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STUDIES ON MARINE *STREPTOMYCETES* UTILIZING SHRIMP SHELL FOR PRODUCTION OF BIOACTIVE COMPOUNDS

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ABSTRACT

This study aimed to explore the antimicrobial and chitinase activity produced by potent marine *Streptomyces* against *Escherichia coli*, MRSA and *Aspergillus terreus*. The powdered shrimp shell (SS) was used as a mono-component medium and tested for their effect on the production of active metabolites against three pathogens and chitinase activity. Shrimp shell was used as production media for optimizing fermentation conditions. The tested medium supported production of bioactive metabolite. Shrimp shell at 5.0 gl^{-1} exhibited significant antagonistic activity against pathogenic microorganisms and produced chitinase in cell-free supernatants at value of 0.56 U/ml/h, while the greatest chitinase activity value of 10.36 U/ml/h was achieved when grown on the shrimp waste as a mono-component medium (40 gl^{-1} shrimp waste in tap water).

Keywords: Marine *Streptomyces*, Shrimp shell, Antimicrobial activity, Chitinase activity

INTRODUCTION

Pathogenic microbes are progressively emerging resistance mechanisms against the latest generation of drugs according to their nature. The imprudent use of antibiotics has created a selective stress that push the emergence and prevalence of multidrug-resistant (MDR) pathogens through developing diverse of resistance mechanisms. MDR microorganisms are among the most important contemporary crisis that threatens mankind. (O'Neill, 2016; van Duin and Paterson, 2016; Genilloud, 2017; Aslam *et al*., 2018). Methicillin resistant *S. aureus* (MRSA) has become one of the most frequently reported nosocomial pathogens worldwide, and is responsible for more than 11,000 deaths annually in the USA alone (Rossiter *et al*, 2017; Liu *et al*, 2019).

Although the urgent need for new drugs is increasing, progress of such agent's faces considerable impasses because of steadily decrease in discovery rate besides the re-isolation of known compounds (O'Neill, 2016; Pye *et al.*, 2017; Liu *et al*, 2019). Such situation encouraged scientists to adopt other strategies for searching novel biomolecules from underexplored and uncommon environments.

Marine sources are not yet exploited and have become a treasure trove for obtaining the remarkable chemical diversity and the novel potent natural products that directly apply or use as lead compounds in drug development (Molinski *et al.*, 2009; Gribble, 2015; Adam *et al.*, 2018; Liu *et al*, 2019; Subramani and Sipkema, 2019; Pedrosa *et al.*, 2020). In particular, microorganisms are the bountiful sources for secondary metabolites; medicines approved by the FDA/EMA estimate at about 35% of natural products or their derivatives, most of them are of microbial origin. More than 50% of clinical antibiotics are of actinomycetes origin and majority from the genus *Streptomyces*.

Streptomycetes have the ability to produce novel secondary metabolites with high biological activities as effective drugs against antimicrobial resistant pathogens (Liu *et al*, 2019; Subramani and Sipkema, 2019) as well as its ability toutilize a complex natural organic compounds such as chitin (Kawase *et al*., 2006; Yu *et al*., 2008).A huge amount of chitinous waste is generated during the shrimp and crab processing all over the world. Traditional methods such as burning, land filling, and ocean dumping for chitinous waste removal becomes a serious environmental and economic issues (Suresh, 2012; Suresh and Chandrasekaran, 1998). Since this chitinous waste is a valuable renewable resource, several studies have been done to convert it into beneficial bioactive compounds to be applied in food, agriculture, medicine, and materials sectors (Muzzarelli *et al*., 2012; Franco and Peter 2011; Ling *et al*., 2011; Rashad *et al*., 2015) by the action of bacteria, particularly S*treptomycetes*.

In response to the challenge and crisis of both chitinous waste and prevalence of resistant pathogens, the focus of the present study is to explore, *in vitro*, the antagonistic activity of a bioactive metabolite from marine sediment-derived *Streptomyces* 10SAE utilizing shrimp shell as a mono-component media against pathogens; to optimize nutritional and environmental conditions for obtaining maximum productivity.

MATERIALS AND METHODS

Bioactive compound(s)-producing strain and growth conditions

The potent strain *Streptomyces* sp. 10SAE was previously isolated, from marine sediment of Alexandria- Egypt, identified and deposited in Microbiological Resources Center (Cairo MIRCEN), Fac. Agric., Ain Shams University and Gene Bank under the accession numbers of EMCC 1919 and Kp064548, respectively. Such strain showed antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria and filamentous fungi. In addition, it has a role as a biocontrol agent against root knot nematode *Meloidogyne incognita* and promotes plant growth due to its ability to produce phyto hormones and also has a biodegradation capability to clean the environment (Rashad *et al*., 2015). The strain was grown on starch casein nitrate broth (SCNB) supplemented with 0.5% NaCl and maintained at -80 °C in the same medium with 20% glycerol.

Optimization of the fermentation condition

A series of experiments was done to optimize the environmental and nutritional conditions, in order to maximize the bioactive metabolites(s) produced by *Streptomyces* 10SAE using SS (2.5% in tap water, Rashad *et al*., 2015) as fermentation media. The flasks were loaded in triplicate with 20% of SS fermentation medium, autoclaved, inoculated with 2% of spore suspension, incubated at 30 °C and 180 rpm for 5 days on a rotary shaker (VISION SCIENTIFIC CO., LTD., Model: VS-8480); each optimized factor was jointed in the following experiments.

Environmental Conditions

We studied seven environmental factors including Seed inoculum age, Fermentation time, Inoculum size, Incubation temperature, working volume, Shaking speed and pH. In order to study the seed inoculum age of *Streptomyces* sp. 10 SAE, starch casien agar slant was inoculated with a loop of *Streptomyces* sp. 10SAE for 1, 3, 5 and7 days, and then the slant cultures were washed with 5 mlof sterile distilled water to prepare inocula. To test the fermentation time by using flasks containing SS medium (20 % v/v) were inoculated with 2 % v/v of the best seed inoculum age for *Streptomyces* sp. 10SAE; and incubated at 1, 3, 5 and 7 days on a rotary shaker. The optimum Inoculum size was studied by preparing different levels of spore suspensions as 1, 2, 3, 4, 5, 6, 8 and 10 % v/v. The spore suspensions were adjusted to ca. 3.4 $\times 10^6$spores/ ml, OD_{550} = 0.17. To optimize the Incubation temperature, the inoculated flasks were incubated at different temperatures 25, 28, 30, 35 and 40°C. The best working volume obtained by using different volume levels of 10, 20, 40 and 60 % (v/v)**.** Testing the Shaking speed at different agitation speed at 50, 150, 180, 200 and 250 rpm. Studying the effect of pH by adjusting to different pHs: 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0.

Nutritional conditions

To determine the ideal concentration of SS as a sole source of carbon and nitrogen in addition to minerals, dried powdered of shrimp shell was mixed with tap water at the concentrations of 0.5, 1, 2.0, 2.5, 3.0 and 4.0%.

Determination of antimicrobial activity

The SS culture filtrate of each optimized factor during the optimization studies was tested for the antimicrobial activity against *Escherichia coli* ATCC 8739, *S. aureus* MRSA ATCC 43300 and *Aspergillus terreus* NRRL using agar well diffusion method (Balouiri *et al.,* 2016).

Estimation of nitrogen and carbon

Carbon and nitrogen contents in the powdered shrimp shell was estimated according to the method of Walklay and Black's (Cotteni *et al*., 1982).

Determination of chitinase activity

SS cultures were centrifuged at 10,000 rpm and 4 °C for 15 min. The supernatants were used as crude enzyme and the colloidal chitin as a substrate for the measurement of chitinase activity according to the method of Somogyi (1952).

Statistical analysis

Data were statistically analyzed using the analysis of variance (ANOVA), and group means were compared by Duncan Multiple Range Test (DMR) using the SAS program.

RESULTS AND DISCUSSION

Optimization of the fermentation conditions

Environmental conditions

The production of antimicrobial substance(s) by *Streptomyces* 10SAE was observed in all treatments inoculated with seed cultures of different ages. However, the highest productivity (P <0.0001) was attained at the 3rd day age; thereafter, a notable reduction in the effectiveness against *A. terreus* was observed by increasing inocula age (Fig. 1a). The onset of the bioactive molecule(s) synthesis was fermentation medium-dependent. As demonstrated by its biological activities, the production of such molecule(s) was started at three days incubation in SS medium and exhibited great activity against MRSA and *E. coli* ATCC

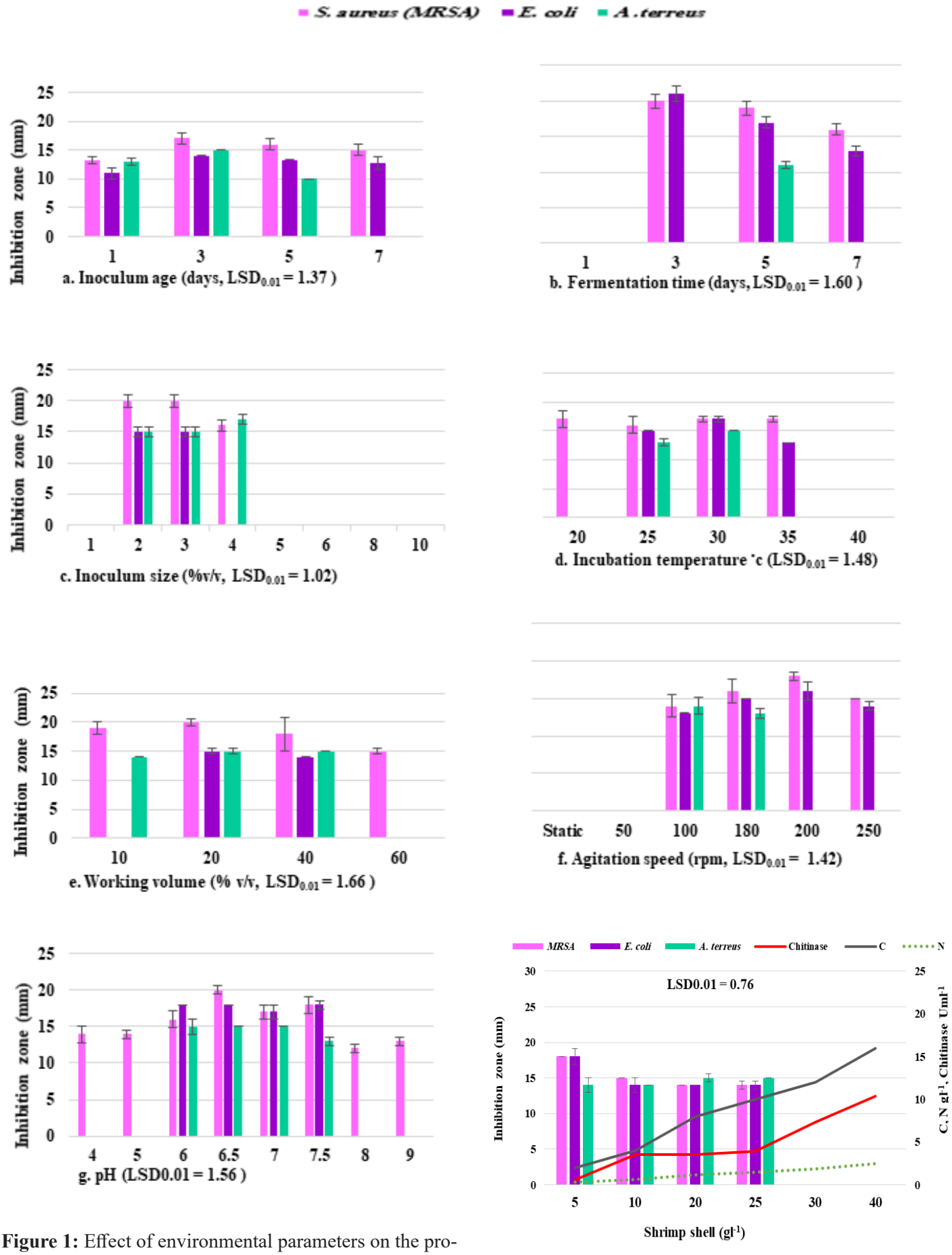

Figure 1: Effect of environmental parameters on the production of the bioactive compound(s) by *Streptomyces* 10SAE in shrimp shell (SS) fermentation media.

Figure 2: Effect of shrimp shell concentrations on production of the bioactive compound(s) by *Streptomyces 10SAE* in shrimp shell medium.

8739; however complete potency against the three target organisms was attained by extending incubation period for 5 days ($P > 0.0001$). The activity against *A. terreus* was not detected either at 3 or at 7 days of incubation (Fig. 1b). The optimum productivity was obtained with an inoculum level of 2 or 3% (v/v), 3.4×10^6 spores ml^{-1}, ($P < 0.0001$). Deviation of the inoculum size less or more than optimum one resulted in reduction of the potency of produced biomolecule(s). However, productivity terminated at higher inoculum levels than 5% (Fig. 1c). *Streptomyces* grew at a wide range of temperature (20 - 35 °C) and generated biomolecule(s); such biomolecule(s) exhibited comparable activities against MRSA and lost its potency against *E. coli* and *A. terreus*at 20 °C (Fig. 1d). The ideal temperature for productivity and potency was 30°C which evidenced by biological activities against all target microorganisms ($P < 0.0001$).The volume of the fermentation medium as well as agitation speed affected both the productivity and effectiveness of the secondary metabolites (Fig. 1e-f). The greatest productivity of elaborated biomolecule(s) was reached at working volume of 20 % and shaking speed at 180 rpm ($P < 0.0001$) as proved by biological activities against tested microorganisms. Such biomolecule(s) lacked its activity against *E. coli* and *A. terreus* when the fermentation media loaded at 60 % (v/v) and lacked its activity against *A. terreus* when the shaking speed increased. Although *Streptomyces* showed good growth at static and/or at low speed rate (50 rpm), no active biomolecule(s) was produced. SS medium allowed the growth of *Streptomyces* strain and subsequently the elaboration of bioactive molecule(s) over a wide initial pH range of 4 up to 9. Highest comparable activity against both *E. coli* and *A. terreus* was attained at pHs 6.0 – 7.5 and 6.0- 7.0, respectively. Maximum potency against MRSA was achieved at pH 6.5, but modest activity was observed when that biomolecule(s) produced at more acidic or alkaline pHs (4.0, 5.0, 8.0 or 9.0). Generally, optimum pH was medium ranged between 6.5 and 7.0(Fig. 1g).

Nutritional requirements

Effect of SS concentrations

Among the different concentrations of SS tested, *Streptomyces* 10 SAE generated the highest productivity of potent antimicrobial molecule(s) against *S. aureus* MRSA and *E. coli* ($P < 0.0001$) at the lowest concentration (5.0 gl^{-1}). Highest activity against *A. terreus* was achieved at a concentration of 20 and 25 gl^{-1}. At higher concentration, the synthesis of antimicrobial molecule(s) ceased as indicated by the absence of antagonistic activities. It seemed that the biosynthesis process is directed for chitinase enzyme production. There was a concomitant increase in synthesizing chitinase with increasing shrimp shell concentration up to 4% in the fermentation medium (Fig. 2).

Discussion

Natural products including those of microbial origin continue to play a central role in contemporary therapeutic drugs for different diseases. They offer better resources than chemical ones in providing structurally and mechanistically new bioactive molecules to meet the challenges resulted from increasing evolution of antimicrobial resistance in the medicinal and agricultural sectors (Watve *et al*., 2001; Bérdy, 2005; Lam, 2006; Smolinski *et al.*, 2003; Pye *et al.*, 2017). Marine actinomycetes and particularly *Streptomycetes* gain special importance due to their capacity in biosynthesis of incredible array of unique bioactive secondary metabolites (SMs) of medicinal, agricultural and industrial importance (Molinski *et al*., 2009; Gribble, 2015; Subramani and Sipkema, 2019; Pedrosa *et al.*, 2020).

Biosynthesis pathways of bioactive SMs are frequently connected and influenced by intermediate metabolites from primary metabolism which serve as precursors for biosynthesis process. Growth and metabolic capacities and subsequently accumulation of SMs by such microorganism are strongly controlled by production of enzymes; such enzymes are encoded by biosynthetic gene clusters (BGCs). Genome sequencing of a given microbe has revealed that it often conceals many “cryptic” BGCs, which are not expressed under laboratory conditions. By fermentation manipulation, expression of “silent” BGCs may be activated; thus production of enzymes and growth of microbes are directly related to the cultivation parameters both nutritional and environmental; such conditions may vary from one species to another (Bode *et al.*, 2002; Van Wezel and McDowall, 2011; Romano *et al.,* 2018).

The elaboration of bioactive SMs found to be directly increased or completely inhibited not only by the changing of cultivation parameters employed, but also when the growth medium switched from solid to liquid (Pickup *et al*., 1993; English *et al.*, 2017; Wu *et al.*, 2018). Aside from the influence on quantity and potency of the generating SMs, slight alterations in the culture medium may influence the overall metabolic figure of an organism (Bode *et al.,* 2002; Scherlach and Hertweck 2009).

In the present work, the mastery of *Streptomyces* 10SAE to synthesize the bioactive SMs was not consistent but found to be depended on fermentation conditions. Shrimp shell at 5.0 gl^{-1}as a mono component medium proved its significance as a cheap suitable medium for maximum production of biomolecules by *Streptomyces* 10SAE. At the highest concentration (30 and 40 gl^{-1}), the biosynthesis of antimicrobial molecule(s) ceased as a result of the catabolite repression from the higher concentration of carbon, nitrogen and phosphorus

(Ibrahim *et al.*, 1999). Under such conditions, the cell machinery have directed for growth and producing the chitinase enzyme.

On the other hand, changes in many environmental factors also found to affect both the growth and productivity of bioactive SMs. *Streptomyces* 10SAE behaved as mesophiles while the highest productivity with highest spectrum of broadness were achieved within temperature range of 30 and 35 °C. Its failure in elaboration of bioactive molecules at both static and low agitation speed might be ascribed to the reduction of dissolved oxygen in the fermentation media; such condition in some cases may cause change in metabolic profile (El-Enshasy *et al*., 2000; Büchs, 2001). The clear-cut time for biosynthesis commencement or obtaining the maximum productivity for bioactive compounds was inconsistent and strain dependent (Sujatha *et al*., 2005). An obvious biphasic pattern of fermentation process verified the suitableness of SS media in supporting the growth for *Streptomyces* 10 SAE. The early onset elaboration of biomolecules in SS medium might be attributed to higher carbon and nitrogen contents 10, 1.5 g l^{-1}, that resulted in faster growth rate and subsequently earlier entrance in production phase. The use of spores rather than vegetative mycelium as inocula reduced the overlap between trophophase and idiophase(Liao *et al*., 1995). Lower inoculum density than optimum level may not be sufficient for producing the required biomass while higher inoculum can cause fierce competition for nutrients which resulted in the exhaustion of the nutrients necessary for productivity, accumulation of toxic substances and the reduction of dissolved oxygen (El-Enshasy *et al*., 2000; Peddi and Donthireddy, 2018). The optimal pH for growth and synthesis of bioactive SMs are medium composition dependent. Considering the strain-specific differences, most *Streptomyces* found to grow optimally near neutral pH with highest productivity of bioactive SMs at initial pHs 6.5 and 7.5 and sometimes at 7-8 or more (James *et al.*, 1991; Ripa *et al.*, 2009; Vijayakumar *et al.*, 2012). Complex nutrients are needed for effective pH regulation and to maintain cytoplasmic pH close to the neutrality over the whole growth pH range (Padan *et al.,* 1981). In the present study, shrimp shell proved its effectiveness in the pH adaptation and thus the growth and biosynthesis of SMs over the pH range of (4-9). Such discrepancy between the obtained results and those obtained by Kontro *et al.,* (2005) might be attributed to strain –specific differences.

CONCLUSION

The bioactive metabolites produced by marine sediment-derived *Streptomyces* 10SAE showed significant antagonistic activity against *Staphylococcus aureus* MRSA, *E. coli* and *A. terreus* as well as chitinase enzyme production. Further studies will be carried out in the next stage of research concerning isolation, purification and identification of different types of bioactive molecules produced in shrimp shell medium.

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