecules were proposed to suppress biofilm production by inhibiting sarA protein activity. This means that the percent of success in drug design was about 60%. This percent is good if considered the hypothetical processes and prediction and modeling of protein and the error percent in each drug design step.

IV. CONCLUSIONS AND RECOMMENDATIONS

Although the study gave an initial view of novel anti-biofilm drugs and need to be completed with *in vivo* studies in future but there are no limitations on using these compounds in other aspects away from human body, as an antiseptic or medical equipment surface protectant.

ACKNOWLEDGEMENTS

This research was supported by the Division of Biotechnology, Faculty of Chemical Engineering, HoChiMinh University of Technology.

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