World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Isolation and characterization of exopolysaccharide from biofilm producing marine bacteria

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Received: 09-06-2014 / Revised: 26-06-2014 / Accepted: 24-07-2014

ABSTRACT

Marine bacterial exopolysaccharides are fascinating industrial uses and presence of bioactive compounds. In this present study, Exopolysaccharide producing *Micrococcus sp* isolated from Arabian Sea and was identified by morphological and biochemical characteristics. Exopolysaccharide characterized by Infra-Red spectroscopy and Antibacterial activity done by disc diffusion method with the clinical isolates. Exopolysaccharide production and quantification were done in different environmental factors like incubation temperature (27^0 C and 37^0C), pH (6,7 and 8) and incubation time (24hr, 48hr, 72hr and 96 hr). From the results, maximum exopolysaccharide production was at pH 8, 27^0C and at 96 hrs of incubation time and exopolysaccharide has antibacterial activity. Exopolysacharide may produce from M*icrococcus* by these method for large scale and small scale industries and has expecting a new bioactive compound.

Keywords: Exopolysaccharide**,** *Micrococcus,* Bio film, Antibacterial activity, Bioactive compound.

INTRODUCTION

Many studies have been carried out to investigate the occurrence of microbial bioactive compounds during last 50 years. Exopolysaccharides (EPS) from bacteria has very important roles in the bacterial attachment and later development of matured complex matrix of bacterial biofilm. EPS are high molecular weight polymers which are sticky in nature and composed of exopolysaccharides, proteins, lipids and nucleic acids [1,2]. Bacterial EPS having novel structures and may have high demand in industrial uses. Exopolysaccharides from bacteria has vital roles in the bacterial primary attachment and development of matured complex matrix of bacterial biofilm in later. Bacterial colonization on artificial surfaces, metal surfaces, abiotic and biotic materials provide some of survival strategies to microbial cells against toxins and antibiotics, improved entry to nutrients, self-protection from predation and some extracellular enzyme activities [3]. Capsular EPS produced mainly in the exponential /log phase of bacterial growth while EPS produced during the stationary phase are slime EPS[4]. Biofilm formation rate is vary depending on the organisms and some environmental factors like pH, ionic strength ,temperature and medium, [5]. Most of these biofilm forming bacteria are resistant characteristics and produce bioactive compounds including EPS with unique structures. The extracellular materials such as polysaccharides, lipids, glycoproteins and lipopolysaccharides can be used as stabilizers, crystallizing agents, adhesives, solidifying agents, emulsifying agents, flocculants and flushing agents in various industries[6].

In this study, 14 bacterial cultures were isolated from ARABIAN SEA and were screened for the biofilm synthesis. A superior biofilm producing strain was selected for further studies and was identified as *Micrococcus sp* (MN) based on morphological, biochemical and physiological characteristics. EPS production was assessed and quantified at different environmental parameters like pH, temperature and at incubation time. Crude EPS was subjected to FT-IR spectroscopy also checked for antimicrobial activity.

MATERIALS AND METHODS

Isolation of bacteria: Water samples collected from ARABIAN SEA (About 1Km from sea shore)

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at Cochin, Kerala, in sterile bottles and aseptically transfer to the laboratory and stored in refrigerator until further study. The isolation of organisms done by standard plate count method on marine agar (Himedia 2216). Experiments were carried out in triplicates.

Screening of biofilm bacteria: Isolated bacteria were screened for biofilm formation by using Glass slides as surfaces. The glass slides sterilized with acetone, immersed in a detergent solution for 1hr, thoroughly washed with distilled water and dried for 1hr at 160^0C. Glass slides were separately immersed in a conical flask containing YMG medium (Yeast Malt Glucose media -yeast extract 3g, malt extract 3g, glucose 10g, peptone 5g, distilled water 500 ml and 500 ml Aged sea water) and inoculated with 14 selected organisms. After 7days of incubation at 37^0C, the glass slides were taken out and washed with phosphate buffer solution to remove unbounded cells. Again, the glass slides transferred to a fresh YMG media which was inoculated with each culture and incubated for 7 days. The glass slides were scrapped, the biomass suspended in 9ml of distilled water and serially diluted. Each dilution was plated and incubated in triplicates and the obtained colonies counted using colony counter. The organisms showing the maximum attachment was selected for this study.

Identification: Based on the morphological, biochemical and physiological characters, the isolate was identified.

Production of EPS: 10 ml inoculum was prepared in of Yeast Malt Glucose broth and incubated for 24 hrs at 25^0C. A volume of 200 µl of culture was inoculated into 50 ml of yeast malt glucose broth and incubated at room temperature for 5 days at 120 rpm. The culture broth was centrifuged at 10,000 rpm for 20 min, after the removal of pellet, supernatant (used as crude EPS for studies) mixed with 3 volumes of isopropyl alcohol and well shaken to prevent local high concentration of the precipitate and left overnight at 4°C. Precipitated EPS weighed after drying at 80°C for 24 hr. EPS extracted according to the method described by [7].

EPS Production in various bio parameters

Estimation of Total Carbohydrate: Crude EPS and dried EPS were subjected to the determination of total carbohydrate content with parameters individually, at pH 6, 7 and 8, at different time of incubation 24,48,72 and 96 hrs and on different temperatures 27°C and 37°C by Anthrone method by using glucose as the standard [8].

Estimation of Total Protein: The total protein estimation of crude EPS and dried EPS were carried out by Lowry's method [9]. The estimations were undertaken at pH 6,7 and 8, different incubation time at 24,48,72 and 96 hrs and at different temperatures 27°C and 37°C.

Characterization by FT-IR: Crude EPS was characterized by FT-IR spectroscopy (Analyzed at STIC-Cochin University-KERALA).

Antimicrobial activity of Crude EPS: Crude EPS (supernatant) was used as a sample to determine the antimicrobial activity and disc diffusion method was followed. Clinical isolates such as *Escherichia coli*, *Klebsiella spp.*, *Salmonella typhi* and *Staphylococcus sp* were used as test organisms collected from nearby hospitals. Zone of inhibition on MHI agar plate (Mueller hinton Infusion Agar) measured in millimeter after 24 -48 hr of incubation at 37^0C.

RESULT & DISCUSSION

Most of the bacterial biofilms are embedded in extracellular polymeric substances. These substances consists of capsular polysaccharides, which form a firmly attached layer or capsule linked to the cell surface, and the exopolysaccharides which form a slime layer loosely attached to the cell surface [10]. Bacterial biofilm matrix which is constructed by producing extracellular polymeric substance during the process of colonization on particular surfaces[11].

In this research work, marine bacterial strain was isolated from ARABIAN SEA. Bio film formation of the isolated strains were checked based on the attachment on glass slides and this result is similar with the studies reported by [12,13]. Related work was reported that glass and stainless steel are the surfaces that provide a greater bacterial adherence [14,15]. Colonies showing more adherence on glass slide were selected for further bio film studies. Bacteria growing in a biofilm on a surface are generally more resistant to many antimicrobial agents than the same bacteria growing in a free-swimming state [16,17,18]. Selected organism MN was identified as *Micrococcus sp*, is an orange pigmented bacterium, gram positive, cocci, an obligate aerobe, non-motile and arranged in tetrads, optimum growth in between 15°C - 30°C. Estimation of total carbohydrate and total protein (Figure:1) of DRY EPS and Crude EPS were quantified in various incubation period, temperature and at pH. From these results, both carbohydrate content and total protein content of Crude EPS were higher at 96 hrs, pH 8 and 27°C and were 96.4 mg/L, 75.1 mg/L respectively.

Lower content of total carbohydrates and total protein were determined in dry EPS when compared to crude EPS but it was also found to be higher at 96 hrs of incubation time, temperature of 27°C, and pH 8 in both EPS. In other growth parameters studied for crude and dry EPS, lesser total carbohydrates and total protein contents were present. [19,20] reported that most of the bacteria were release the largest quantity of EPS during stationary growth phase in laboratory culture. Similar results were found in this study. The maximum production of EPS from MN was in the temperature of 27°C and 96 hrs of incubation time and pH 8 and were the optimum conditions for EPS production.

Antibacterial activity of crude EPS was shows zone of inhibition against *Escherichia coli*, *Klebsiella sp*, *Salmonella typhi* and *Staphylococcus sp* (Table :1) after 24hr of incubation time. Antimicrobial activity among marine bacteria is very much familiar and has been studied in a number of research works [21,22].Similar studies were reported ,Crude EPS of marine *Halomonas sp* has an antibacterial activity against some of organisms[23].

FT-IR characterization of the Crude EPS (Figure: 2) , the value 3366.07 cm^{-1} indicates the several primary and secondary amides proteins of bacterial cell , 2107.00 cm^{-1}–C≡C–stretch alkynes,1631.75 cm^{-1} indicates N-H bend primary amines and 1001.36 cm^{-1} indicates C–O stretch and 724.28 cm^{-1} indicates C-H alkanes.

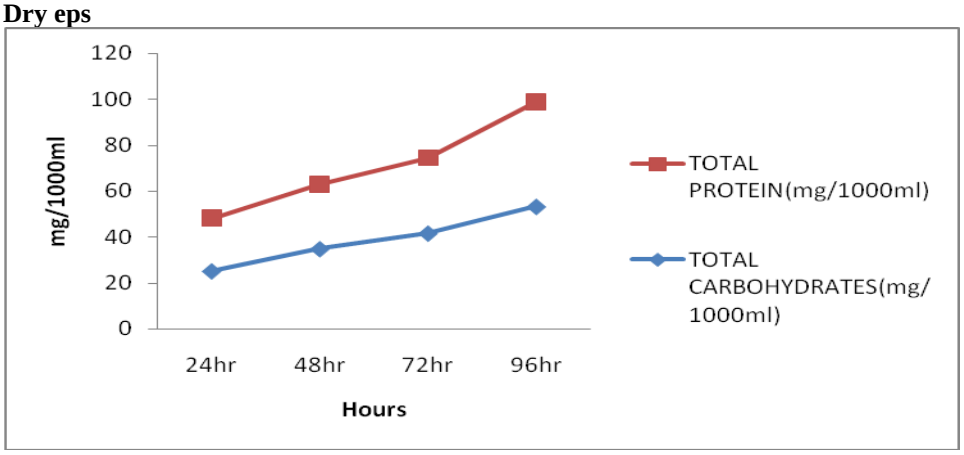
CONCLUSION

Marine *Micrococcus sp* (MN) can capable to attach and grow on surfaces and produce biofilm. MN has a potent producer of exo polysacharide. The optimum conditions for higher EPS production from MN was at 96 hr, 27°C and pH 8. These conditions may used to produce industrially valuable EPS in future in mass. FT-IR analysis of crude EPS indicates the presence of cell wall protein, 1˚, 2˚amines , alkanes and N–H and C-O groups. EPS production was increased in the log phase and maximum at stationary phase that was in 96hr of incubation . Crude EPS isolated from MN has an antimicrobial activity against some of pathogens and may the presence of bioactive compounds. This is the primary research work on *Micrococcus sp* (MN) in EPS optimization and identification of the antibacterial compound. Further studies are needed to evaluate the biofilm exopolysaccharides application and identification and purification of a new bioactive compound.

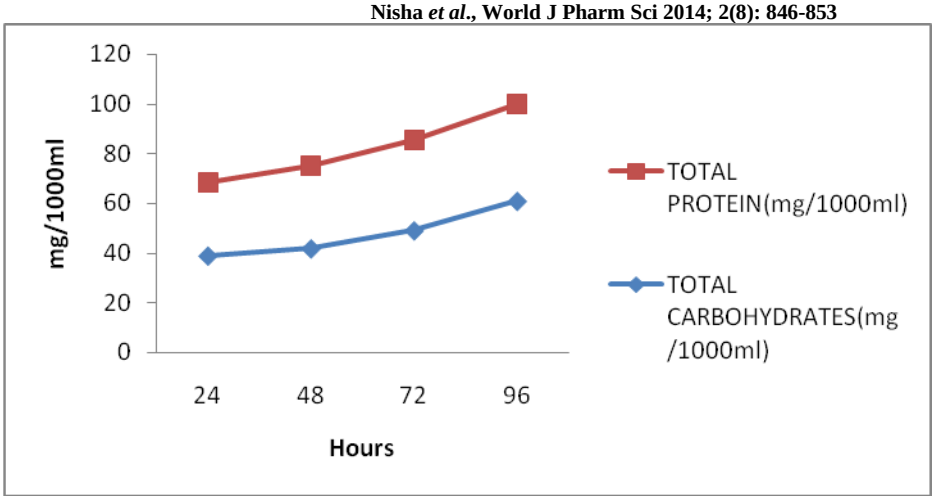
Table: 1-ANTIMICROBIAL ACTIVITY OF CRUDE EPS

Sample	Test Organisms	Zone of Inhibition
CRUDE EPS	*Escherichia coli*	10mm
	Klebsiella sp	9mm
	Salmonella typhi	8mm
	Staphylococcus sp	9mm

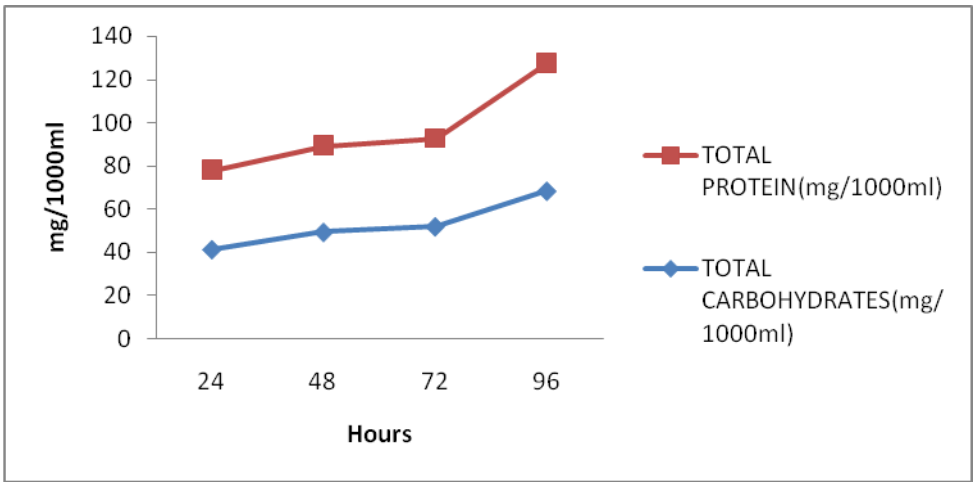
Figure : 1 - Total Carbohydrate & Total protein estimation
Dry eps



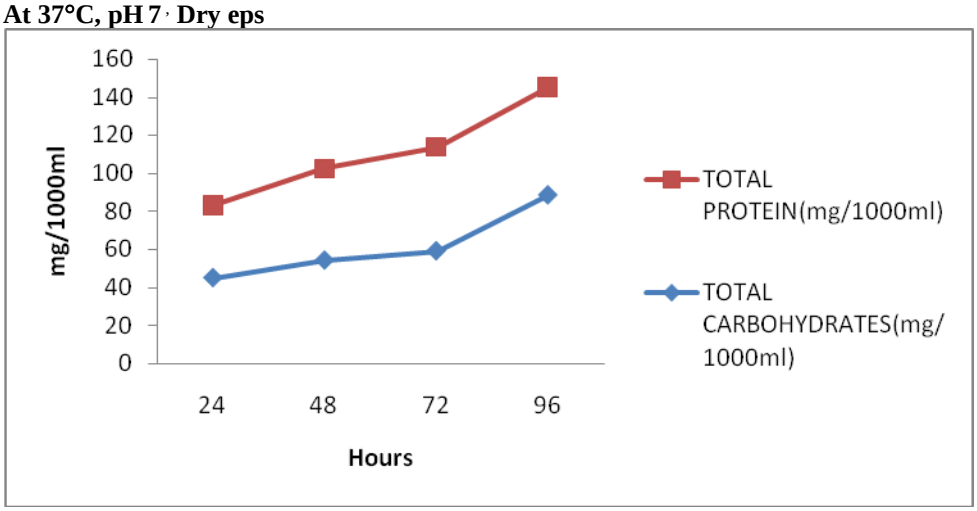
At 37°C, pH 6, Dry eps



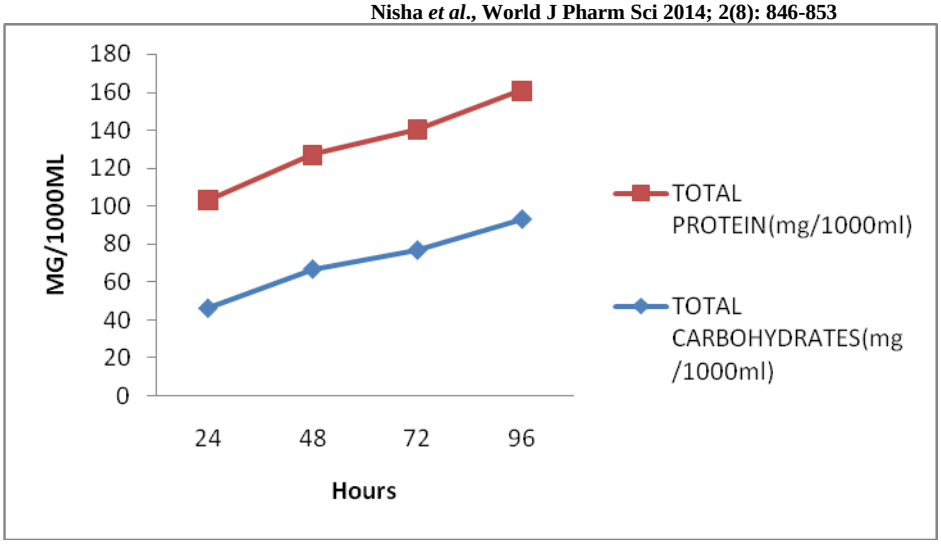
At 27°C, pH 6, Dry eps



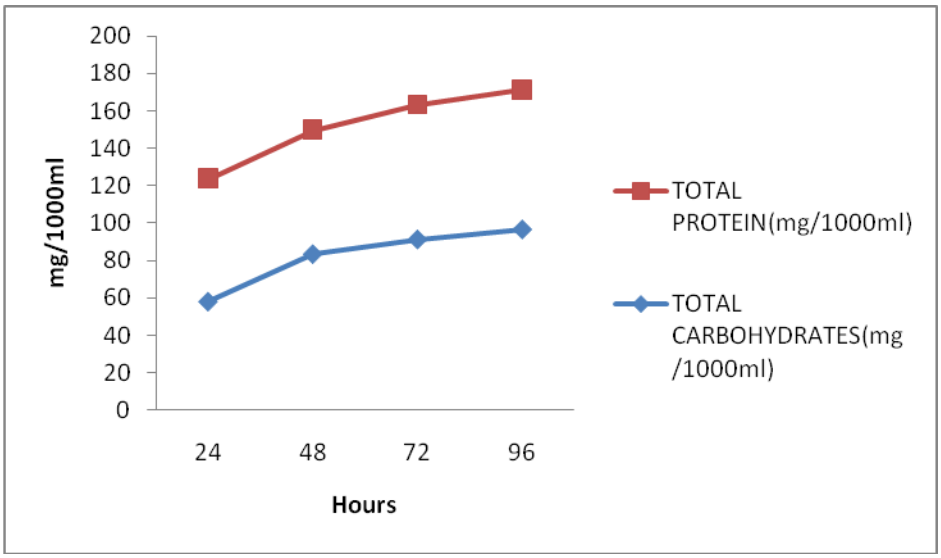
At 37°C, pH 7, Dry eps



At 27°C, pH 7, Dry eps

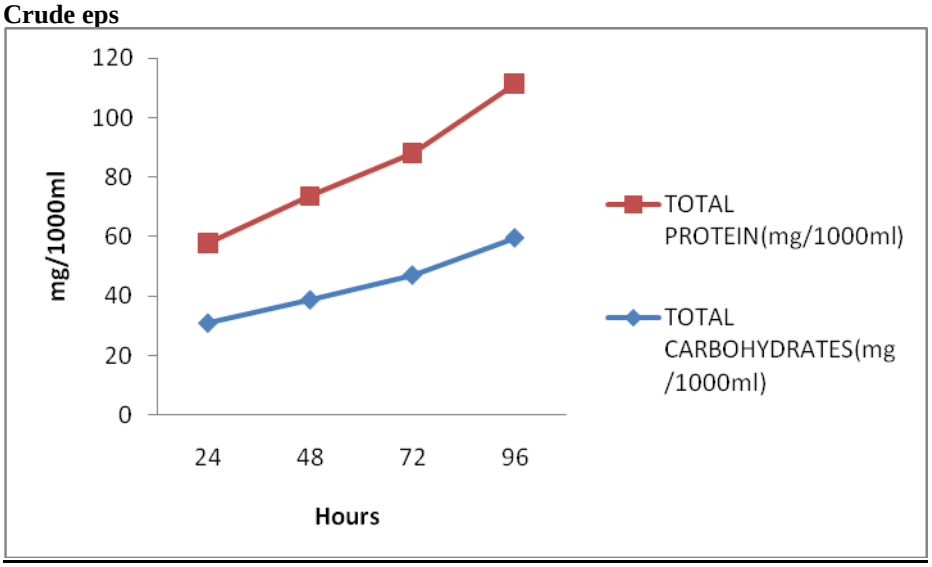


At 37°C, pH 8, Dry eps

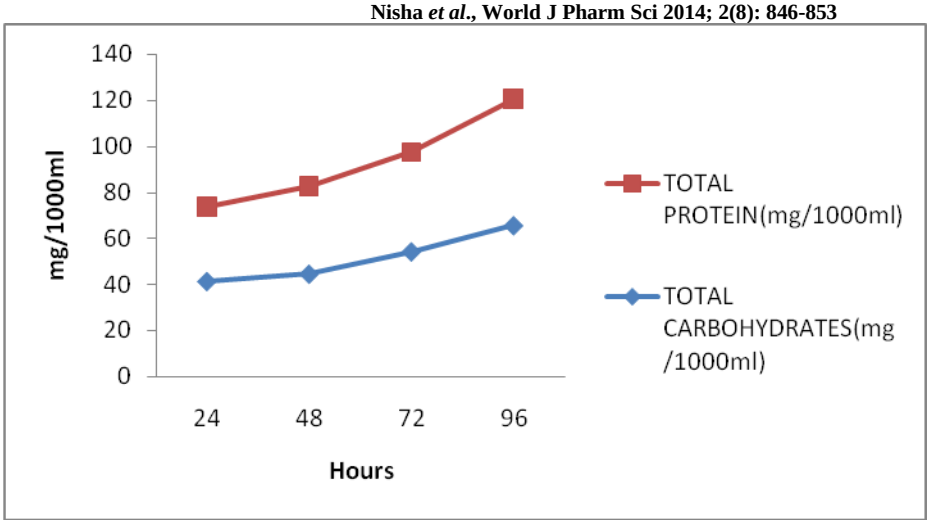


At 27°C, pH 8, Dry eps

Crude eps



At 37°C, pH 6 ,Crude eps



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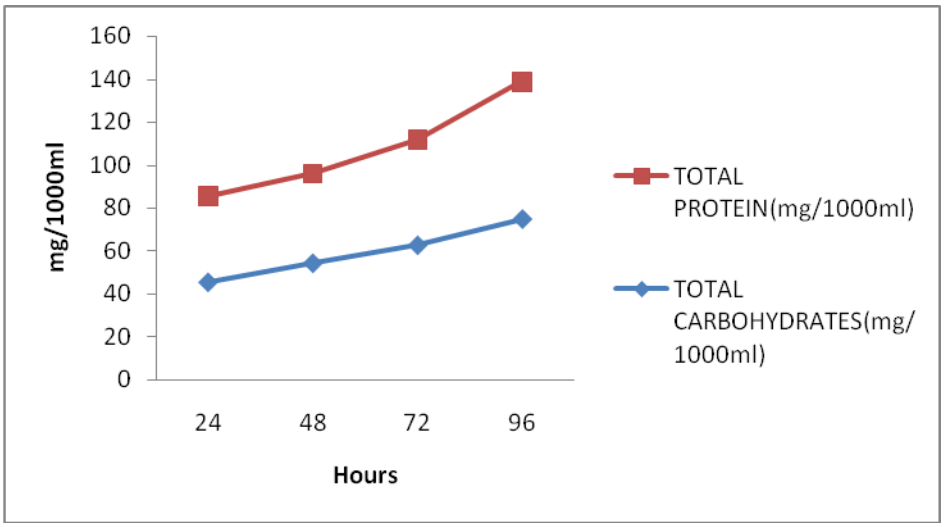

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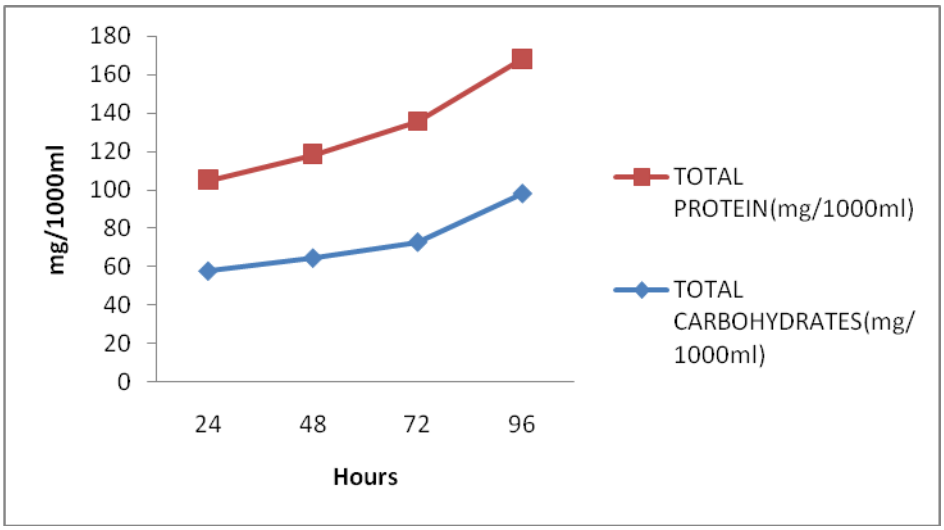

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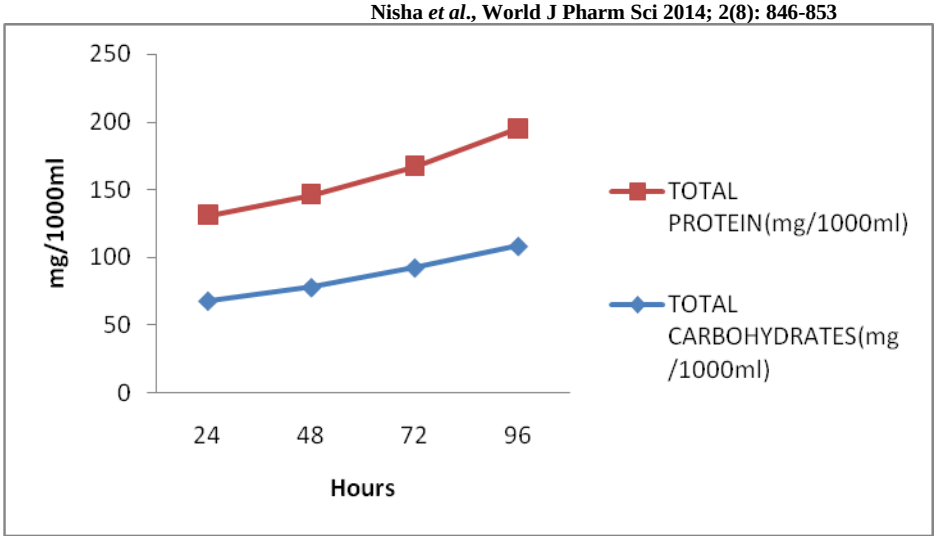
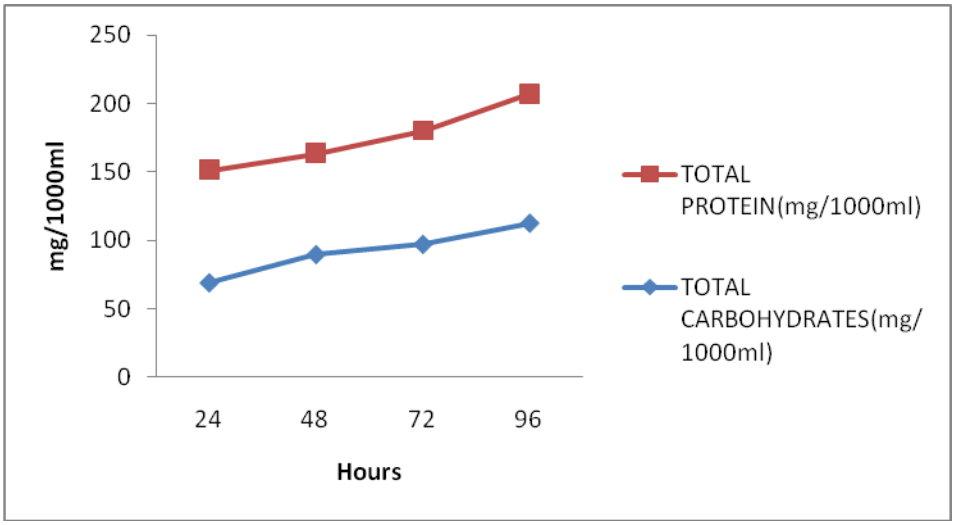


At 37°C, pH 8 , Crude eps



At 27°C, pH 8 , Crude eps

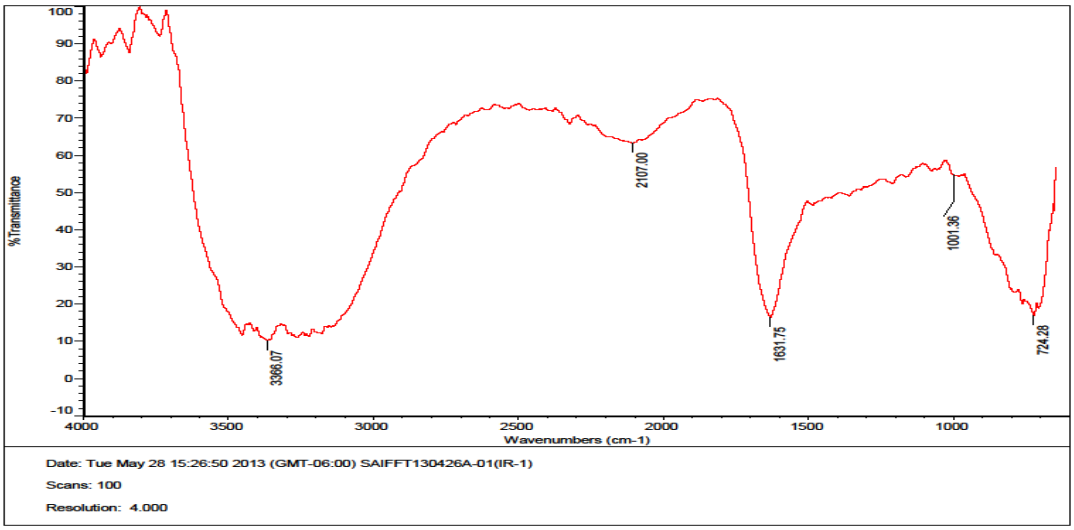


Figure : 2—FTIR of Crude EPS

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