RESEARCH COMMENTARY

Microwaves, Salt-loving Bacteria, Music, and the Antivirulence Approach: A Short Commentary on Research Carried Out in Our Lab and Some Lessons Learned *en route*

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INTRODUCTION

Through this issue of *Current Biochemical Engineering*, I take the opportunity to share what research I have been doing along with my students over the last few years. Besides describing the work done by us, I have also added few lines about the field under which it falls, to create some sort of context for the readers. Our research over the period of last ten years can broadly be described under four different headings, which I am putting below in the order in which we started working in the respective research area. I hope, the readers of *Current Biochemical Engineering*, particularly the early and mid-career researchers will find this commentary interesting, which aims to provide a light scientific reading with no heavy emphasis on full technical details of the experiments mentioned.

BIOACTIVE NATURAL PRODUCTS

In the year 2007, we started screening plant products for their potential antioxidant and/or antimicrobial properties. For preparing the extracts of plant materials (mainly seeds) selected by us, we developed a rapid *microwave assisted extraction* (MAE) protocol. MAE is believed to be a fast extraction method, largely suitable for heat-labile phytocompounds too. At the onset, we optimized the parameters like microwave power, seed: solvent ratio, heating and cooling cycles for different solvents, total extraction time, etc. Using this MAE protocol, we were able to obtain an appreciable extraction efficiency of 17% with *Annona squamosa* seeds, extracted in chloroform: methanol (2: 1), by applying microwave heating just for 50 seconds [1]. Since then, we have employed MAE for preparation of many bioactive plant extracts with good success, without much modification, except that initially we were doing this in flasks covered with glass lid, and later we shifted to screw-capped glass bottles, which helped in minimizing the solvent evaporation during microwave heating. In one of our studies, while comparing various extraction methods, we found MAE to be a better method for extraction of antibacterial plant compounds [2]. In this study, extraction efficiency was found to have no notable correlation with any of the efficacy parameters assayed. This is important to realize that high extraction efficiency always is not synonymous with high efficacy.

Over the years, we have identified quite a good number of natural products (of plant or microbial origin) with one or more of the following properties: antioxidant, antibacterial, antifungal, anti-quorum sensing, anti-beta-lactamase, etc. One notable among them is the methanolic extract of *Tamarindus indica* seeds, which exerted appreciable *in vitro* bactericidal effect against *Staphylococcus epidermidis*, at a minimum inhibitory concentration (MIC) of 53 µg/mL, and minimum bactericidal concentration (MBC) of 56 µg/mL [3]. Through another interesting experiment, we demonstrated the protection conferred on cabbage leaf against *Xanthomonas campestris* by application of *Pheonix sylvestris* hydroalcoholic extract [4]. In the same study, curcumin was found to be capable of halting growth of three different phytopathogenic bacteria in the MIC range of 30-70 µg/mL. Quercetin could inhibit *X. campestris* at 40 µg/mL. Quercetin (along with gallic acid) was also demonstrated by us to be present in the antimicrobial extracts of the *Syzygium cumini* seeds [5]. Some of the seed extracts screened by us were able to exert *in vitro* microbistatic effect on *Malassezia furfur* (a dandruff-associated yeast), and *Propionibacterium acnes* (a bacterium associated with acnes) [6]. During this study, we also came across the phenomenon of *post extract effect* (PEE), about which the experimenter should be cautious, and must confirm its absence while labelling any test product as microbicidal. This PEE is parallel to the *post antibacterial effect* (PAE)/*post antifungal effect* (PAFE) described in context of the conventional antibiotics [7].

Following demonstration of the antimicrobial potential in certain plant extracts, from the year 2013, we shifted our focus more towards *anti-virulence (anti-infective)* properties of the natural products, particularly those which can interfere with bacterial *quorum-sensing* (QS). The widespread problem of antibiotic-resistance among pathogenic microorganisms has persuaded researchers to design novel strategies to deal with virulent microbes. One of such promising approaches is to target virulence rather than only aiming at killing the pathogen. Among the many virulence traits which can be the possible target of the anti-infective agents, the most attractive seems to be the QS, which is a means of intercellular communication among bacteria that regulates expression of many genes including those involved in production of virulence factors, biofilm formation, efflux pump

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activity, toxin production, pigment production, etc. Consequently, QS is increasingly being viewed as an attractive target for the development of novel anti-infective measures that do not rely on the use of antibiotics. Anti-QS seem to be a promising strategy to combat bacterial infections as it is less likely to allow bacteria to develop resistance, since it does not impose that strong selection pressure [8]. A good example of QS modulatory polyherbal preparation of demonstrable therapeutic value is the *Panchvalkal* extract, which contains extracts from bark of five different plants. Broad spectrum antimicrobial potential of this formulation described in various *Ayurvedic* texts including *Charak Samhita*, was recently shown by us to stem from its QS modulatory capacity [8a]. Preliminary results of *in vivo* efficacy assays for this polyherbal formulation using the nematode *Caenorhabditis elegans* as a model host are encouraging, as this herbal cocktail seems to be protecting the nematode worm from the test pathogen, with no toxicity to the former. Full details of our experiments with this extract against multiple gram-positive and gram-negative bacteria are likely to be published next year. A number of anti-QS approaches have been documented, and natural as well as synthetic products are being studied in this context. Screening the natural products and/or large combinatorial chemistry libraries for potential anti-QS property may pave the way for development of novel anti-virulence leads, and can help in dealing with the problem of antibiotic-resistance among bacterial pathogens. We are currently using *Chromobacterium violaceum, Serratia marcescens, Staphylococcus aureus,* and *Pseudomonas aeruginosa* as the test organisms, while screening the natural products for their potential QS-inhibitory (QSI) potential. Soon, we shall be adding few more organisms (perhaps some phytopathogenic bacteria too) as test organisms. Doing so, will help us investigate how broad the QSI spectrum our test products possess. All the four bacteria mentioned above produce different pigments, whose production is QS-regulated. While working with them, we learned an important lesson [8b] that wavelengths conventionally used for quantifying bacterial growth in microbiology labs, may not necessarily be suitable for quantifying the OD of suspensions of pigmented bacteria, and hence an appropriate wavelength needs to be selected for measuring cell density in such pigmented bacterial suspensions, at which pigments do not interfere significantly.

Besides plant extracts, we have also demonstrated QSI poperty of a microbial pigment (prodigiosin), and the antibiotic streptomycin at sub-MIC level [9]. This indicates that antibiotics may have functions other than just killing the competitors in nature. Recently we have also identified QSI potential in *Punica granatum* peel extracts, which seem to be acting by interfering with the signal-reception machinery of the target bacterium. We are also employing molecular docking to get some hints on possible mode of action of these QSI natural products. Once we identify few natural products with good *in vitro* QSI potential, we plan to evaluate *in vivo* efficacy of these products using *Caenorhabditis elegans* as model host [10], and also to perform transcriptional profiling for identifying the many bacterial genes whose expression is modulated under the influence of our QSI extracts. This may possibly result in identification of new drug-targets. Very recently (this summer only), we have also ventured into investigating utility of plasma (fourth state of matter) treatment for modulating biofilm formation (one among many QS-regulated traits) by bacteria.

BIOLOGICAL EFFECTS OF MICROWAVES

As we noted in [11], "Microwaves (MW) are a part of electromagnetic spectrum with multiple applications. Thermal effects resulting from high-power microwave radiation are well established, but considerable controversy surrounds the possible microwave specific athermal effects. Reports suggesting its presence or absence keep accumulating in literature. Exact mechanism through which low-power microwave radiation exerts its effect on different life forms including microorganisms yet remains to be elucidated. For this effective experimental strategies needs to be devised, where thermal and athermal effects can be studied separately. Identifying suitable biological model(s) for such studies is also required."

Our experiments regarding the biological effects of microwaves remained confined to the 2450 MHz microwave radiation. Our studies [12-15] indicated that microwave exposure could alter growth/ enzyme activity of the test organism(s). The MW-induced effects observed in these studies were measured not directly on the cells which received the MW treatment, but on their daughter cells obtained after inoculation of MW-treated cells in appropriate growth media, allowing for transfer of MW-induced changes into next generation(s). Results of these studies prompted us to employ MW as potential mutagenic agent [16, 17]. Identifying mutagenic frequencies of MW radiation can pave way for large scale screening programmes employing MW mutagenesis as a tool for strain improvement, and yield genetically stable overproducing mutants. We attempted microwave mutagenesis of *Brevibacillus parabrevis* for enhanced cellulase production (data yet unpublished, going through peer-review). Though microwave treatment could alter the cellulase activity of the test bacterium, none of the mutants obtained were found to be genetically stable. Thermal stability of the *B. parabrevis* cellulase was also investigated. This enzyme was found to be capable of retaining its activity even after heat treatment (50-121˚C, for 30-60 min). Fluorescence spectrum revealed a *red shift* in the emission maxima of the heat-treated enzyme preparations, indicating some structural change upon heating, but no major loss of activity was observed. This enzyme was found to be active over a broad temp range, with 90˚C as the optimum temp, which is interesting as the producing organism is a mesophile.

At present a large part of general population is exposed chronically to non-thermal MWs from different types of mobile communication. In light of recent reports about MW effects (particularly athermal effects) on different life forms, the safety standard of exposure needs to be re-evaluated. Further research is needed on the exact mechanism(s) behind non-thermal effects of MW. Significance of acquiring reliable information regarding the effects of low power MW needs to be well acknowledged by the society.

HALOTOLERANT BACTERIA

In the year 2010, we isolated five halotolerant bacteria from saline soil, and identified them as *Staphylococcus epi-*

dermidis, *Bacillus atrophaeus*, *Halomonas shengliensis*, *Halomonas koreensis*, and *Virgibacillus salarius* [18]. Among all isolates *Virgibacillus salarius* exhibited better metal tolerance/resistance. Such organisms can serve as a good model for study of stress response among prokaryotes, and can also be explored for their potential of bioremediation of metal polluted saline sites with alkaline pH. We found *V. salarius* to be capable of hydrocarbon metabolism, too [19]. Presence of catechol 2,3 dio-xygenase, and chlorocatechol 1,2 dioxygenase activity was indicated in it. Catechol 2,3 dioxygenase activity in this organisms was more susceptible to increase in salinity of the growth medium than chlorocatechol 1,2-dioxygenase activity. To the best of our awareness, it was the first description of catechol metabolism in *V. salarius*.

MICROORGANISMS AND SOUND

The topic of how microbes respond to the audible sound, has remained a neglected area of research till now. Higher organisms like animals have well-developed ears, and hence they can clearly receive and respond to the sound stimuli. In case of microorganisms, it seems that mechanosensory channels may be involved in sensing and responding to sound. We have recently started some research in this field, and published our preliminary findings [20, 20a], which indicate that sound stimulation can notably influence microbial (bacteria and yeast) growth, metabolism, antibiotic susceptibility, and membrane permeability. More manuscripts from our lab in this area are under preparation, wherein we shall be reporting our findings indicating that certain sound frequencies can induce higher response from the bacteria, than others. In these studies, the test sound (mono-frequency as well as multi-frequency) pattern(s) used were capable of affecting quorum-sensing regulated production of pigments by *Serratia marcescens*, *Chromobacterium violaceum,* and *Pseudomonas aeruginosa*. Transcriptome profiling of our sound-exposed bacterial samples is also underway, which shall provide us with an insight into the molecular basis of microbial response to sound, by comparing the gene expression profile of the sound stimulated culture with that of control (unexposed to sound). For this we have been sanctioned a research grant from Gujarat Council on Science and Technology (GUJCOST).

Bacteria not only can respond to sound, but they can produce it too [21]. Till now whatever little work we have done in this area, we have found that different microbes may respond differently to the same sound pattern, and even among the similar responses, the magnitude of response vary. Developing any microbiological application utilizing sound stimulation as a tool will be possible only after sufficient fundamental research in this area is done. Unfortunately, not many scientists seem to be attracted to this field, presently. Many interesting questions remain to be attempted e.g., is the microbial response to sound, frequency dependent; Whether there are any frequencies which can elicit higher microbial response than others; Will different microbes respond differently to the same sound, etc. With deeper understanding of bioacoustics, some microbiological applications may also be developed. For example, use of music in fermentation industry for increased production, or accelerating waste-treatment by microbes.

LESSONS LEARNED

During this rewarding research journey, I have learned few good lessons (many more remain to be learned yet), which I consider worth sharing here:

1. You always need not have to have a large number of Ph.D. students working under you to do publishable research (More than half of my publications have M.Sc. students as my co-authors). Explanatory comments for the same can be found in [22].
2. Once a logical research theme has been designed, simply implement it. Do not allow undue criticism from any source to deviate you (while remaining open to formal or informal, but valid critical peer-review).
3. Working with natural products can be a complicated issue at times, because these are always not *easy-to-handle* pure products. This is not a matter of worry, but one should be prepared for troubles inherent to such work. Relevant explanatory comments can be found in [23].
4. Pigmented bacteria makes a task as simple (seemingly) as taking OD of bacterial suspension, bit tricky. Further, many of them may lose the property of pigment formation over multiple subcultures, making it necessary to always safely maintain the original master culture. We have found the simple and almost-no-cost method of storing these bacteria in sterile distilled water at room temperature in screw capped vials, to be effective for this purpose.
5. Early-career researchers should focus on getting some papers published in reasonable (but not predatory) journals, as soon as possible, rather than targeting high-profile journals straight-away. That helps them establishing that they are productive, which is very important in the initial phase of career (in later phases, people evaluating you become somewhat considerate looking at your previous productivity). Citations can be earned by publishing in any (preferably open access) peer-reviewed indexed journal(s), irrespective of journal's empirical reputation [24].
6. It is more satisfactory to work on ideas of one's own, rather than those *donated* (owing to a variety of complex intentions) by someone else, however glamorous the latter may be.

I hope readers of this issue of *Current Biochemical Engineering* will find this commentary worth of their attention. Any suggestions/comments from the learned readers are welcome through e-mail.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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