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The Effect of Heat Shock on Production of Recombinant Human Interferon Alpha 2a (rhIFN α-2a) by *Escherichia coli*

Rajesh K. Roy*, Shreekant M. Sapatnekar and Ranjana A. Deshmukh

Dept. of Virology, Haffkine Institute for Training, Research and Testing, Acharya Donde Marg, Parel, Mumbai, 400 012 India

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ABSTRACT

Recombinant human interferon alpha 2a (rhIFN α-2a) production and cell growth were monitored in a set of genetically modified *E. coli* strains (MSD1519, MSD1520, MSD 1521, MSD 1522, MSD 1523) producing rhIFN α-2a. The growth was followed at OD 600 nm, changes in cell physiology were detected by pyrolysis mass spectrometry (PyMS) of cell biomass and recombinant protein production was determined by SDS-gel electrophoresis. The heat stress applied was minimal (50 ℃ for 5 minutes) but the effects were detected in most of the strains. All the strains except MSD 1520 showed a significant increase in the quantity of the rhIFN α-2a secretion at 25 hour growth under the heat shock condition, quantitated by the Bio-Rad Molecular analyst software, MultiAnalyst from the digital image of gels captured using a Fluor S image analysis system. In the main fermentation system at T7 hour, only MSD 1523 showed an increase in the rhIFN α-2a secretion under the heat shock condition, at T8 hour MSD 1520 and MSD 1523 had an increased rhIFN α-2a secretion, and at T10 MSD 1521 and MSD 1522 had an increased rhIFN α-2a secretion under the heat shock condition. The PCCV ordination diagrams obtained from the PyMS result showed, a considerable effect of heat shock on the MSD 1519 strain at T5 hour. For the other strains, the result largely agreed with both the growth curves and the rhIFN α-2a production that had a limited effect on *E. coli* cultures. The increase of temperature in the main fermentation during the log phase of the bacterial culture during rhIFN α-2a expression depends on the strain specificity. This situation could definitely lead to over expression of the gene and higher intracellular accumulation of rhIFN α-2a molecule. *Iran. Biomed. J. 9 (4): 155-162, 2005*

Keywords: Recombinant protein, Recombinant human interferon alpha 2a (rhIFN α-2a), Heat shock protein (Hsp), Heat stress

INTRODUCTION

The mechanism of an organism's adaptation to high temperatures has been investigated intensively in recent years. It was suggested that the macromolecules of thermophilic micro-organisms (especially proteins) have structural features that enhance their thermo stability [1, 2]. Most cell types from all known organisms respond to thermal stress by synthesizing a small number of evolutionarily conserved polypeptides that have come to be known as heat shock proteins (Hsp) [3-5]. Hsp belong to a larger class of proteins called stress proteins that are induced by a wide variety of physiological insults. At least two of the stress proteins (HSP60 and HSP70) function as molecular chaperons that facilitate correct folding of newly synthesized polypeptides during normal cell growth [6-9]. *Escherichia coli* is a versatile organism for the production of recombinant proteins. Often, however, the recombinant protein does not reach its native, biologically active conformation within the bacterial

*Corresponding Author; E-mail: rkr3@rediffmail.com

cell but deposits as inclusion bodies [7-11]. The heat-shock chaperones, a group of polypeptides omnipresent in all kingdoms of life, form a network to assist proper folding of cellular proteins by binding to exposed hydrophobic residues, prevent their deposition and can even dissolve deposits of misfolded proteins formed during environmental stress conditions such as excessive heat [5, 7-9, 12]. Coproduction of individual chaperones with the target protein can also reduce deposition of the recombinant protein into inclusion bodies [3, 5]. Hsp comprise a group of ubiquitous polypeptides whose expression is induced when cells are subjected to stressful conditions such as increasing temperatures, high pressures, or toxic compounds. The induction of Hsp correlates with the abundance of unfolded polypeptide chains, which suggest a protective physiological role for these proteins. Hsp are involved in the assembly and disassembly of multimeric protein structures, the translocation of proteins across membranes, secretion and degradation of proteins and also stimulate protein glycosylation [1, 3-5, 7-10]. Although the stress response is well documented in a wide range of organisms, its regulation and molecular mechanism are presently not very clear. The present study was designed to observe whether potentially beneficial effects of stress response could be evaluated by applying a mild heat shock to recombinant *E. coli*, containing different ribosome binding sites cloned with the rhIFN α-2a gene, into a expression vector pZen 0148. The plasmid contained a constitutive trp promoter as well as the ribosome-binding site, a multiple cloning site derived from M13mp18 and an ampicillin resistance marker.

MATERIALS AND METHODS

The recombinant *E. coli* strains used in this study were developed by Zeneca Pharmaceuticals (UK) to produce rhIFN α-2a. The coding sequences were cloned into expression vector pZen0148 and transformed into *E. coli* MSD 462 [13]. The protein expression system is under the control of a constitutive trp promoter and includes alternative ribosome binding site sequences to vary the level of protein production. The strains used in the fermentation experiments are shown in Table 1.

Table 1. Strains used in the fermentation experiments.

Strain	Host	Plasmid	Description
MSD 1519	MSD 462	pZEN1371 :trp RBS 7	*rhIFN α-2a* (4-155)
MSD 1520	MSD 462	pZEN1372 :trp RBS 8	*rhIFN α-2a* (4-155)
MSD 1521	MSD 462	pZEN1373 :trp RBS 9	*rhIFN α-2a* (4-155)
MSD 1522	MSD 462	pZEN1374 :trp RBS 11	*rhIFN α-2a* (4-155)
MSD 1523	MSD 462	pZEN1375 :trp RBS 13	*rhIFN α-2a* (4-155)

Inoculum. For each growth, a loopful of the strain was grown overnight on M9 agar plate (1.5% agar and 50 μg/ml of ampicillin) and then inoculated 50 ml of M9 medium supplemented with 50 μg/ml ampicillin The cultures were grown at 37℃ for approximately 15-16 h at 150 rpm to give OD_{600} = 1. This culture was used for inoculation of the growth experiments.

Growth. The inoculated 250ml culture flasks were incubated at 37℃/150 rpm and samples were taken every 1 h for 13 h. A sample of the inoculum was taken at inoculation and a sample was taken after growth overnight. OD 600 nm was measured for each sample. A sample volume equivalent to 1.0ml of culture of OD 600 nm 1.5 was taken and centrifuged for 30 minutes at maximum speed (13.226-28,341 ×g Approximately). Where the volume to be centrifuged was greater than 1.5 ml, the cells were centrifuged in 15 ml centrifuge tubes. The supernatants were discarded and the samples were immediately frozen at -20℃ for analysis by SDS gel electrophoresis [13] and pyrolysis mass spectrometry (PyMS) [14, 15]. The experiment was repeated three times under similar conditions.

Heat shock. At the first sample time following a culture biomass level of 0.5 OD 600 nm the duplicate flask for each culture was heat shocked in a water bath at 50℃ for 5 minutes. This step was performed to generate heat stress response within the strains.

Heat shock effect. To determine the time required to affect an initial heat stress response MSD 1519 was heat shocked for 5 different times at 0.5 OD 600 nm at 50℃ for 2 minutes, 5 minutes, 10 minutes, 15 minutes or 20 minutes, leaving one flask without any

heat shock as control. The rest of the experiment was identical to the previous experiment.

Bio-Rad's precast Ready Gel was used to assess protein production [13]. β-Mercaptoethanol (50 µl) was added to 950 µl of sample buffer prior to use. The sample (biomass equivalent to 1.0 ml of OD 600 nm = 1.5) was resuspended in 20 µl of distilled water, 15 µl was used for PyMS and 5 µl was used for SDS gel electrophoresis. The sample was diluted to 50 µl with sample buffer and heated at 95℃ for 10 minutes to lyse the cells and dissolve the sample proteins before loading 40 µl into the gels. Protein standard marker (Bio-Rad low range molecular weight marker) was used. Gels were run according to the manufacture guidelines (but at 150 V rather than 200 V, for 35 minutes approximately). Gels were washed 3 times for 5 minutes in 200 ml distilled water and then stained with 50 ml Bio-Safe coomassie stain (Bio-Rad). The gel was kept shaking for 1 h on the shaking platform of a biometra hybridization oven. It was then rinsed with 200 ml of distilled water. A digital image of gels was captured using a Fluor S image analysis system. The images were analyzed using the Bio-Rad Molecular analyst software, Multi Analyst. The rhIFN α-2a peak and the amount were identified by comparison with the molecular weight markers. The amount of rhIFN α-2a was determined relative to the peak of one of the prominent *E. coli* protein bands. The relative rhIFN α-2a present in each sample was calculated compared to the reference protein band normalized to the reference band in reference sample T8, from heat shocked MSD 1519 (Table 2).

$$\text{Relative rhIFN } \alpha\text{-2a amount} = \frac{\text{Area of rhIFN } \alpha\text{-2a peak} \times \text{Area of reference peak of (T8 sample)}}{\text{Area of reference Peak (of the cytoplasmic protein)}}$$

Pyrolysis mass spectrometry. The pellets collected during the growth period of the strains MSD 1519-1523 were resuspended in 20 µl of distilled water, 5 µl of suspended biomass was pipetted onto each of triplicate foils for PyMS [14-17]. This was to ensure that the total mass ion count was above 500,000, most total ion count were above 1 million.

Data analysis. The analyses of the data were carried out with the help of statistical routines incorporated in the RAPyD software (Horizon instruments) [15]. Data handling was carried out off line on an IBM compatible computer using the RAPyD software and statistical analysis by the commercially available package GENSTAT [14, 15]. The result of the combined analysis is the production of 3-dimensional graph where each sample analyzed is placed in a point of space according to the relationship to the other samples included in the data set.

Table 2. Relative amounts of rhIFN α-2a produced in heat shocked and non-heat shocked cultures of *E. coli* strain MSD 1519, MSD 1520, MSD 1521, MSD 1522, MSD 1523. Samples T7, T8, T10 and T25 taken at 7,8,10 and 25 hours growth. H, heat shock.

Strains	IFN T7	IFN T8	IFN T10	IFN T25
MSD 1519	106.23	100.00	63.63	71.90
MSD 1519 H	85.04	52.67	56.17	91.38
MSD 1520	27.90	75.87	28.80	46.26
MSD 1520 H	18.41	86.65	15.90	23.32
MSD 1521	49.39	77.56	12.60	8.22
MSD 1521 H	9.96	22.34	18.35	17.59
MSD 1522	141.99	70.06	15.45	79.95
MSD 1522 H	66.12	11.44	80.37	82.26
MSD 1523	61.38	172.18	79.57	57.18
MSD 1523 H	77.96	179.00	55.40	59.58

RESULTS AND DISCUSSIONS

The effect on growth by heat shock varied depending upon the genetically modified *E. coli* strain used in the study. *E. coli* strain MSD 1519 showed no effect of heat shock and also showed a non-significant difference in the growth curve (Fig. 1A) during growth preceding the heat shock. Immediately after the heat shock, the growth was slowed in the heat shocked flask, at sample T5 (5 h growth) following heat shock at T4 (4 h), but recovers by T7 (7 h). On the other hand, the strains MSD 1520-22 showed a considerable affect on the growth curves as seen in the (Fig. 1B, C and D) unlike the strain MSD 1523 (Fig. 1E). It can be predicted that the immediate effect of heat shock leads to cessation of cell growth for a few h followed by an increase in the growth rate. It has been found that, strong production of recombinant proteins also interferes with cellular processes in many ways. Drainage of precursors and energy urges the cell to readjust metabolic fluxes and enzyme composition, stress responses are induced, and hence the cellular activity is shifted from growth to reorganization of biomass. This may result in inhibition of growth or low level of product

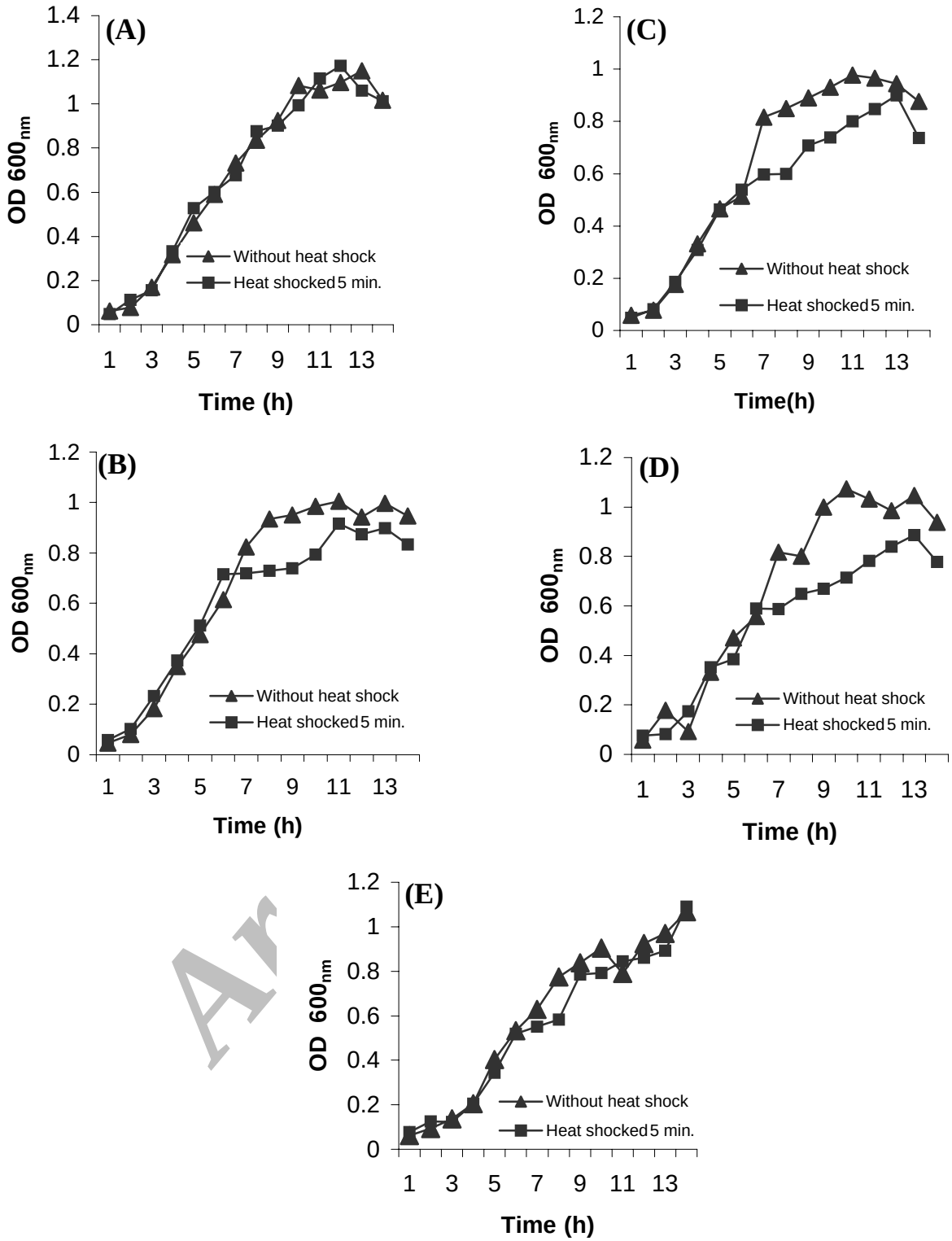

Fig. 1. (A), Growth of *E. coli* strain MSD 1519 with and without heat shock; **(B),** Growth of *E. coli* strain MSD 1520 with and without heat shock; **(C),** Growth of *E. coli* strain MSD 1521 with and without heat shock; **(D),** Growth of *E. coli* strain MSD 1522 with and without heat shock; **(E),** Growth of *E. coli* strain MSD 1523 with and without heat shock.

accumulation [7-9, 18-20]. Bacteria are known to respond to unfavorable conditions, e.g., exposure to toxic chemicals and physical agents, nutrient limitation, or sudden increase in growth temperature, by rapid expression of regulons related to the heat shock, SOS, and oxidative stress responses [7-9, 11]. The SOS responses allow the bacteria to overcome the detrimental condition and helps in adapting to the stressful situation. Due to this reason, most of the *E. coli* strains used in the study might have resumed the original growth pace after a preliminary set back preceding the heat shock and did not show a permanent effect at least in first 12 h. A drastic response of heat shock could not be observed in the final OD 600 nm values for the heat shocked and without heat shocked readings, but still in the OD 600 nm values immediately after the heat shock, it could be seen that the growth curves showed a significant difference for a few h from the non-heat shocked curve. The effect of heat shock on the production of rhIFN α-2a was determined using Coomassie blue staining of SDS protein gels and quantitated using the Multi Analyst image analysis software. The accuracy of this type of determination is poor, typically +/- 10-20%. However, results suggest that either there is an increase or decrease in the rhIFN α-2a production in the main fermentation i.e. between T7 hours to T10 hours after the heat shock to the cultures. Only in MSD 1519, the relative amount of protein produced at 25 hours (overnight) was found to be 91 rhIFN α-2a for the heat shocked culture higher than 72 for non-heat shocked control. Recent investigations have revealed that the cellular content of many direct participants in protein synthesis (e.g., initiation factors, elongation factors, even RNA polymerase) are decreased when *E. coli* enters stationary phase as the result of nutrient and energy starvation. In particular, the amount of active ribosome is drastically different between fast- and slow-growing cultures [21]. This could indicate, at that condition, the cells might lead to lesser production of the desired recombinant protein contradictory to the findings that simultaneous expression of Hsp genes increases the expression of recombinant protein [22]. But in our study, in the main fermentation system at T7 hours MSD 1523 showed an increase in the rhIFN α-2a secretion under the heat shock condition, at T8 hours MSD 1520 and MSD 1523 had an increased rhIFN α-2a secretion, and at T10 MSD 1521 and MSD 1522 had an increased rhIFN α-2a secretion. This definitely indicates that the strains are capable of producing increased amount of rhIFN α-2a biomolecule at the stationary phase under the heat-shocked condition. Though, unfortunately in the present study the *E. coli* growth curves (Fig. 1) does not show the lag, log, stationary and the death phase distinctively. From relative rhIFN α-2a calculation, with the exception of *E.coli* strain MSD 1520, all other strains have produced rhIFN α-2a in larger amounts in the heat-shocked culture. Under conditions of stress hsps constitute a major portion of prokaryotic cellular proteins [11, 18]. Thus, in the present study, the role of heat shock proteins generated due to the heat shock to the *E. coli* cultures in the increased accumulation of rhIFN α-2a molecule at the different growth hours could definitely not be ignored.

The pyrolysis mass spectrometric analysis was used as a sensitive detector of differences in the physiology and metabolism of *E. coli*. Pyrolysis is the thermal degradation of complex material in an inert atmosphere or a vacuum. It causes molecules to cleave at their weakest points to produce smaller, volatile fragments called pyrolysate [17]. Curie- point pyrolysis is a particularly reproducible and straightforward version of the technique, in which the sample, dried onto an appropriate metal is rapidly heated to the Curie- point of the metal. A mass spectrometer can then be used to separate the components of the pyrolysate on the basis of their mass- to- charge ratio (m/z) to produce a pyrolysis mass spectrum [23], which can then be used as a "chemical profile" or fingerprint of the complex material analyzed. There is a need for the development of rapid, efficient and reliable methods for the analysis of microbial cultures (over) producing biomolecule of interest. The ideal method for such analyses would have minimum sample preparation, would analyse samples directly (i.e. be reagent less), would give information about recognizable chemical characters, and would prove to be rapid, automated, quantitative and (at least relatively) economical. PyMS is an automated, instrument-based technique, which possesses the above properties. It is reagentless, rapid (the typical sample time is less than 2 minutes) and may easily be automated. A particular goal lies in the rapid and quantitative analysis of recombinant protein production in (bacterial) hosts over-expressing the gene of interest, since existing

chromatographic and electrophoretic methods remain far from ideal. Therefore, PyMS was considered as a suitable method for analysis of the genetically modified *E. coli* strains producing recombinant proteins.

The data from the PyMS experiments, in which *E. coli* strains MSD 1519-1523 were grown in matching heat shocked and non-heat shocked cultivations, were analyzed in sets which consisted of the samples from each of the heat shocked and non-heat shocked samples for each *E. coli* strain. The data are plotted as ordination diagrams of the first 3 PCCVA axes. The ordination diagrams have been rotated to align the growth pattern for each of the strain to progress from left to right. Figure 2 shows the 2D view of the 3D ordinations for strains MSD 1519-1523. However, the

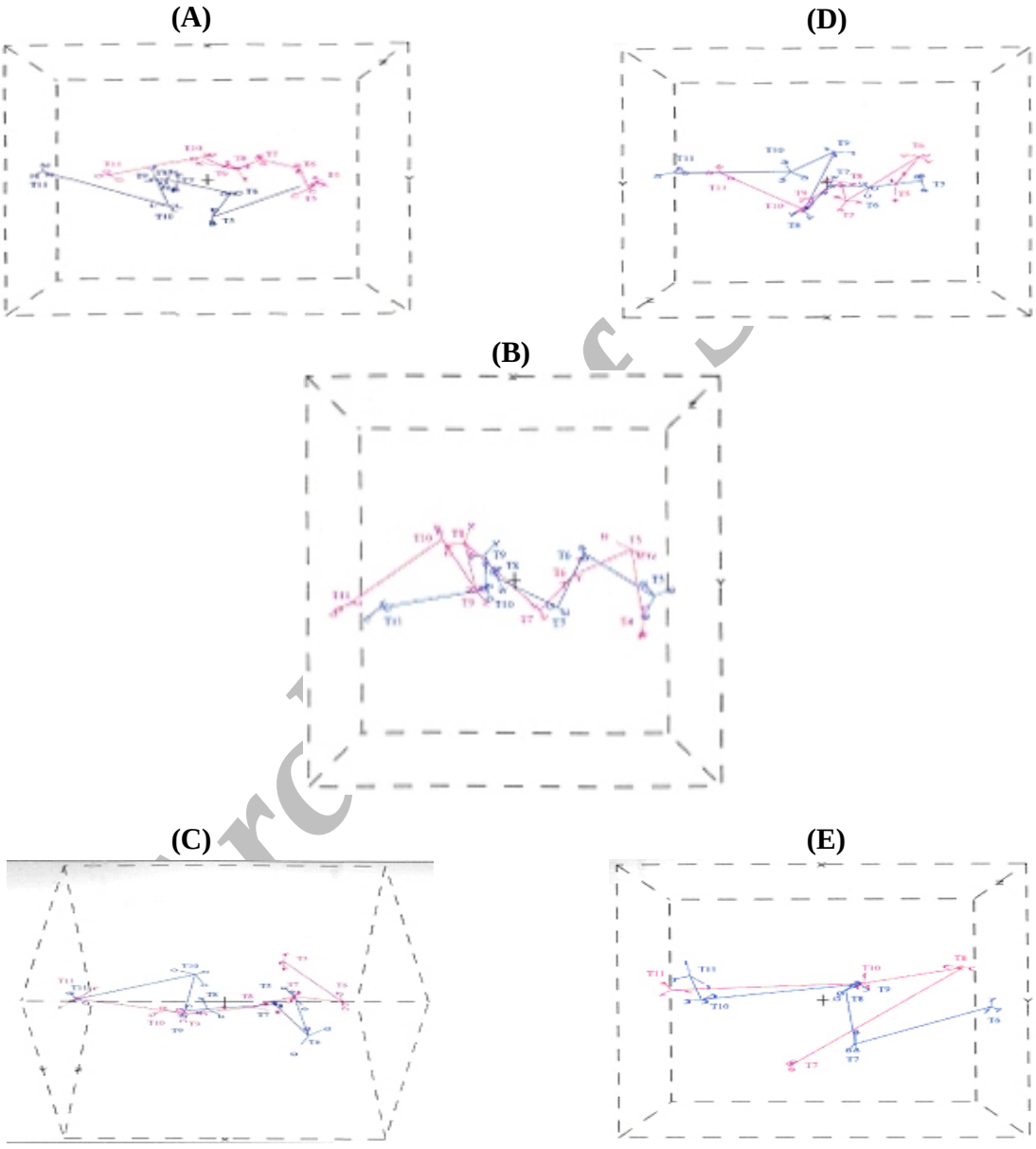

Fig. 2. **(A)**, Pyrolysis mass spectrometry of *E. coli* strain MSD 1519. **(B),** *Pyrolysis* mass spectrometry of *E. coli* strain MSD 1520. **(C),** Pyrolysis mass spectrometry of *E. coli* strain MSD 1521. **(D),** Pyrolysis mass spectrometry of *E. coli* strain MSD 1522. **(E),** Pyrolysis mass spectrometry of *E. coli* strain MSD 1523; Blue line, heat shocked.

ion counts for the samples from the different times of heat shock were too low to enable a comparison of the effect. In the case of the experiments in which a heat shock for 5 minute was used, the results largely agree with both the growth curves and the rhIFN α-2a production in showing a limited effect on the *E. coli* cultures. Therefore, in MSD 1519 strain, a considerable effect of heat shock can be seen at T5 hours. Otherwise, there was an obvious progression with time on all the PCCV ordination diagrams when analyzed by various strains (Fig. 2). This corresponds to the growth phases, lag, log, and stationary phase.

Irrespective of the specific characteristics of the genetically modified *E. coli* strains used in this study, an increase in the temperature is the main fermentation system during the log phase of the culture could definitely lead to an increase expression of the rhIFN α-2a gene resulting to an increase intracellular accumulation of rhIFN α-2a protein molecule. Thus the above approach could be a solution for the development of a cost effective rhIFN α-2a molecule leading to an affordable rhIFN α-2a biomolecule for the drug market. The expression level of the rhIFN α-2a gene (as seen in the present study) under the control of constitutive (trp) promoter and with different ribosome binding sites gives an insight of the choice of vectors essential to set up a successful fermentation condition for the development of rhIFN α-2a biomolecule

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