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Enhancement of Oxytetracycline Production by Heat Shock using Microwave

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The microwave exposed cultures during 48 hr. accumulated more oxytetracycline (OT) titers which was highest having an antibiotic titre of 380 μg/ml as against 24 hr exposed sample and control with 310 μg/ml of antibiotic titre. There is distinct change in the mode of action of antibiotic, with a clear spheroblast formation at the periphery of the zone of inhibition of the test organism *Bacillus cereus* as evidenced by scanning electron microscopic studies. The present report represents the first description of the microwave exposure inducible property and as such provides some insight into the stress response as evidenced by the heat shock protein profile and physiologically complex *Streptomyces species*, which produces oxytetracycline, a secondary metabolite.

Keywords: Oxytetracycline, Heat Shock, Microwave, *Streptomyces sp.*

Microwave irradiation as efficient thermal energy source is becoming a standard technology in various fields of chemistry. Spectacular results have been obtained giving clear indications on the potentialities and advantages of this new technology; when compared to conventional methods in synthetic organic chemistry. It was thus shown that a great number of organic reactions (nucleophilic substitutions, esterifications, rearrangements, Diels and Alder Claisen and ene reactions etc) can be considerably accelerated when conducted in Microwave oven. It was generally concluded that performing reactions in such conditions resulted in faster and Cleaner reactions due to less thermal decomposition of products and minimization of secondary processes[1].

Vela and Wu[2] studied the mechanism of microwave bactericidal action under dry conditions at a subbactericidal temperature concluded that the dry or lyophilized microorganisms are not capable of absorbing microwave energy and are not damageable by the microwaves. Weyland *et al.,*[3] However, used microwaves to heat the dry spores in comparison with conventional thermal heating and found that the thermal and electromagnetic non-thermal functions of microwaves are interdependent. Effects of microwaves on biological systems other than chemical bond cleavage have been reported. If the non-thermal effects of microwaves can cause bactericidal and sporicidal activities, they must be a result of non-thermal effects other than chemical bond cleavage. Electromagnetic energy in the microwave region (225 Mhz to 100 GHz, typically 2,450 MHz) is extensively studied as one of the alternative energy sources for sterilization.The efficiency of a Microwave sterilization is essentially a function of both the electromagnetic field strength and the exposure time[4].

Although studies to differentiate the UV thermal and non-thermal effects of microwaves on microbiological systems have been attempted,

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a few recent studies have conflicting conclusions. Olenson *et al.,*[5] believed that the non-thermal effects of microwaves play a role in inactivation of microorganisms in suspension via formation of H_2O_2 and other chemical transformation of small molecules such as chemical bond cleavage. The first description of Non-nutritional stresses such as heat shock effect on production of antibiotic as such provide the lore of stress response in the morphologically and physiologically complex *Streptomyces*. Production of novel polyketide antibiotic jadomycin B by *Streptomyces sp.* by heat shock has been reported by earlier workers[6]. We have been working on different antibiotics such as Penicillin[7,8] Oxytetracycline[9] and Gentamycin[10,11] for enhanced production by whole cell immobilization, various feeding strategies in bioreactors and nutritional requirements. The present paper describes the effect of microwave heat shock on fermentation broth at different time intervals on antibiotic production and its induction capabilities. This efficient use of microwave in organic chemistry has prompted us to use this non-nutritional stress on antibiotic production.

MATERIALS AND METHODS

Microorganism

Streptomyces varsoviensis (NCIB 9522) was used throughout the study. The growth medium contained per liter glucose 4g, yeast extract 4g, Malt extract 10g, $CaCO_3$ 2g, pH 7.2-7.4. The production medium for shake flask cultures contained per liter Maltose 70g, corn steep liquor 9g, $CaCO_3$ 6g, $(NH_4)_2SO_4$ 3.5g, NH_4Cl 3g, $MgSO_4$, $7H_2O$ 1g, $MnSO_4$ H_2O 0.1g, $CaCl_2$ 0.005g. pH was adjusted to 7.2 before sterilization.

A 50 ml of production media in 250ml Erlenmeyer flask was inoculated with *S. varsoviensis* and incubated at 28^{0}C in a rotary shaker at 150 rpm. Every day samples were collected and centrifuged at 8000 rpm for 10 min. Supernatant was used for testing oxytetracycline concentrations by cup well method[12] using *Bacillus cereus* as test organism. Standard Oxytetracyline (Himedia, Bombay) has been used in all the assay plates to calculate the amount of antibiotic present in the sample. The results presented are average of triplicates.

Heat shock treatments

Microwave oven was used for heat shock. Microwave oven (Model BPL, BMD800 IS) used in this study is an industrial unit capable of upto 800W of microwave output power at 2,460MHz. The maximum experimental power output was 150W. Constant power output (150W) was used throughout the study. Depending upon the time of exposure, temperature may be varied. Heatshock was given to growing culture (5-7 day old). Production media in all the three flasks were exposed to microwave oven for different time periods such as 20, 40 and 50 sec. and the corresponding temperatures recorded in flasks are 40, 45 and 50°C ± 2°C respectively with optimum antibiotic titre at 45°C (Table 1).

Production media (50ml) in 4 different flasks was taken and inoculated with 5% inoculum of 48-hr old culture. Heat shock was given to these flasks at different incubation periods after 24, 48 and 72 hours for 40 Sec (45 ± 2 °C in a microwave). These flasks were incubated on shaker at 150 rpm for seven days. Samples were collected everyday and antibiotic assay was carried out using cup well method.

Table 1. Time and temperature variations during microwave exposure of 5 day old cultures*

S. No.	Heat shock time in Sec	Temperature in ^{0}C
1	0	27 ± 2
2	20	40 ± 2
3	40	45 ± 2
4	60	50 ± 2

*Antibiotic conc. is 340-350 mg/ml

RESULTS AND DISCUSSION

Initially a preliminary experiment was carried out to see the temperature and time variation in seconds in the microwave oven. Table 1 shows the effect of microwave heat shock at different time intervals such as 20-60 seconds. At each exposure time, the temperature of broth is measured using thermometer. The temperature measured at 40 seconds exposure showed 45 ±2°C with a maximum antibiotic concentration of 340 µg/ml. The culture broths are exposed to heat shock during 5-7 day period, where the antibiotic concentration was around 350 µg/ml.

Based on the above experimentation, the following experiment was conducted for 40 sec at 45±2°C of heat shock in the microwave oven. Four different sets of production media are prepared and exposed to heat shock at different growth periods of the growing culture, such as 24, 48 and 72 hrs. One flask served as control. Table 2 shows the effect of microwave heat shock on Oxytetracycline titre and pH at different time intervals when compared with unexposed control samples. In general maximum oxytetracycline is produced during the 5th day of fermentation. Fig. 1 shows the flask 2, which was exposed for 48 hrs. recorded maximum antibiotic concentration of 380 µg/ml, 24 hr old exposed culture recorded an amount of 340 µg/ml. 72 hrs old exposed fermentation broth recorded 230 µg/ml of antibiotic which is less when compared with all other exposed cultures (24 hrs and 48 hrs) and also control.

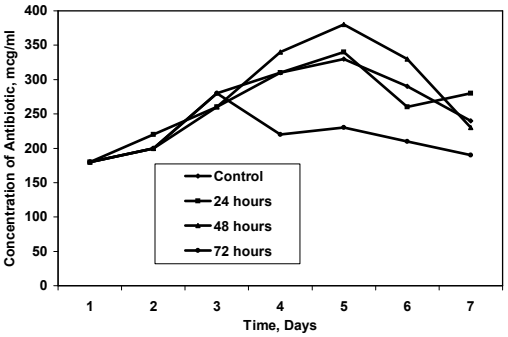

Fig. 1. OT production in control and heat shock treated fermentation broth at different time intervals

Table 2. Effect of Microwave heat shock on Oxytetracycline production and pH by *Streptomyces varsoviensis*

Day	Control		24 hours		48 hours		72 hours	
	OT Conc (µg/ml)	pH	OT Conc (µg/ml)	pH	OT Conc (µg/ml)	pH	OT Conc (µg/ml)	pH
1	180	6.5	180	6.5	180	6.5	180	6.5
2	200	6.3	220	6.2	200	6.3	200	6.3
3	280	7.5	260	6.3	260	7.9	280	7.5
4	310	7.7	310	6.4	340	7.5	220	6.7
5	330	7.6	340	6.3	380	7.6	230	6.4
6	290	7.5	260	6.2	330	7.4	210	6.3
7	240	7.0	280	6.0	230	7.2	190	6.2

The increase in production of antibiotic in flask 2 during 48-hr old culture is because of the metabolic state of the culture, which is in late logarithmic, primary metabolic stage and before the onset of secondary metabolism. Earlier workers have reported that the heat treatment increases the level of culturability of a spore population and a brief heat shock response and temperature up-shift above the normal growth range resulted in a set of highly conserved stress proteins that may be the result of altered metabolism[13,14]. It could be explained that the decrease in antibiotic production during 72 hrs old exposed fermentation broth might be due to its late metabolic phase. A general finding is that production of antibiotic compounds rarely occurs during periods of rapid growth in rich media. The onset of their biosynthesis generally decides with periods of growth rate reduction owing to exhaustion of carbon, nitrogen or phosphate[15].

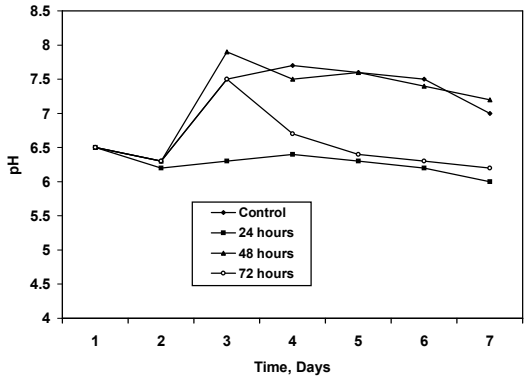

Fig. 2. pH variations during time course of fermentation of control on heat shock treated cultures

Fig. 2 shows the pH variations during time course of fermentation. During 1-2 days period, the pH is around 6.5. During further course of fermentation the pH shifts towards alkalinity (around 7.5) in control and 48 hrs exposed culture broths. The pH remained same at 6.3 in 24 hrs and 72 hrs exposed samples.

Fig. 3 shows the maximum antibiotic concentration and pH during the 5th day of fermentation. Maximum antibiotic concentration is achieved in 48 hours treated culture, where the pH recorded is 7.6. Antibiotic titre is less in control when compared with 48 hours sample. The biosynthesis of antibiotic largely depend upon the pH of the fermentation broth as shown by earlier workers. The pH shock greatly enhanced the production of Kasugamycin by applying non-nutritional stress[16].

Spheroblast formation was observed at the edge of inhibition zone in heat shock samples, which is due to elongation of bacterial cells resulting in the chain formation (Spheroblasts). This implies the change in mode of action of antibiotic, whereas control sample has not shown spheroblast.

The scanning electron micrograph has been taken to distinguish *Bacillus cereus* cells (test organism) in the periphery of zone of inhibition. Fig. 4 clearly shows individual cells taken from the periphery of the control samples and Fig. 5 shows cell elongation (long chains), which resulted in distinct spheroblasts in the periphery of the inhibition zone for all the heat

Table 3. UV maxima of control and heat shock treated samples

Sample	UV maxima range
24 hrs	258
48 hrs	263
72 hrs	255
control	270

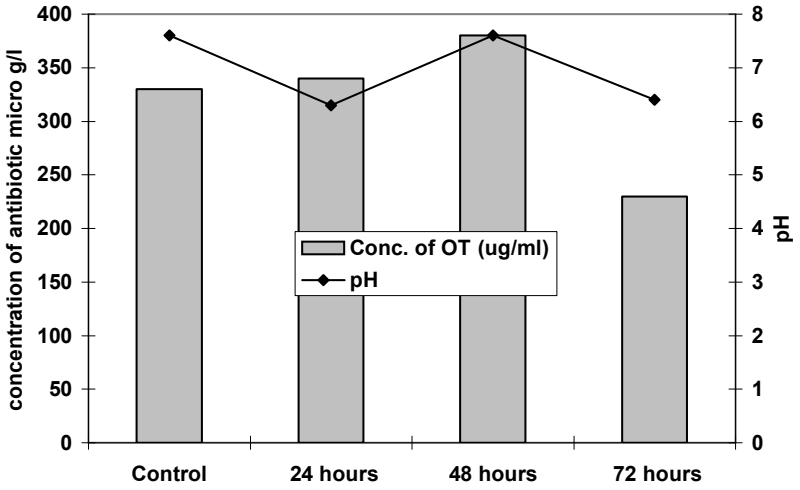

Fig. 3. OT concentration and pH during 5th day fermentation in control and heat shock treated samples

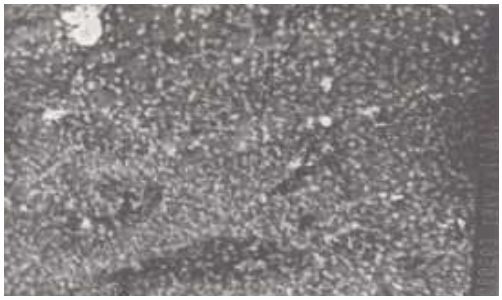

Fig. 4. Scanning electron micrograph showing individual cells taken from the periphery of the control sample

Fig. 5. Scanning electron micrograph showing clear cell elongation (long chains)

shock treated samples. This indicates that the stress given by microwave heat shock has resulted in the change of metabolism which in turn resulted in increased / decreased antibiotic production and its mode of action. Many workers have studied this metabolic change due to non-nutritional stress such as pH and heat shock. Enhancement of Kasugamycin production by pH shock in batch cultures of *Streptomyces kasugaensis* has been reported recently[16].

To further assess the change in antibiotic, UV maxima of all the samples have been recorded on Beckmann's spectrophotometer. Oxytetracycline has UV maxima of 270 [17]. Table 3 shows UV maxima of control and heat shock treated samples. The control sample that produced Oxytetracycline, has shown exactly 270 as per the earlier studies. There is a decrease in UV maxima of all the treated samples. 48hrs exposed samples recorded 263 while 24 hrs and 72 hrs exposed samples recorded 258 and 255 respectively. This clearly indicates that there may be a change in the biosynthesis of antibiotic.

CONCLUSION

The present work represents the first description of effect of microwave heat shock as a non-nutritional stress in *Streptomyces varsoviensis* for the production of Oxytetracycline. Further work is in progress to assess the structural variation in the Oxytetracycline molecule.

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