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Immobilization of *Escherichia coli* Mutant Strain for Efficient Production of Bioethanol from Crude Glycerol

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ABSTRACT
Glycerol, generated in large amounts during the production of biodiesel, present an excellent opportunity to convert it into bioethanol. A native, nonpathogenic bacterial strain such as *Escherichia coli* (*E. coli*), able to ferment glycerol to useful products under anaerobic condition is very desirable. The objective was to develop mutant strains of *E. coli* (ATCC 11505) that are capable of withstanding high glycerol concentration as well as resisting ethanol-inhibition. The ability of this mutant, vis-à-vis the original strain, in utilizing glycerol in a high glycerol containing medium, with the concomitant bioethanol yield, was assessed. A glycerol-utilizing mutant *E. coli* strain for production of bioethanol using low power microwave have been developed (frequency 2.45 GHz and irradiation time 5 min pulse irradiation). In the present work, the mutant *E. coli* were permeabilized and then cells were immobilization on glutaraldehyde cross-linked chitosan for bioethanol production from glycerol. The fermentation runs was carried out with glycerol as substrate in 2.0 L as worked volume containing 100 g of immobilized cells coated with chitosan cross linking with glutaraldehyde. The Scanning Electron Microscopy (SEM) images confirmed that the bacteria cells were entrapped in the porous network of cross-linked chitosan beads. The results showed that the chitosan beads-cross-linked with glutaraldehyde immobilized with permeabilized mutant *E. coli* showed great potential for use during the bioethanol production, which boosted up the production 2.9-folds comparing with the reactor with free cells. Particularly, the research confirmed the effectiveness of cells immobilization in chitosan beads reflected by the high yield of bioethanol (96.7 g L^{-1} compared of those using free cells) and the ability to utilize glycerol in high glycerol containing medium.

Key words: Chitosan, permeabilization, *Escherichia coli* (ATCC 11105), immobilization, microwave irradiation, glycerol, bioethanol

INTRODUCTION

Energy crisis, climate changes such as rise of atmosphere temperature due to increase of greenhouse gases emission and the Kyoto Protocol restrictions in generation of these gases, coupled with high oil prices, limitation and depletion of fossil fuels reserve make renewable energies more appealing (Mousavi, 2012). Renewable energy resources are energies generated from natural resources such as wind, sunlight, tide, hydro, biomass and geothermal, which are naturally replenished. Biofuel are renewable energy with rather high efficiency and its sustainability and biodegradability contribute to the reduction of carbon dioxide emission and other harmful gases during combustion (Janaun and Ellis, 2010; Girard and Fallot, 2006). The EU Directive on Renewable Energy (2009/28/EC) (EC., 2009) has set a target for 10% of transport energy to be met with renewable sources by 2020, a target which requires significant growth in the biofuels arena. The growth of biofuels to date has been largely directed by policy (Zah and Ruddy, 2009), which initially widely promoted

biofuels. However, concerns over environmental damage (e.g., deforestation) and social issues (e.g., food prices) have led greater inclination to renewable energies utilization among the developed countries. The production capacity and market values of biodiesel and bioethanol are gaining lots of attention than other categories of biofuels (Deenanath *et al*., 2012).

In the industrial scale production of biodiesel, the economical method applied is the homogeneous base-catalyze trans-esterification of triglycerides from vegetable oils, animal fats, or waste cooking oils with methanol (Kolesarova *et al*., 2011). During biodiesel production, glycerol is produced as a byproduct at levels of approximately 10% (w/w) of the total biodiesel generated (Johnson and Taconi, 2007). In recent years, the mandatory demand of biodiesel has flooded the market with excessive crude glycerol. Such abundant supplies of raw glycerol have not only greatly disturbed the market for traditional glycerol in terms of price, but have also created a significant environmental problem because glycerol cannot be discharged without treatments (Da Silva *et al*., 2009). Thus, considerable efforts have been directed toward development of methods to refine glycerol from a low-cost feedstock into industrially valuable materials including fuels such as bioethanol (Yu *et al*., 2010). Using glycerol as feedstock for bioethanol production is desirable since it can utilize almost all the waste glycerol produced by the biodiesel industry. In this context, glycerol is used as a substitute for common, traditional substrates such as sucrose, glucose and starch (Suhaimi *et al*., 2012). The set back is that glycerol is non-fermentable by most microorganisms, with the exception of a group of bacteria including *Bacillus*, *Clostridium*, *Enterobacter*, *Klebsiella* and *Lactobacillus* species (Dharmadi *et al*., 2006; Zheng *et al*., 2008). However, these microbes cannot be used in industrial application due to their pathogenicity. Recently, Gonzalez *et al*. (2008) demonstrated anaerobic fermentation of glycerol by *Escherichia coli* (*E. coli*), a species that had long been considered to be incapable of glycerol utilization; however, the yield was very low. Increased yield of bioethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology (Brooks, 2008). The interest in using the *E. coli*, as a host strain for the production of bioethanol from glycerol, is because it is very amenable to industrial applications, easy to handle and grows well. The potentials for using *E. coli* at the industrial level could overcome issues such as pathogenicity, requirement of strict anaerobic conditions, need of supplementation with rich nutrients and unavailability of the genetic tools necessary for their effective manipulation. However, the wild type *E. coli* strains are rarely used for glycerol-derived bioethanol production as they are incapable of glycerol utilization as carbon source. The task now, is to find effective non-pathogenic microorganisms capable of utilizing glycerol for bioethanol production. A native, nonpathogenic strain of *E. coli* able to ferment glycerol to useful products under anaerobic condition without the need of genetic engineering is being developed currently by many researches.

Immobilization is a process by which whole cells are confined to a phase distinct from the one in which the substrates and the products are present. The interest in cell immobilization for bioethanol production has increased in the last decades due to several advantages presented by the using of cell immobilization, such as increased productivity, reduced risk of contamination, biocatalyst recycling, rapid product separation and the stability of cell activity (Hsieh *et al*., 2008; Kuhad *et al*., 2011; Sembiring *et al*., 2013). Other advantages of immobilized cells compared to free ones: protection against harsh environmental effects such as pH and toxic compounds, high cell loading density, retain high cell densities inside the reactor, a relatively easy cell separation process, reusability and lower susceptibility to contamination by other microorganisms, greater volumetric productivity, tolerance to higher concentrations of substrate and products, minimal clogging under continuous-flow conditions and relative easiness of downstream processing (Hasan and Srivastava, 2009; Takei *et al*., 2011; Sembiring *et al*., 2013). Several natural and synthetic polymers are available for immobilization of cells. Among natural polymers, chitosan is known for its biodegradability, biocompatibility and non-toxic properties, low cost and large-scale availability (Alves and Mano, 2008; Kim *et al*., 2008). Chitosan is the product obtained from deacetylation of chitin, which is the second most abundant biopolymer after cellulose (Kim *et al*., 2008). One of the important applications of chitosan is as support for immobilization of whole cells as it is non-toxic and user-friendly and has protein affinity (Krajewska, 2004). Chitosan has great potential due to its poly-cationic properties, which are unique among abundant polysaccharides and natural polymers (Vold *et al*., 2003). The amino (-NH_2) and hydroxyl (-OH) group serve as the coordination and reaction sites (Chang and Juang, 2004). However, the weak mechanical properties and the solubility of chitosan in acidic media are the major obstacle for using chitosan as support medium (Ngah *et al*., 2011). In this case, cross-linking modification of raw chitosan with glutaraldehyde is a good way to improve the chemical stability of chitosan in acid solution (Ngah *et al*., 2002, 2005; Chen and Chen, 2009; Rosa *et al*., 2008). Both hydroxyl and amino groups found in chitosan can be modified either by using physical or chemical modification (Ngah *et al*., 2005). Physical modification increases the sorption properties; gel formation and involves an expansion of the porous network. Chemical modification, on the other hand, increases the sorption properties of chitosan while preventing the dissolution of chitosan in strong acids. Cross-linking can change the crystalline nature of chitosan and enhance the resistance of chitosan against acid, alkali and chemicals and also increase the sorption abilities of chitosan (Ngah *et al*., 2005). Another problem with the whole cells biocatalysts is the limitation in diffusion of substrate and products through

cell wall (Bernardino *et al*., 2007; Adikane and Thakar, 2010). The permeability barrier of the cell envelope often causes very low diffusion for the substrates. Permeabilization of the cellular membrane without complete cell disintegration promises to solve low permeability problem of whole cells to a certain extent. Permeabilized cells can be used as whole-cell biocatalyst to perform biotransformation mediated by their intracellular enzymes since the bacterial outer membrane becomes permeable allowing small molecules to freely enter and leave the cell while keeping the morphology of the cell intact (Somkuti *et al*., 1998). This method permits keeping most cellular structures, protein-protein interactions and most of intracellular enzymes in their original environment inside the cell.

Microwave is electromagnetic wave between radio wave and infrared ray, with frequency between 300 MHz and 300 GHz. Microwave is non-ionizing electromagnetic wave and has various interactions such as reflection, penetration and absorption with materials. Microwave can affect ion move, dipole rotation but cannot change the molecular structure. When dielectric constant polar solvent meets microwave, the temperature rises in a short time. Microwave irradiation is an efficient heating source for chemical reactions where reactions that require several hours under conventional conditions can often be completed in a few minutes with very high yields and reaction selectivity (Lidstrom *et al*., 2001; Larhed and Hallberg, 2001). Microwave chemistry or microwave-assisted chemistry is focused around the world, because microwave is absorbed in polar molecule, improves reaction rate and yielding rate and it is easy to control and operate. There are two mechanisms that microwave energy interacts with materials. In case of polar molecule with dipole moment, dipole tries to arrange to electric field of microwave. On exposure of dipole molecules to electromagnetic wave within microwave range frequencies, polar molecules try to follow the field and align themselves in phase with the field. However, owing to inter-molecular forces, polar molecules experience inertia and are unable to follow the field. This results in the random motion of particles and this random interaction causes molecular friction and collisions which gives rise to dielectric heating (Lidstrom *et al*., 2001; Larhed and Hallberg, 2001). In case of ion (ionic conduction), back and forth under the influence of the electric component of microwave irradiation. They collide with their neighboring molecules or atoms and these collisions cause agitation or motion, creating heat (Lidstrom *et al*., 2001; Larhed and Hallberg, 2001). In contrast to specific thermal microwave effects, an existence of specific non-thermal microwave effects is also suggested. Essentially, most non-thermal effects result from a proposed direct interaction of the electric field with specific molecules in the reaction medium and electrostatic polar effect (Larhed and Hallberg, 2001). The microwave irradiation has been extended for use in the chemical modification of polysaccharides such as starch and chitosan. Ge and Luo (2005), reported the carboxymethylation of chitosan in aqueous solution under microwave irradiation, because no organic solvents are required, represents a more environmentally-friendly method for modification of chitosan.

Efficient bioethanol production requires a rapid fermentation to get high bioethanol concentrations; therefore a bacteria strain must have a good specific growth rate and good specific bioethanol production rate at high ethanol concentration (Choi *et al*., 2011; Oh *et al*., 2011). Recently, glycerol-utilizing mutant *E. coli* strain is reported for production of bioethanol using low power microwave technique (Nomanbhay and Hussain, 2014). The present work was therefore carried out to apply immobilization technology by using the chitosan-cross linked with a glutaraldehyde for the production of bioethanol using permeabilized mutant *E. coli*. The application of immobilized *E. coli* on swollen beads is likely applicable to enlarge the bioethanol production from waste crude glycerol by reusing the cells for batch fermentation process.

MATERIALS AND METHODS

Bacterial strain and chemicals: Glycerol, major byproduct produced from palm oil industry, was supplied by Sime Derby biodiesel mill (Carey Island, Klang Malaysia). Lyophilized *Escherichia coli* (ATCC 11105) strain was obtained from American Type Culture Collection (ATCC). For long-term bacteria storage, the bacterial stock (in 20% glycerol medium, v/v) was kept at the -80°C. The Mueller Hinton Broth and physiological saline, acetic acid, sodium hydroxide, monobasic sodium phosphate, dibasic sodium phosphate, glucose and rich media (10X MOPS) buffer minimal media kit were purchased from (Sigma-Aldrich, USA). Chitosan (75% deacylated, meduim molecular weight), glutaraldehyde and N-cetyl-N,N, N-trimethyl Bromide (CTAB) were purchased from (Merck, Germany). Deionized water used was supplied from ELGA Lab Water (UK), ultrapure water purification system in the laboratory. All the chemicals used were of analytical grade and used as received.

Apparatus: Equipment used throughout this study were FTIR spectrophotometer-model IRPrestige-21 (Shimadzu Corparation Japan), microwave oven (Samsung CE2877-N, Korea), rotary shaker (Lab Companion Shaker-300, Korea), autoclaving bench top centrifuge (Model No.25X-2, All American Company), table top centrifuge (Hettich Zentrifugen Rotofix 32, Germany), Bio Gene bioreactor fermenter model (Biogene T type, India), Seven-gauge needle and model 100 push-pull syringe pump (Sigma-Aldrich Company, U.S.A.), UV-Vis Spectrophotometer- Lambda 35 (Perkin Elmer, U.S.A) and Scanning electron microscope to scan surface morphology (Phillips XL-20, Japan).

Pretreatment of crude glycerol: Crude glycerol obtained from a local biodiesel production plant (Sime Derby biodiesel mill), that utilizes palm oil as the raw materials via the alkali catalyst mediated trans-esterification process. As obtained, the

crude glycerol was a sticky dark brown solution with pH of 11.7. Prior to use, the crude glycerol was slowly heated to around 55-60°C and was pre-treated according to the procedure as described by Saifuddin *et al.* (2014). Typically during the pre-treatment process the crude glycerol was purified using microwave assisted acidification by the addition of H_2SO_4 to a pH 1.0 and then followed utilized by a bio adsorbent synthesized from dead yeast cells immobilized on 5.0 g cross linked chitosan (cross linking by glutaraldehyde). The pre-treatment allowed partial purification of crude glycerol with reasonable high glycerol content (~65%) and with relatively low contaminant levels. This partially purified glycerol was used subsequently for all fermentation processes for bioethanol production.

Medium preparation and cultivation of bacteria: Lyophilized *Escherichia coli* (ATCC 11105) were used throughout this study. Before experimental use, the strain was sub-cultured in broth media by incubated in a rotary shaker at 150 rpm at 37°C for 72 h. Batch pre-cultures were conducted in 900 mL of glass bottle closed with blue cap with 250 mL as working volume, containing sterilized medium of Mueller Hinton Broth composed of (Beef infusion solids 2.0% (g L^{-1}), Starch 1.5% (g L^{-1}) and Casein Hydro lysate, 17.5% (g L^{-1})). Sterilization performed separately by autoclaving the media for 20 min at 121°C. Cell growth was monitored by measuring the optical density of culture at 600 nm, using spectrophotometric method. The active growth phase culture broth (OD_{600} = 1.0) were harvested by transferring a 1.5 mL micro centrifuge tube and centrifuged at (8000 rpm, 10 min) to retain the cells and to decant the supernatant. The cells pellet was washed thoroughly with sterilize 0.9% (w/v) sodium chloride solution, followed by distilled water. Finally the cell mass was suspended in sterilize sodium chloride (10 mL) and was store at 4°C for further use.

Microwave irradiation induced mutation of *Escherichia coli* strain: Microwave irradiation-induced mutation on *Escherichia coli* cells have been explored by Nomanbhay and Hussain (2014). In their work, cells of wild type *E. coli* were exposed to microwave irradiation at frequency 2.45 GHz, with output power 180 W. The total exposure time was 5 min with intermitted pulse. The resulting cells suspension was immediately kept at on ice (0°C) and then quickly revived in enrich media before being used for the fermentation with the glycerol (as carbon source).

Bacteria reviving using enrich meduim: Microwave irradiation-induced mutant *E. coli* cells were revived after the microwave treatment by immediate addition of the cells into 250 mL Erlenmeyer flasks containing 100 mL of Mueller Hinton Broth consist of (Beef infusion solids 2.0% (g L^{-1}), Starch 1.5% (g L^{-1}) and Casein Hydro lysate, 17.5% (g L^{-1})) and 2 g L^{-1} of glucose. The flasks were kept on a shaker at 120 rpm rotation speed for 2 h. The final cells suspension was used for the permeabilization process and finally used as immobilized *E. coli* mutant cells in the fermentation with the partially purified waste glycerol as carbon source.

Permeabilization of *Escherichia coli* with N-cetyl-N, N, N-trimethyl ammonium bromide: Permeabilization of the cellular membrane without complete cell disintegration is useful to solve the low permeability problem of whole cells biocatalyst to a certain extent. Permeabilization of the microwave irradiation-induced mutant *E. coli* cells (after revival phase as mentioned), was performed according to Bagherinejad *et al.* (2012) using N-cetyl-N, N,N-trimethyl Bromide (CTAB). Approximately 100 mL of the revived *E. coli* cell suspension was added to a solution containing 0.1% (w/v) CTAB in 100 mL of phosphate buffer (pH 7.0, 0.1 M). The cells were incubated (45 min) in this solution at room temperature and occassionally stirred with a magnetic stirrer. At the end of the incubation, cells pellets were collected by centrifugation at 8000 rpm, for 10 min. The pellet was washed twice with phosphate buffer and the washed cells were store in vial, containing of 15 mL of phosphate buffer. The resulting suspension was used later for the immobilization on chitosan beads.

Immobilization of the mutant *E. coli* (permeabilized) on macroporous chitosan beads: Chitosan beads were prepared as reported elsewhere with some modification (Chandy and Sharma, 1993; Shu and Zhu, 2001; Wei *et al.*, 1992). Typically, about 3.0 g chitosan was dissolved in 100 mL of 2% (v/v) acetic acid. The viscous solution was well stirred at room temperature and left overnight before adding the solution to ensure all of the chitosan flakes were dissolved. The permeabilized cells which are were added to chitosan solution were mixed and stirred for 30 min to get a uniform mixture suspension. The solution was slowly dropped through a seven-gauge needle into 2.0 M sodium hydroxide solution and the gelled spheres formed instantaneously. This process was accomplished by using a model 100 push-pull Syringe pump. The formed chitosan beads coated cells were kept in the sodium hydroxide solution for 5 h and washed with distilled water until the washing solution became neutral. After filtering the beads were stored in distilled water for later use.

Cross-Linking of *E. coli* immobilized chitosan beads using glutaraldehyde and microwave irradiation: Cross-linked chitosan beads were prepared using glutaraldehyde as the cross-linking agents following a novel microwave irradiation method modified from the previously mentioned method (Wei *et al.*, 1992; Guo *et al.*, 2004). A solution containing 500 mL of 0.067 M sodium hydroxide (pH = 12) with 15 mL of 25% of glutaraldehyde was prepared. Freshly prepared 20 g of wet chitosan beads coated with *E. coli* cells were put into a flask solution and stirred slowly to allow proper mixing. The mixture was subjected to several (about 12) short burst of microwave irradiation using a microwave oven at frequency

of 2.45 GHz at power output of 200 W. Each short burst of irradiation lasted for 1 min. The mixture was cooled (50 sec at room temperature) between each irradiation. This ramp/cool cycle was repeated 12 times. After the 12th cycle (total time required about 22 min), the cross-linked beads were filtered and washed extensively with distilled water to remove any unreacted glutaraldehyde until the washing solution became neutral (the neutrality measured by universal indicator study).

Fermentations in batch bioreactor: Batch bioreactor fermentations were conducted in an anaerobically sealed a 3 L bioreactor (Bio Gene bioreactor fermenter model - Biogene T type, India), in which the working volume was 2.0 L. The reactor's glass chamber was autoclaved before the fermentation process. The fermentation media used in this experiment consist 1 L of 10X MOPS (3-N-Morpholino propanesulfonic acid) buffered minimal media (100 mL 10X MOPS concentrate; 1 mL 1.32 M K_2HPO_4; 5 mL 1.90 M, NH_4Cl; 1 mL 0.276 M, K_2SO_4; 2.5 mL 4 mg mL^{-1} thiamine) supplemented with 1% 0.132 mM N_2HPO_4 and 0.1% 1 μmol sodium selenite. The minimal media was filtered using 0.2 μm millipore filter. The media was also supplemented with 5 g L^{-1} yeast extract and 10 g L^{-1} tryptone. The partially purified glycerol (65%) was used as the sole carbon source. Glycerol solution containing 770 mL of partially purified glycerol (filtered with 0.2 μm millipore filter) made up to 1 L with ultra-pure water was added to the minimal media. The final volume of the fermentation media was 2 L and the concentration of glycerol was 250 g L^{-1}. In the previous study (Nomanbhay and Hussain, 2014), there was addition of enrich media (10 g L^{-1} Mueller Hinton Broth) hence, glycerol was a co-carbon source. However in this particular study no addition of enrich media was done; hence, glycerol was the only sole carbon source. One hundred grams of crossed-linked *E. coli* coated chitosan beads was added to the media. Agitation performed at 130±5 rpm, the incubation temperature was 38°C and pH was maintained at 6.5 by automatic controlled by addition of 5 M NaOH. To achieve anaerobic conditions the medium was purged with pure N_2 for 10 min before inoculation with *E. coli* coated chitosan beads and for 10 min after inoculation.

Analytical methods

Determination of cells concentration: Biomass was measured by measuring dry weight which was in turn correlated with optical density following the modified method reported by Chaudhary *et al.* (2011). One bead of chitosan coated cells was transferred into centrifuge tube containing of 1 mL of 2% (v/v) of acetic acid solution and then smashed until become solution form at pH = 5. The sample was collected and the optical density measured at 600 nm in spectrophotometer. Similarly, one bead of chitosan (pure; without cell coating) was also dissolved with acetic acid. This solution of dissolved pure chitosan bead was used to zero the spectrophotometer. After the OD measurement, both the samples were weighed out by using electronic analytical balance and the values were recorded. It was then dry in oven at 50°C for 36 h (or more) until constant weight was achieved. The difference in the dry mass of the two samples was recorded as the dry weight of cells and was correlated with the OD value.

Determination of glycerol concentration: The glycerol concentration was determine using glycerol assay kit (Megazyme, K-GCROL) by the absorbance the difference of (A_1-A_2) for both blank and sample. $\Delta A_{glycerol}$ was obtained by taking the absorbance differences of the blank from the absorbance of the sample. The concentration of glycerol can be calculated as follows:

$$C = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{glycerol} \ (g\ L^{-1})$$

Where:
V = Final volume of glycerol assay (mL)
MW = Molecular weight of glycerol (g mol^{-1})
ε = Extinction coefficient of NADH at 340 nm
= 6300 ($1 \times mol \times cm^{-1}$)
d = Light path (cm)
v = Sample volume of the fermentation broth (mL)

Determination of bioethanol concentration: The bioethanol concentration was determine using ethanol assay kit (Megazyme, K-ETOH) by the absorbance the difference of (A_2-A_1) for both blank and sample. $\Delta A_{ethanol}$ was obtained by taking the absorbance differences of the blank from the absorbance of the sample. The concentration of bioethanol can be calculated as follows:

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 2} \times \Delta A_{ethanol} \ (g\ L^{-1})$$

Where:
V = Final volume of ethanol assay (mL)
MW = Molecular weight of ethanol (g mol^{-1})
ε = Extinction coefficient of NADH at 340 nm
= 6300 ($1 \times mol \times cm^{-1}$)
d = Light path (cm)
v = Sample volume of the fermentation broth (mL)
2 = 2 moles of NADH produced for each mole of ethanol

Fourier Transformed Infrared (FTIR) spectra measurement: Chitosan, chitosan coated with permeabilized cells of *E. coli* and chitosan cross-linked with glutaraldehyde samples were examined by FTIR spectrophotometer. The FTIR spectra measurements were performed using the Shimadzu FTIR spectrophotometer-model IRPrestige-21 (Shimadzu Corparation Japan) equipped with temperature controlled DLATGS (deuterated, L-alanine doped triglycine sulfate) detector. The scan settings were set as follows; resolution: 8 cm^{-1}, accumulation: 20 scans, measurement

mode: transmittance (T%), wave number 4000-650 cm^{-1}. In a typical analysis, sample was placed on the surface of the horizontal Attenuated Total Reflectance (ATR) crystal disc (Diamond Type II crystal) at controlled ambient temperature (23°C). A background measurement of air spectrum was performed. Spectra were processed using IR solution-window based software version 1.4 (Shimadzu). After every scan, a new reference air background spectrum was taken. After trialing, the sample was removed and the surface of crystal disc was washed with acetone and finally it was dried and cleaned with tissue.

Surface morphological characterization: The surface morphology of the chitosan beads and glutaraldehyde cross-linked chitosan were examined using (Phillips XL-20, Japan) with operating voltage 10 kV. The beads were taken after coating the surfaces of bead samples with a thin layer of gold by using Sputter Coater under air atmosphere. Magnifications at 300 X were applied to each sample in order to estimate the surface morphology of the bead. The SEM was performed in National Nanotechnology Directorate Division (NND), Technology Park Malaysia (TPM), (Kuala Lumpur, Malaysia).

RESULTS AND DISCUSSION

Effect of microwave irradiation power and exposure time on *E. coli* strains: Fermentative utilization of glycerol by *Escherichia coli* for the production of biofuels has been getting a lot of attention in recent years. *Escherichia coli* is generally considered an easy and safe species to work with for various industrial processes. Wild-type *E. coli* cannot grow anaerobically on glycerol, because the redox potential is imbalanced with accumulation of high levels of NADH (Murarka *et al*., 2008). The *E. coli* is also not efficient in converting glycerol to bioethanol due to the existence of many inefficient pathways. In this study and also in the previous reported work (Nomanbhay and Hussain, 2014), normal wild-type strain of *E. coli*, which is known as a poor microbe for bioethanol production from glycerol (as sole carbon source) was subjected to short microwave (MW) irradiation treatment (1 min) at a frequency of 2.45 GHz and output power was at 180 W in cyclic manner (total 15 cycles) in order to produce mutant *E. coli* with good bioethanol production yield (Nomanbhay and Hussain, 2014). Duration of MW exposure seems to be a major determinant of MW effect on living cell. The time of exposure and power density are correlated in a way that decrease in power density could be compensated by increase in duration of exposure. From present study and also previous report (Shamis *et al*., 2011), it was shown that the specific MW effect was not bactericidal under these experimental conditions. Accordingly, there is a possible non-thermal effect involved with the microwave irradiation. The non-thermal effect by the microwave irradiation could arise from the interference of cell metabolic activities and energy absorption and DNA/RNA molecule rotation in response to the microwave. The approach for detecting MW-induced conformational changes of *E. coli* was by means of Attenuated Total Reflection Fourier-Transform Infrared (ATR-FTIR). Figure 1 (a and b) shows the results obtained from the previous study on FTIR absorption spectra of *E. coli* and its modified mutant strain with bands at 2000-800 cm^{-1} (Nomanbhay and Hussain, 2014). The MW irradiation at (2.45 GHz; 180 W) for 5 min pulse, had induced some changes in the *E. coli* strains. The typical signals of the polysaccharides were exhibited at 1650 cm^{-1}. The MW radiated *E. coli* showed distinct strong signal stretching at 1650, 1400, 1250 and 1100 cm^{-1}. The FTIR spectra of the MW induced *E. coli* showed that the stretching vibration band at 1650 cm^{-1} was narrowed compared to the wild type *E. coli*. The main functional groups of mutant *E. coli*, including the C = O stretching at 1650 cm^{-1}, δ O-H bending at 1400 cm^{-1}, δ O-H bending at 1250 cm^{-1}. The band at 940 cm^{-1} corresponds to the pyranose units of the polysaccharide and proves that the cyclic pyranosyl rings were not destroyed by microwave radiation. As low intensity MW are believed not to possess sufficient energy for breaking chemical bonds directly; the effect vibrational energy generate from microwave irradiation, penetrate into intercellular compounds to cause the mutation without destruction to the total structure of the cell (Nomanbhay and Hussain, 2014).

Permeabilization of mutant *E. coli*: The bacteria *E. coli* have been chosen because of their industrial and/or fermentation importance. Most bacteria have a rigid cell wall. The cell wall is an essential structure that protects the cell protoplast from mechanical damage and from osmotic rupture or lysis. The cell wall is made of peptidoglycan. All gram-negative bacteria (such as *E. coli*) possess an additional membrane known as Outer Membrane (OM). This membrane lies over and covers both the cytoplasmic membrane and the cell wall. It functions mainly as a protective layer to prevent entry of toxic substances into the cell. The outer membrane has an asymmetric distribution of lipids with lipopolysaccharide located in the outer surface of the membrane. The outer membrane forms a proficient barrier against hydrophilic macromolecules and hydrophobic substances due to a lipopolysaccharide layer on its surface (Kumar and Pundle, 2009). Thus, outer membrane resists the movement of substrate and product by imposing limits on diffusion, however, this resistant can be circumvented by permeabilizing the cells wall with certain types of treatment (Canovas *et al*., 2005). It has been shown that permeabilization can disrupt the integrity of the outer membrane resulting in loss of the barrier function but lacking direct bactericidal activity (Helander and Mattila-Sandholm, 2000). Cell permeabilization is needed to overcome the permeability barrier of the outer membrane to substrates and products of enzymatic reactions. The method used to permeable the *E. coli* cells was treatment with N-cetyl-N,N,N-trimethyl Bromide (CTAB); which is a tertiary ammonium compound capable of dissolving proteins ant fatty acids in outer membrane and inner membrane and make them porous.

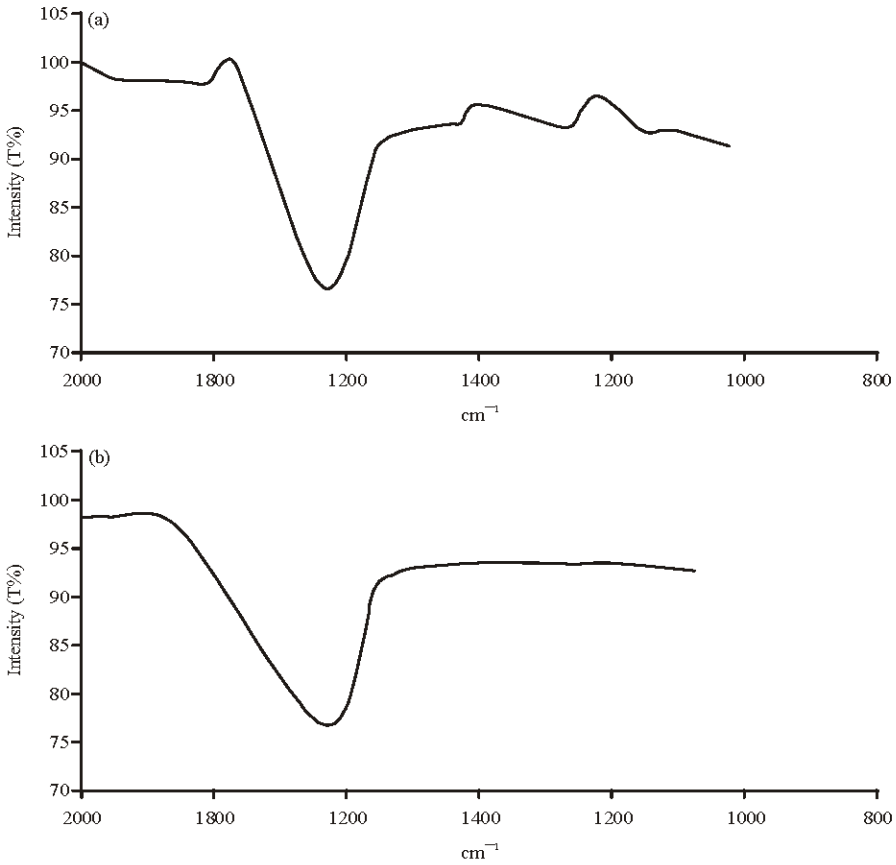

Fig. 1(a-b): Representative FTIR spectra of *E. coli* (a) Mutant type (MW irradiated) and (b) Wild type (non-MW) as reported by Nomanbhay and Hussain (2014)

FTIR spectra can be used as a rapid detection and identification method of any changes in the biochemical structure and composition of the cellular constituents that include water, fatty acids, proteins, polysaccharides and nucleic acids of microbial cells. Figure 2, shows the FT-IR spectroscopy results of *E. coli* cells permeability based on the optimal concentration of 0.1% (w/v) of CTAB. The chemical permeabilization treatments were performed with CTAB (b) and compared with the intact cells (a). According to Abraham and Bhat (2008), the concentration of detergent plays an important role in determining whether the cell is being permeabilised during the treatment with detergent. Moreover, differential affinity of the detergent to the outer membrane could affect the permeabilization process.

The FTIR spectrum (Fig. 2a) of intact bacteria outer membrane exhibited major broad bands at around 3350 cm^{-1} (N-H and O-H stretching vibration of polysaccharides and proteins). The band at around 1750 cm^{-1} denotes the C = O of ester group stretching for lipids and triglycerides. The amide 1 band due to C = O stretching of amide groups of protein appears at around 1660 cm^{-1}. The band around 1400 cm^{-1} is assigned to C = O symmetric stretching of COO-group in amino acids, fatty acids and P = O asymmetric stretching of phosphodiesterase in phospholipids (around 1320 cm^{-1}). After the treatment with CTAB, the FTIR spectrum of the cells showed relatively minor compositional and structural differences in bacterial outer membrane and composition. These suggest that there have been no major conformational changes and/or changes in composition or bonding of biomolecules, which is apparent from only minor change in stretching or vibration signal pattern. The notable difference was the absorption bands from stretching of PO_2^- double bonds which was the region around 1330-1220 cm^{-1}. The decreasing intensity of this region in the CTAB treated *E. coli*, which demonstrated that the phosphoryl group in lipopolysaccharide of the outer membrane participated in the interaction with $-NH_3^+$ of CTAB. Cationic detergent mainly acts on the lipopolysaccharide layer of the bacterial outer membrane, causing changes in protein conformation and forming a channel in the membrane, thereby cell permeability increases (Nagalakshmi and Pai, 1994; Felix, 1982). When cells are permeabilized with an appropriate detergent, their cytoplasmatic membrane becomes highly permeable, allowing small compounds such as metallic ions and cofactors to diffuse out of the cells while keeping the macromolecules inside (Fonseca and Cabral, 2002). Similarly, it was reported that when the barrier permeability of the outer membrane is altered, the intra/extracellular mobility increases and allows access to

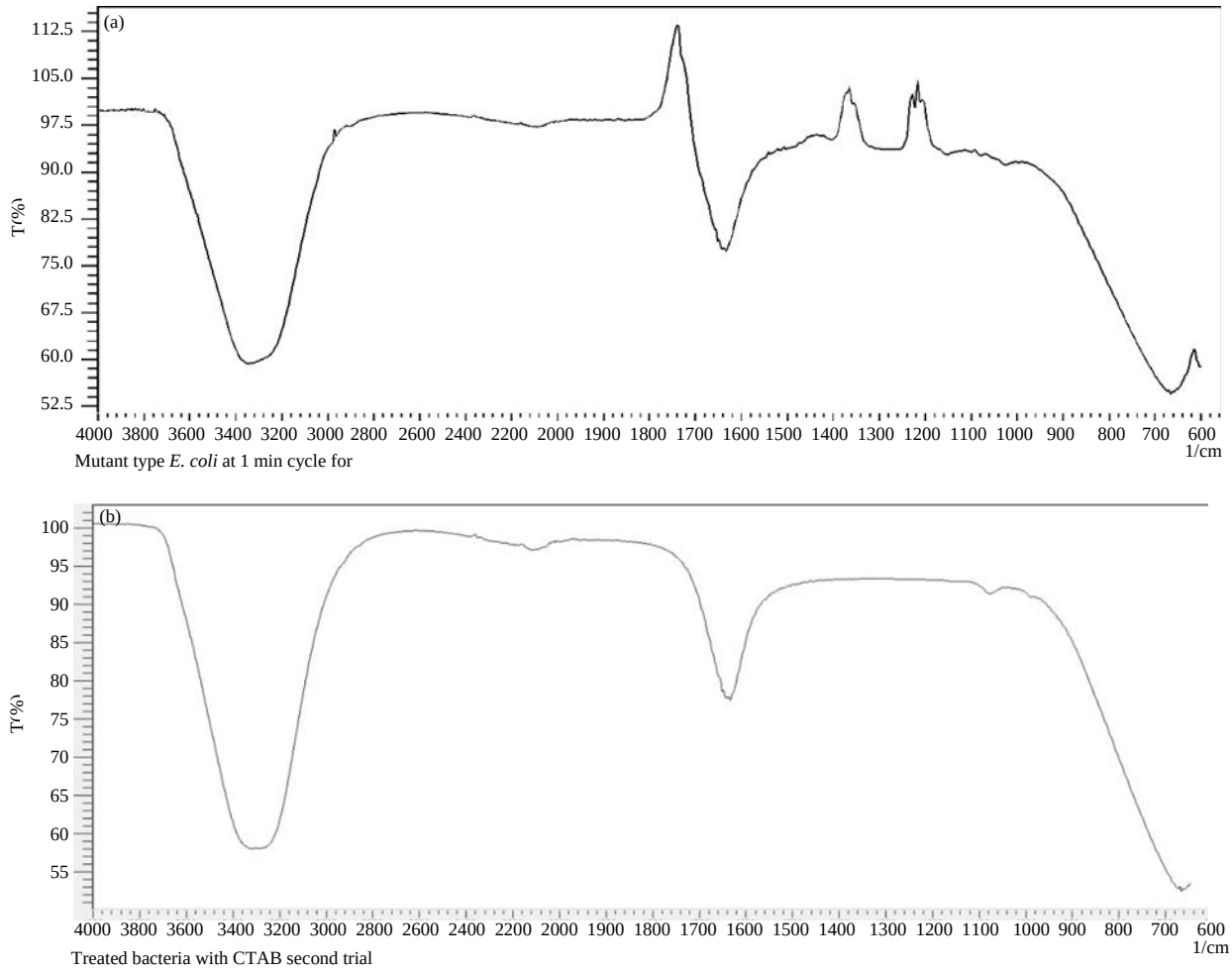

Fig. 2(a-b): FTIR spectrum for (a) Untreated *E. coli* and (b) Treated *E. coli* with CTAB, respectively

a large numbers of intracellular enzymes (Abraham and Bhat, 2008; Doolan and Wilkinson, 2009). Hence, the enzymes retained by the cellular wall may be easily accessed by substrates.

Characteristics of chitosan coated bacteria cells and cross-linked chitosan coated bacteria cells

Scanning electron microscopy analysis: Materials with pores can be divided into macro porous (pore diameter >0.05 μm), meso porous (2 nm<pore diameter<0.05 μm) and micro porous (pore diameter <2 nm). In this study, chitosan beads were prepared by phase inversion. Uniform beads of particle size (2.25±0.05 mm) of the macro porous chitosan beads were obtained. Figure 1 depicted the surface morphologies of the immobilization of the mutant *E. coli* attached on (a) chitosan beads and (b) cross-linking chitosan (with glutaraldehyde) at 300x magnifications obtained from the SEM analysis. Pure chitosan beads covered with cells showed relatively regular shape, homogenous and opaque with some wrinkles and fissures. After reinforcing the structure with glutaraldehyde, the chitosan beads revealed a texture modification. From the images (Fig. 3) of the crossed-linked chitosan it can be seen that the surfaces of the beads are tighter, fluffy and smoother then the non-crossed-linked chitosan. Thus, it is obviously that the surface of the cross-linked chitosan becomes less porous, darker, decreasing in the size of macro spheres and with good structural integrity due to greater cross-linking of the pores. The influence of glutaraldehyde (cross-linking agent) caused stiffening of the polymeric matrix.

Chitosan is highly hydrated in an aqueous environment. When glutaraldehyde is exposed to chitosan, the amino groups of chitosan develop imine bonds with the aldehyde groups of glutaraldehyde (Dini *et al*., 2003). Chitosan beads formulated with a glutaraldehyde develop a greater number of covalent bonds, thus the polymeric matrix becomes stiffer. Chitosan are easily soluble in dilute acetic or formic acid solutions to yield a hydrogel in water. Therefore, the crosslinking treatment of chitosan reinforces its chemical stability in organic acidic media and enhances the physical strength of the chitosan beads. The cross-linking treatment increases the rigidity and also the chemical stability of the beads.

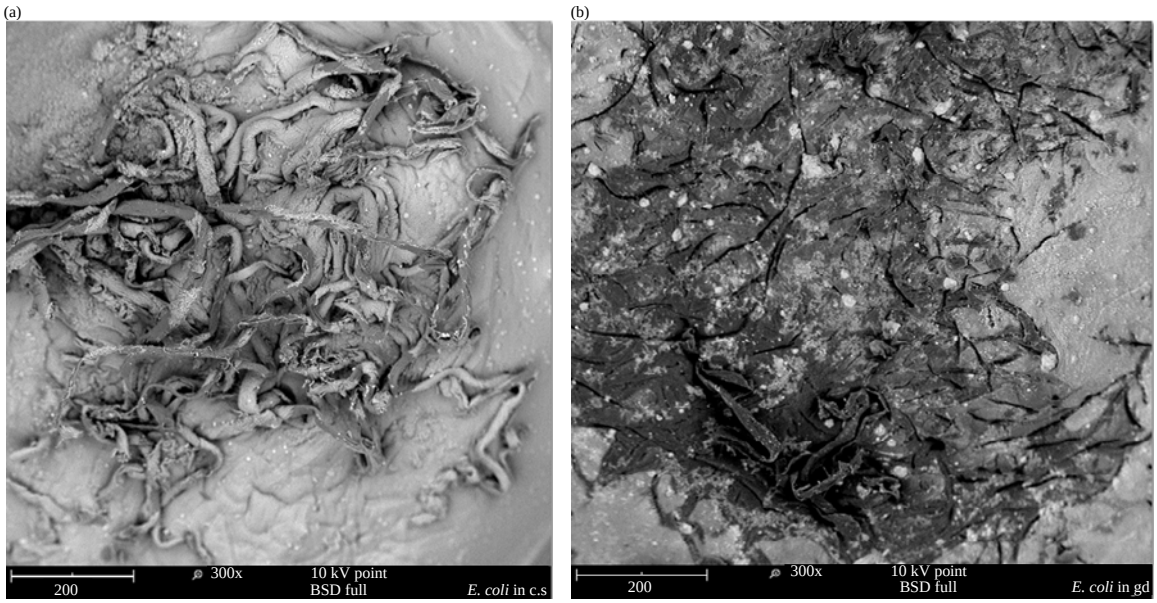

Fig. 3(a-b): SEM images of chitosan coated (a) Mutant bacteria cells and (b) Cross-linked chitosan coated with mutant bacterial cells (b) at 300x magnifications

Fourier transform infrared spectra for chitosan and cross-linked chitosan beads: The FT-IR spectra of (a) Non-cross linked chitosan and (b) Cross linked chitosan beads, in the range of 4000-600 cm^{-1} is presented in Fig. 4. The FTIR study was conducted to monitor the chemical modifications structure on chitosan coated cells, after cross linking with 25% of glutaraldehyde. The major peaks for chitosan are around: The broad peak at 3348-3287 cm^{-1} was assigned to the (N-H and O-H stretching vibration). The peak at 2959-2928 cm^{-1} was ascribed to (CH_3 symmetric stretch). The peaks at 1636 cm^{-1} and 1582 cm^{-1} indicated the (C = O stretching vibration). The peak at 1485 cm^{-1} was attributed to (C-N stretching vibration), (CH_3 bending vibration) was illustrated by the band at 1385-1361 cm^{-1}. Furthermore, the peak at 1261 cm^{-1} corresponded to the C-O group of chitosan. The broad peak at 1150 cm^{-1} indicated (C-O-C bending vibration). Chitosan showed a saccharide structure due to the presence of peaks at 1065, 1034 cm^{-1}.

Similar observations were reported by Dhawade and Jagtap (2012). However, notable characteristic changes due to of the alteration of chemical groups, have been observed in the chitosan spectrum after modification with cross linking. The narrowing of the band at wavelength 1767 cm^{-1} is seen clearly in chitosan modify by the cross linking. The C = O stretching vibration at 1636 cm^{-1} remained same in modify and unmodified chitosan structure. The C-N stretching vibration at 1485 cm^{-1} shifts to 1416 cm^{-1}, (CH_3 bending vibration) at 1362 cm^{-1} shifts to 1323 cm^{-1}, the peak at 1261 cm^{-1} corresponded to the C-O group of chitosan, shifts to 1258 cm^{-1}. The broad peaks at 1150 and 1034 cm^{-1} indicated (C-O-C bending vibration), polysaccharide structure, respectively, continue to appear in chitosan crosslinking with less sharper peaks, which indicate that the peaks, has less flexibility to respond to the external energy. The band at 1582 cm^{-1} (amide II) was found in FITR of chitosan crosslinking, but not in FITR of chitosan (Fig. 4). The result from this study is consistent with many previous studies (Mitra *et al*., 2012; Jabli *et al*., 2012; Yao *et al*., 2012) which have demonstrated that there was a difference in FITR profile between chitosan and chitosan-crosslinking with glutaraldehyde.

The appearance of the peak at 1582 cm^{-1} can be attributed to the crosslinking reaction of chitosan and glutaraldehyde. Ramachandran *et al*. (2011) found that the new sharp peak at 1610 cm^{-1} represents stretching vibrations of C = N in Schiff's base formed by the reaction of glutaraldehyde and chitosan. Crosslinking of chitosan with glutaraldehyde has been studied by several authors (Kildeeva *et al*., 2009; Gupta and Jabrail, 2006a; Beppu *et al*., 2007; Ruiz *et al*., 2000). It is suggested that the crosslinking mechanism is based on the formation of imine bonds between the amine groups of chitosan and the aldehyde groups of glutaraldehyde (Ngah *et al*., 2002). It results in the appearance of a new band which corresponds to the C = N vibrations of the Sciff base (Zielinska *et al*., 2011). Furthermore, Knaul *et al*. (1999) found that the chitosan film that reacted with glutaraldehyde exhibits a strong absorbance at 1664 cm^{-1}, while Gupta and Jabrail (2006b) reported that the IR spectra have shown a strong absorption band at 1660 cm^{-1} in the glutaraldehyde cross-linked chitosan microsphere. In addition, Monteiro and Airoldi (1999), revealed that the increase of glutaraldehyde in the sequence of these modified chitosan caused a successive increase in

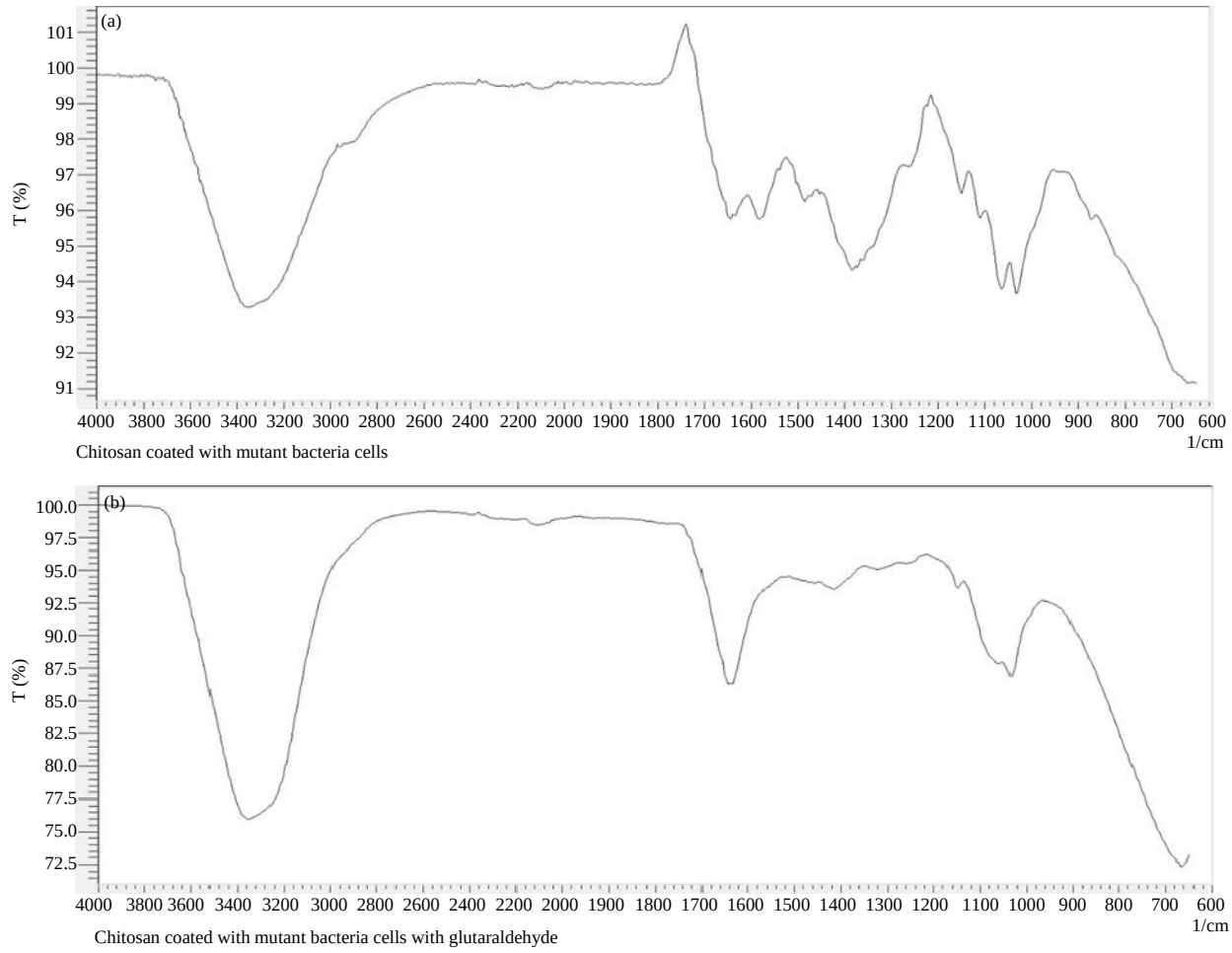

Fig. 4(a-b): (a) Chitosan beads and (b) Cross-linked chitosan beads coated with permeabilized mutant bacteria cells, respectively

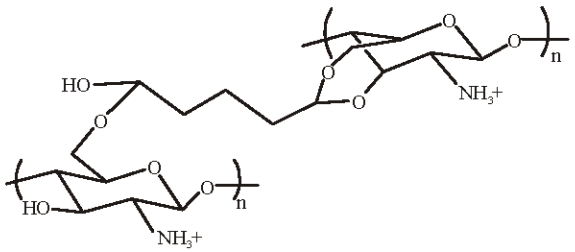

Fig. 5: Schematic drawing of chemical structure of cross linked chitosan (Xiong *et al*., 2009)

intensity of ethylenic bond frequency at 1562 cm^{-1}. The result from this study indicated that the amide II band at 1582 cm^{-1} that may contribute to the amine-NH_2 group was specific for chitosan crosslinking. The change in the characteristic shape of the chitosan spectrum after cross-linking at 1560 cm^{-1} appears to be stretching vibration spectra of the amide group of chitosan (Tripathi *et al*., 2009). Zhang *et al*. (2003) reported that the intensity at 1557.19 cm^{-1} reveals the quaternary-amino band which is due to the positive charge NH_2^+ after crosslinking with glutaraldehyde. Thus, the positive charge NH_3^+, is expected to interact with gram-negative bacterial surface that predominantly consisted of anionic components, such as lipopolysaccharides, phospholipids and lipoproteins. Indeed, previous studies have indicated that this charge interaction could disrupt the organization of the outer membrane in bacteria and increase its permeability (Lou *et al*., 2011; Li *et al*., 2008). Additionally, Chan *et al*. (2013) attributed that the peak at 897 cm^{-1} was related to the C-H bending vibration. The schematic drawing of chemical structure of cross linked chitosan is shown in Fig. 5 (Xiong *et al*., 2009).

Comparison of the bioethanol production by immobilized and suspended-free cells: Glycerol is a good carbon and energy source for many microorganisms and therefore, can be an invaluable feedstock for industrial fermentations. The

maximum theoretical yield of ethanol and hydrogen (or formate) from glycerol dissimilation is 1 mol each of bioethanol and hydrogen (or formic acid) per mol of glycerol utilized (Nwachukwu *et al*., 2012):

$$C_3H_8O_3\ C_2H_5OH \text{ or}$$

$$C_3H_8O_3\ C_2H_5OH$$

This means that 50% glycerol is theoretically converted to bioethanol. As presented in our previous investigation (Nomanbhay and Hussain, 2014), the mutant *E. coli* have been considered as an appropriate strain for raw glycerol conversion into a bioethanol product. The biotechnological production of bioethanol using glycerol as substrate has mostly been reported for cultures using suspended free cells. To perform a more economical and environmentally friendlier fermentation process, the performance of immobilized mutant *E. coli,* was compared to that of the free mutant *E. coli* in two different batch bioreactors, under anaerobic conditions, using partial purified glycerol as sole carbon source. A typical profile for bioethanol, 1, 3 propenediol (1, 3 PDO) and dry weight biomass produced during fermentation of glycerol by free *E. coli* is shown in Fig. 6a. With free mutant *E. coli* cells; there was a progressive formation of bioethanol, reaching the highest concentration, which was 39.5 g L^{-1} ethanol, with the productivity 0.33 g L^{-1} h. The production of 1, 3 PDO was slow with maximum amount of production at 13 g L^{-1} after 120 h. The production of bioethanol is lower compared to our previous result because in this study there was no addition of other carbon source (only glycerol) whereas previously (Nomanbhay and Hussain, 2014) the glycerol was supplemented with Mueller Hinton Broth enrich media (10 g L^{-1}) as co-substrate. The present study validates the ability of the strain to tolerate with highly elevated amounts of raw glycerol, since satisfactory cell growth was observed even at cultures with 250 g L^{-1} partially purified glycerol as sole carbon source. It also suggests that any impurities found into the partially purified waste glycerol (by-product from biodiesel production) did not have negative effect upon the microbial metabolism. Likewise, the ability of the mutant *E. coli* strain, to have noticeable growth at elevated concentrations glycerol, is indeed an important point to note as it will minimize the reactor size and running cost during industrial applications using crude glycerol from biodiesel processing plants.

Remarkable differences were detected in terms of bioethanol, cell growth and final 1,3 PDO production when immobilized mutant cells of *E. coli* were used, suggesting that immobilized cells fermentation by *Escherichia coli*, could be feasible for potential scale-up commercial production. An initial amount of glycerol (250 g L^{-1}) was converted to significant quantities of the end-products, predominantly bioethanol, while low quantities of 1,3 PDO was observed (96.7 and 5.2 g L^{-1} respectively) after 100 h of fermentation (Fig. 6b). This result indicated that substrate inhibition did not occur in the bioethanol production and that the *E. coli* mutant strain was able to achieve conversion to bioethanol up to around 77% of the theoretical yields. The productivity of bioethanol production was 0.967 g L^{-1} h. Table 1 summaries the bioethanol yield obtained in this study.

Table 1: Bioethanol production from partially purified crude glycerol (250 g L^{-1})

Fermentation parameters	Values
Bioethanol yield (mole bioethanol/mole glycerol)	0.77:1
Productivity of bioethanol from Immobilized cells (g L^{-1} h)	0.967
Productivity of bioethanol from free suspended cells (g L^{-1} h)	0.33

Compared to the other studies, this study showed good production of bioethanol. Substrate concentrations below 50 g L^{-1} were used in the majority of previous studies, in contrast to the relatively high substrate concentration used in our study. In addition, the mutant *E. coli* was able to produce nearly 96.7 g L^{-1} of bioethanol, corresponding to a yield of 0.77 mol/mol. The ability of the new mutant *E. coli* strain to convert glycerol to bioethanol at effectiveness of up to 0.77 mol bioethanol/mol glycerol, in high glycerol containing medium, is potentially of great value to biofuels industry. Nwachukwu *et al.* (2012), reported that *E. aerogenes* was able to utilize high amounts of glycerol and effectively convert same to bioethanol (mutant strain converted 30 g L^{-1} glycerol into 16 g L^{-1} bioethanol in 48 h). Yang *et al.* (2007) utilized high glycerol concentrations (60 g L^{-1}); however, the bioethanol yield was relatively low and the microbe used was genetically modified. Dharmadi *et al.* (2006) reported that within 84 h, 80% of the glycerol initially present in the medium was consumed. This result is different from the results obtained by Zhu *et al.* (2002) who reported that high levels of glycerol significantly inhibit cell growth. This difference may have occurred due to the different strains of *E. coli* used. Besides these reports, majority of other studies have reported good yield of bioethanol production but using other microbes. The maximum concentration of bioethanol production from glycerol obtained thus far has been reported by a newly isolated *Kluyvera cryocrescens* strain, with bioethanol quantity achieved at around 27 g L^{-1}, with productivity value of 0.61 g L^{-1} h^{-1} (Choi *et al*., 2011). Recently Rossi *et al.* (2012) isolated a strain of *K. pneumoniae* able to convert raw glycerol to 6.1 g L^{-1} of bioethanol, under anaerobic conditions, while Metsovitia *et al.* (2012) in their investigation have demonstrated a newly isolated *Citrobacter freundii* strain that produced about 15 g L^{-1} of bioethanol from waste glycerol.

The data presented in this study demonstrate that batch bioethanol production from partial treated glycerol using immobilized mutant *E. coli* cells entrapped in chitosan cross linking with glutaraldehyde is promising. While the majority of the reports implicated usage of genetically modified bacterial strains to improve the bioethanol production, this study has shown a new mutant *E. coli* produced by simple microwave irradiation that has good potential for bioethanol production from glycerol. To the best of knowledge of this study gives among the highest bioethanol production yield reported by *E. coli* strains utilizing raw glycerol as carbon

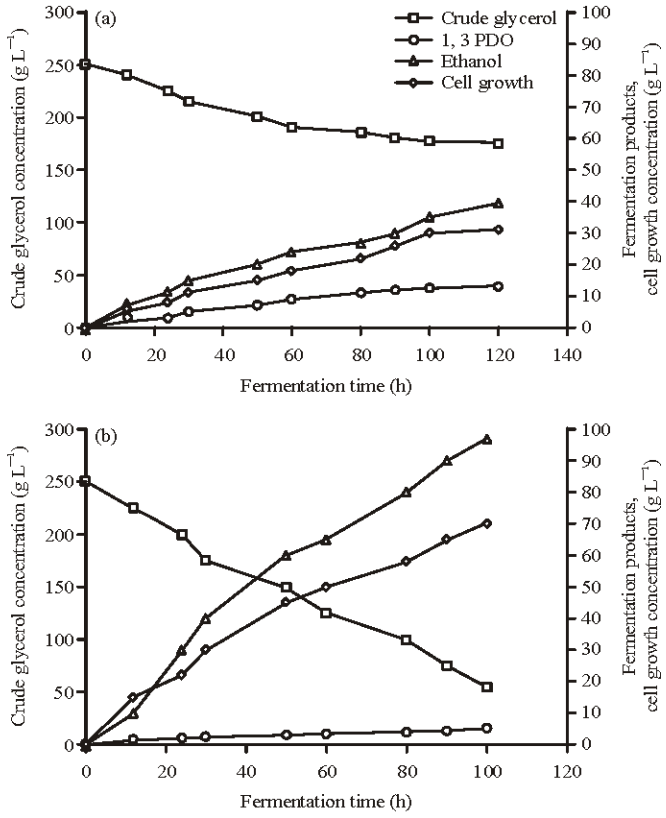

Fig. 6(a-b): (a) Bioethanol production by free cells and (b) Immobilized of *Escherichia coli*, respectively

source. Although others have reported higher yield of bioethanol (above 0.92 mol), the amount of glycerol used was not more than 60 g L^{-1} (Nwachukwu *et al*., 2012; Adnan *et al*., 2014). Hence, the slight drop in yield this study was still acceptable since very high glycerol concentration used. These results show that for upscaling of the process (250 g L^{-1} glycerol), efficiency will be affected to a certain extent but immobilization will be able to minimize the decrease in efficiency. Compared to the batch free fermentation cells, higher bioethanol productivity (2.9-fold) was obtained by fermentation with the immobilize cells. The robust performance of the immobilized cells in the cross-linking chitosan beads during batch bioethanol fermentation, was clearly evident from the higher yield obtained. In addition, the immobilized cells strain has ability to attain a faster conversion, more resistant to high levels of bioethanol produced in the fermentation process when compared to the free strain. The result also indicates higher tolerance of immobilized cells (as compared to free cells) toward inhibition at high initial glycerol concentration.

It has been pointed out that the use of immobilized cell systems increases bioprocesses productivities due to the effects of high cell densities and cell protection against environmental stresses. According to Hamdy *et al*. (1990) and Lee *et al*. (1983) immobilization increases the biological stability of cells against environmental variations of pH, temperature, nutrient concentration and reduces the effects of products toxicity, increasing the fermentation efficiency. Immobilization provides high cell concentrations and cell reuse. According to Kapu *et al*. (2013), high cell density can provide effective fermentation at high substrate concentrations. A 77% bioethanol yield was achieved using yeast strain LYCC 6469 after 48 h at high cell density. Meanwhile, Kleman and Strohl (1992), hypothesized that high cell densities could enhance the formation of important products. They found that a high cell density improved bioethanol production even at high glycerol concentrations. Other benefit of immobilized cells is that it eliminates washout problems at high dilution rates and the costly processes of cell recovery and cell recycle. Mariam *et al*. (2009) stated that the immobilized yeast cells were able to last for re-use in the consecutive six batches. However, the bioethanol production decreased significantly after four batches due to gel beads becoming fragile and deformed in shape. Similarly, in this study, the bioethanol production is expected to decrease after a few (4th onwards) consecutive batches although no analysis was done in this regard. Repeated batch fermentation by using immobilized cells has advantage of improving bioethanol productivity reducing the time of inoculum preparation (Carvalho *et al*., 1993; Kourkoutas *et al*., 2004; Ikram-ul-Haq *et al*., 2005). High volumetric productivities can also be obtained with the combination of high cell concentrations and high flow rates. Immobilization may also improve genetic stability (Nichols *et al*., 2005).

CONCLUSION

High yield and productivity of the desired product are the major objectives for optimizing microorganisms used in an industrial scale bioprocess. There is considerable interest in using crude glycerol for fine chemical production due to its many advantages, including low price and ample supply. The present study shows the utilization of non-conventional microwave irradiation method as a clean and green approach toward the development of modified *E. coli* strains to maximize bioethanol production from glycerol. The present study has shown that high yields of bioethanol can be achieved by using a simple microwave technique to produce mutant *E. coli*. To further improve the fermentation of bioethanol, the immobilized cell technique was applied. The result indicated that biofuel production was increased significantly by using immobilized mutant *E. coli* cells under high glycerol concentration. Glycerol was successfully converted to bioethanol with production of up to 77% of the theoretical yield. In this study, pretreated crude glycerol was proved as a suitable carbon source for bioethanol production. The maximum yield of bioethanol (96.7 g L^{-1}) was achieved after 100 h with immobilized mutant *Escherichia coli* strain cultivation. Lower production of bioethanol (39.5 g L^{-1}) was observed after 120 h by free mutant *E. coli* cells. This study has successfully demonstrated the feasibility of bioethanol production by mutant *E. coli* using high glycerol concentrations without any substrate inhibition. Future study is required for the possibility of cell reutilization and optimization of other parameters. The bioethanol yield obtained in this study is among the best in terms of the yield obtained at high glycerol concentrations. Thus, this mutant *E. coli* strain produced in this study is considered as a potential glycerol-fermenting bacterium for the bioethanol industry as glycerol is a cheap and abundant resource derived from the biodiesel industry. The results from this study provide further references when applying microwave irradiation to alter the enzymes activities in the bacteria strain and hence the phenomenon of non-thermal microwave effect could be appreciated clearly.

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