Chemosphere 152 (2016) 166–172

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

The synergetic effect of starch and alpha amylase on the biodegradation of n-alkanes

M. Karimi, D. Biria*

Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, Hezar Jarib Ave., Isfahan, Iran

HIGHLIGHTS

- Addition of starch enhanced the biodegradation efficiency of hydrocarbons.
- Alpha amylase can degrade alkanes in a microorganism free system.
- Negative binding energies resulted from in silico docking of enzyme with normal alkanes.

ARTICLE INFO

Article history:
Received 6 June 2015
Received in revised form
22 February 2016
Accepted 28 February 2016
Available online 9 March 2016

Handling Editor: Chang-Ping Yu

Keywords:
Biodegradation
n-Alkanes
Starch
Alpha amylase
Cometabolism

ABSTRACT

The impact of adding soluble starch on biodegradation of n-alkanes (C_{10}–C_{14}) by *Bacillus subtilis* TB1 was investigated. Gas chromatography was employed to measure the residual hydrocarbons in the system. It was observed that the efficiency of biodegradation improved with the presence of starch and the obtained residual hydrocarbons in the system were 53% less than the samples without starch. The produced bacterial enzymes were studied through electrophoresis and reverse zymography for explaining the observations. The results indicated that the produced amylase by the bacteria can degrade hydrocarbons and the same was obtained by the application of a commercial alpha amylase sample. In addition, in silico docking of alpha-amylase with n-alkanes with different molecular weights was studied by Molegro virtual docker which showed high negative binding energies and further substantiated the experimental observations. Overall, the findings confirmed the catalytic effect of alpha amylase on n-alkanes degradation.

1. Introduction

Among various environmental contaminations, organic pollutants especially petroleum hydrocarbons are of great importance. The activities of the petroleum related industries, pipelines and reservoirs leakages as well as inevitable transport accidents are the main causes of the hard to clean hydrocarbon contaminations of soil and water (Das and Chandran, 2011). The research conducted over the past fifty years has supplied comprehensive information on biodegradation of hydrocarbons by a plenty of bacteria, yeasts and fungi capable of growing on these organic compounds (Wentzel et al., 2007). Although a large number of microorganisms which can potentially grow on hydrocarbons have been suggested, a certain microorganism alone has not the required potency to degrade all or even the majority of organic substances in polluted sites so much so some of these materials will remain intact (Rehm and Reed, 2008). In addition, the hydrocarbons biodegradation process is usually slow and incompetent because of several unfavorable factors such as low water solubility of hydrocarbons, their high concentration toxicity, inaccessibility to enzymes and insufficient enzymes activity. In fact, the main target of engineering strategies for improving bioremediation of oil pollutants is to overcome the factors causing tenacity (Rittmann and McCarty, 2001).

In recent years, there has been an increasing amount of literature on the application of surfactants, optimization of culturing media, use of a consortium of microorganisms, stimulation of indigenous microorganisms and design of special bioreactors and contactors to improve the efficiency of biodegradation (Huang et al., 2013; Tomei and Daugulis, 2013; Barin et al., 2014; Suja et al., 2014; Lan et al., 2015; Montagnolli et al., 2015). Among the suggested improvements for contamination bio-removal,

* Corresponding author.
E-mail address: d.biria@ast.ui.ac.ir (D. Biria).

http://dx.doi.org/10.1016/j.chemosphere.2016.02.120
0045-6535/

cometabolic bioremediation can be used for treating a few recalcitrant compounds. Cometabolic bioremediation can be defined as the biotransformation of an organic compound (pollutants) without nutritional profit in the presence of another substrate as the energy and carbon source. For example, biodegradation of polycyclic aromatic hydrocarbons (PAH) through cometabolism has been frequently reported (Hadibarata and Kristanti, 2012; Nzila, 2013). Moreover, it has been successfully applied to several sites such as the bioremediation of chlorinated hydrocarbons at Moffett Federal Airfield in California. In this case, methane, ammonia, toluene and phenol were used as the induced substrates to biodegrade dichloroethylene (DCE), trichloroethylene (TCE) and vinyl chloride as cosubsrates (Lee et al., 2006).

Indubitably, alkanes are one of the main fractions of the crude oil composed of a mixture of components including linear, branched or cyclic saturated hydrocarbons most of which can be degraded by microorganisms. However, their biodegradability decreases with molecular weight increase. In fact, the high molecular weight linear alkanes have a similar structure to polymers like poly ethylene which are too hard to decompose in the environment. One of the successful methods in accelerating the biodegradation of such polymers is the addition of natural polymers such as aliphatic polyesters to the synthetic polymer matrix (Zuchowska et al., 1999 and Nakamura et al., 2005; Ammala et al., 2011). For instance, it has been frequently reported that the addition of starch to poly ethylene can make it more susceptible to biodegradation. Accordingly, the present study was inspired by the structural resemblance between polyolefins and alkanes (linear and branched) and the positive effect of the presence of natural polymers (e.g. starch) on polymers biodegradation. In fact, the main objective was to investigate if the addition of starch had any positive impact (e.g. cometabolic biodegradation) on alkanes bioremoval from the system.

2. Materials and methods

2.1. Chemicals

Triton X-100 was from Applichem and $(NH_4)_2SO_4$ from Carlo-Erba. Glycine was supplied by Dae-Jung, Nutrient agar by Gentaur, n-paraffin by Iran Chemical Industrial Investment Company and Tris–HCl by Loba-Chemie. Materials like Acetic acid, Acryl amide, bis-acryl amide, $CaCl_2$,Coomassie blue G 250 and R 250, H_3PO_4, I_2, K_2HPO_4, KI, Maltose, Methanol, $MgCl_2$, Mg SO_4, Na H_2 PO_4, NaOH, Na_2HPO_4, Na_2 SO_4, Na_3PO_4, $(NH_4)_2S_2O_8$, Peptone, Sodium dodecyl sulfate (SDS) and Starch were obtained from Merck. Alpha-Amylase Liquozyme SC DS was Novozymes production. Substances such as agar, bovine serum albumin (BSA), Chloroform, dialysis bag, 3, 5-Dinitrosalicylic acid (DNS), $FeCl_3.6H_2O$,KCl, KH_2PO_4,KNO_3, Potassium sodium tartrate tetra hydrate, 3′,3″,5′,5″-tetra bromophenol sulfonphthalein, Tetra methyl ethylene diamine (TEMED), were obtained from Sigma Aldrich. Nutrient broth was from Thermo scientific.

2.2. Microorganism

Bacillus subtilis TB1 which was previously isolated from an oil contaminated soil sample was used to study the hydrocarbons biodegradation. It is a gram positive, motile, facultative bacterium that can grow on hydrocarbons as the sole carbon source (Barin et al., 2014). Moreover, its capability to produce alpha-amylase was verified by growing on starch agar medium and lugol solution as the marker (Mishra and Behera, 2008). The 370 bp of 16S-rDNA gene of this strain was deposited in GenBank under the accession number KC309409.

2.3. Culturing media and growth condition

Nutrient broth and nutrient agar were utilized for the provision of inoculums and counting of colony forming units (CFU) respectively. Bacterial degradation of hydrocarbons was investigated in Bushnell-Hass(BH) medium prepared by dissolving $MgSO_4.7H_2O$ 0.2 g, $CaCl_2$ 0.02 g, $FeCl_3$ 0.05 g, KH_2PO_4, K_2HPO_4 and $(NH_4)_2NO_3$ 1 g in 1 L distilled water (Bushnell and Haas, 1941). Normal paraffins mixture (mainly C_{10}–C_{14}) was added to BH medium as the carbon source by a total concentration of 1% (v/v). All the mentioned culturing media were sterilized in autoclave at 121 °C for 15 min.

To obtain bacterial crude enzyme (amylase) solution, the starch broth comprising peptone 0.05%, KCl 0.01%, $MgSO_4.7H_2O$ 0.05%, $(NH_4)_2SO_4$ 0.01%, NaH_2PO_4 0.01% and soluble starch 2% was used (Mishra and Behera, 2008). It was inoculated by bacteria (10^8 cells/mL) and CFU enumeration was employed to control the bacterial count of inoculates. The mixture was incubated at 37 °C and 160 rpm for 72 h. Then, the culture was centrifuged at 4000 rpm and 4 °C for 10 min. To ensure the complete bacterial removal, the supernatant was filtered through a 0.45 μm syringe filter. The resultant liquid was used as the bacterial amylase solution.

Bacterial growth on n-paraffins with the presence of both starch and n-paraffins was investigated. The growth conditions were established based on the procedure mentioned above. The growth curves were obtained by measuring the cultivated biomass dry weight every 12 h. The residual starch in the medium was measured after filtration of the broth to remove bacteria from solution. The paraffins were eliminated through solvent extraction with chloroform. The starch content of aqueous phase was measured by addition of Lugol's solution and spectrophotometry at 550 nm.

2.4. Enzyme assay

There are several types of amylases produced by microorganisms which can be assayed by relevant methods. Exoamylases like glucoamylases participate in starch degradation reaction by eliminating glucose from the non-reducing starch chain ends, thereby decreasing the length of accessible starch chain to iodine binding and providing glucose units which are incapable of binding to iodine. In contrast, endoamylases such as alpha-amylase cleave α-(1,4) glycosidic bonds of starch chains randomly. A major portion of end products of endoamylases are oligosaccharides which are able to make blue starch–iodine complexes. Fuwa (Fuwa, 1954) represented a method to determine enzyme activity on the basis of the residual starch while Miller (Miller, 1959) suggested an approach to measure the activity on the basis of the produced sugars. Comparing the results of Fuwa and Miller methods, provides a simple technique to distinguish between endoamylases and exoamylases (Xiao et al., 2006).

2.5. Protein analysis

Total Concentration of the solubilized proteins in the crude enzyme solution was determined by the method of Bradford, applying BSA as the standard protein solution. The sample absorbance was measured at 595 nm after incubation at 25 °C for 5 min (Bradford, 1976).

Ammonium sulfate salt precipitation was used to separate proteins in crude enzyme solution. 52 g salt was added to 100 (mL) crude enzyme solution during stirring for 60 min at 4 °C. Next, the suspension was stirred for 24 h to permit solvent-protein equilibration. The mixture was then centrifuged at 11,000 rpm (7500 g) and 4 °C for 30 min and the obtained wet precipitate was added to phosphate buffer solution at pH 7. The salt was eliminated by

dialyzing wet precipitate by using 10,000 molecular weights cut off dialyzing tubing which had been prepared according to Sigma Aldrich instructions. The dialyzing bag was submerged in a flask containing a large quantity of sterile distilled water (100 times the volume of sample protein) and hereafter the water was gently stirred at 4 °C overnight. The contents of flask were replaced by fresh sterile distilled water every 60 min eight times.

Native polyacrylamide gel electrophoresis (PAGE) was performed for separating the complex mixture of the purified bacterial enzymes with 8% polyacrylamide gel by the method of Laemmli (Laemmli, 1970). The Chromatein Prestained Protein Ladder, the product of Vivantis Technologies PR0602, used as a protein marker, includes 11 proteins within the limit of 10–175 KDa. It allows estimation of molecular weights of proteins in bacterial crude enzyme by SDS-Page method. Two parts of samples were diluted with 1 part of native sample buffer. The gels were operated on the 4-gelMini-PROTEAN® Tetra Cell (Bio-Rad Laboratories, Singapore). Reverse electrophoretic zymography was executed for estimating molecular weight of effective enzymes on both starch and hydrocarbons (Manchenco Genandy, 2003). After enzyme separation by Native-PAGE, SDS was removed by the non-ionic detergent, Triton X-100. This stage permits the enzymes to partially return to their original conformation. In reverse gel zymography, the reaction of an enzyme and a substrate can exactly be visualized. For instance, hydrolytic enzymes such as amylases can be identified by their capability to degrade a specific substrate (e.g. starch).

2.6. Hydrocarbons biodegradation

100 mL of BH medium containing 1 mL n-paraffin was inoculated by a pre-culture broth to have 10^8 cells per mL biomass concentration. Flasks were incubated in an orbital shaker at 37 °C and 160 rpm for 72 h. The effect of enzyme on biodegradation was investigated in cell free flasks containing the above mentioned medium supplied with either 5 mL crude enzyme solution (0.4 activity fuwa) or 0.5 mL of Alpha-Amylase Liquozyme (enzyme concentration was equal to 9.97 (μg/mL) and enzyme activity based on Fuwa method was 20.19 (U/mL)). Proper blanks were considered for each case.

Biodegradation of hydrocarbons in both the microbial and enzymatic conditions were analyzed through solvent extraction followed by gas chromatography. At the end of cultivation time, the whole content of each flask was centrifuged (10,000 rpm, 4 °C, 10 min) to remove biomass from the broth. After that, solvent extraction was employed to assess residual n-alkanes in the system. Chloroform was added to a decanter funnel containing the sample in 25:1 volumetric ratio and vigorously mixed. Then, the organic phase was taken by phase segregation. The solvent phase was dehydrated by passing through anhydrous Na_2SO_4. The obtained samples were analyzed by gas chromatography in an Agilent system HP6890 gas chromatograph equipped with HP5 capillary column and a FID detector. Injector and detector temperatures were set to 280 °C and 300 °C respectively. The injection volume was equal to 1 μL. Nitrogen was used as the carrier gas at the constant flow rate of 2 mL min^{-1}. The split ratio was set at 20:1. The oven temperature was programmed to increase from 50 °C to 210 °C at a heating rate of 20 °C min^{-1} followed by a 3 min holding time and a rise to 270 °C at the rate of 10 °C min^{-1}. It was kept constant at this temperature for 20 min. Biodegradation of n-alkanes was quantified by comparing the peak areas at the identical retention times related to each alkane with the blank. The biodegradation of n-paraffins was replicated five times for each sample.

2.7. In silico docking analysis

Molegro Virtual docker (MVD 5.5, 2012) software was employed to predict the complex formation of hydrocarbons with amylase as a nonspecific enzyme and the potential binding sites of the enzyme molecule. The three dimensional structures of alpha-amylase (PDB ID: 1BAG) and maltopentaose (tetrahexoses) based on their x-ray crystal structures were prepared from Protein Data Bank (www.rcsb.org/pdb) (Fujimoto et al., 1998). The three dimensional coordinates of hydrocarbons such as decane, dodecane, thridecane, tetradecane, pentadecane and hexadecane were obtained from the ZINC database http://zinc.docking.org/search/. Water molecules were eliminated from docking workspace. A maximum number of seven cavities were determined by MVD for alpha-amylase (PDB ID: 1BAG). The grid resolution, number of runs, maximum iterations, maximum population size and the energy threshold for pose formation were equal to 3 Å, 50, 2000, 50 and 100 (Kcal/mol) respectively and were kept fixed during all docking stages. The MolDock score function (E_{score}) used by MVM has been derived from piecewise linear potential (PLP) which has been modified by adding hydrogen binding and electrostatic terms and can be defined by the following formulation. The complete description of docking algorithm in MVD has been reported by Thomsen et al (Thomsen and Christensen, 2006).

$$E_{score} = E_{inter} + E_{intra} \quad (1)$$

where E_{Inter} is the interaction energy of ligand-protein and E_{Intra} is the ligand internal energy in kcal/mol. Docking algorithm was set to MolDock SE.

3. Results and discussion

3.1. Bacterial growth on starch and hydrocarbon

The effect of addition of starch to the hydrocarbon enriched BH medium on the bacterial growth has been shown in Fig. 1. As can be seen, when the carbon source has been limited to hydrocarbons, the growth begins at about 12 h and continues for more than 84 h. In contrast, there are two lag phases in bacterial growth with the presence of starch which can be related to the required updating time for the expression and production of necessary enzymes to consume the second substrate (i.e. hydrocarbons). This observation is in agreement with starch consumption trend in the Bushnell–Haas medium at the presence of n-paraffins (Fig. 2). The

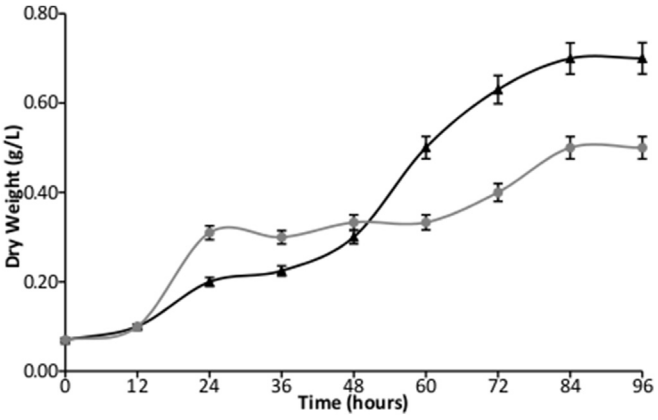

Fig. 1. The growth curve of *B. subtilis* in (▲)Bushnell–Haas (n-paraffin(10 mL/L) as carbon source) and (●) Bushnell–Haas (N-paraffin (10 mL/L) and Soluble Starch (2 g/L) as Carbon sources).

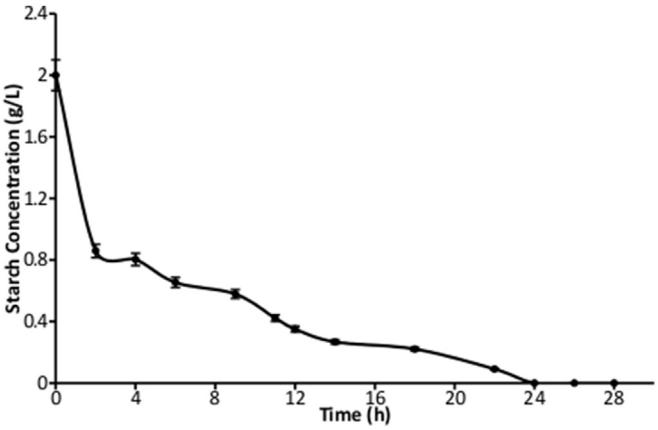

Fig. 2. The starch utilization curve in Bushnell–Haas by the presence of n-paraffin with concentration (10 mL/L) and Soluble Starch (2 g/L).

results demonstrated that starch was completely consumed as the primary substrate at the first 24 h after inoculation of *B. subtilis* TB1.

The overall produced biomass in the starch containing system was higher, and interestingly, the exponential growth of bacteria mainly began after 36 h which was after the consumption of starch. This means that the utilization of starch in the system has a positive impact on the biodegradation of hydrocarbons by bacteria and biomass production even after its consumption. This observation is in agreement with the results of Al-Hadhrami et al. (1996) that shows an enhancement in alkanes biodegradation at the presence of molasses in the system. The results of measuring hydrocarbon content of the two mediums in successive time steps verify the point. The chromatograms have been supplied as supplementary materials Figs. 1s and 2s. The hydrocarbon biodegradation has gradually continued in the system without starch while with the presence of starch the biodegradation has occurred steeply after 60 h. The remaining hydrocarbon content of the starch containing system was about half of the sample without starch after 72 h. This observation can be explained in several ways. Firstly, it was assumed that starch may help to remove the hydrocarbons mass transfer constraints through some kinds of surfactant activity. Clearly, hydrocarbons are hydrophobic molecules and their degradation will be enhanced with the presence of surfactants. It is reported that soluble starch can act as an emulsifying agent or emulsion stabilizer with the presence of n-paraffins (Bertoloini, 2010). However, measuring the surface tension in both systems had not shown any significant distinction. Moreover, starch has been completely consumed by the bacteria in the first 24 h. Therefore, the surfactant activity of starch was rejected.

Secondly, *B. subtilis* is a well known biosurfactant producing microorganism and its production yield depends on the carbon source type in the system (Mukherjee and Das, 2005). Consequently, the presence of starch might affect the biosurfactant production yield which in turn can enhance the biodegradation of hydrocarbons. This interpretation was examined through measuring the biosurfactant concentration at both the systems with and without starch. In fact, the produced biosurfactant is an anionic lipopeptide compound (Barin et al., 2014) and the two-phase mixed indicator titration method could be used to determine its concentration (Li and Rosen, 1981). The obtained concentrations of surfactant vs. incubation time have been shown in Fig. 3s in supplementary materials. Results indicated that the produced biosurfactant at the presence of starch is slightly higher than the starch free medium which cannot be considered as the main reason for the significant biodegradation improvement observed in the starch containing medium.

Thirdly, we thought the starch-stimulated enzymes produced by the bacteria can be responsible for the higher cell density and biodegradation rate in the system. In fact, the major consequence of the presence of starch in the culturing medium is the production of hydrolysis enzymes by *B. subtilis*. Therefore, the bacterial enzymes were studied carefully. The results have been discussed in the following section.

3.2. Enzyme assay and protein concentration

Analysis of the crude enzyme solution by Fuwa and Miller methods showed that both types of exoamylases and endoamylases have been produced by the bacteria (Fig. 3). In fact, the enzyme activity based on Miller was about twice of Fuwa method from 36 to 72 h of incubation. After 132 h, exoamylase activity fell to the level of endoamylase.

Fig. 4 shows the concentration of proteins in the crude enzyme solution by incubation time. As can be seen, the obtained protein concentrations have not matched the amylase activity trend. The protein concentration has a peak at 84 h while the amylase activity is maximum between 48 and 60 h. Therefore, the other proteins produced in the system might affect the biodegradation. It should be noted that the TB1 strain can utilize n-paraffins as the sole carbon and energy source. This means that it is capable of producing appropriate enzymes to catabolize this type of substrate. Therefore, the impact of amylase on biodegradation of hydrocarbons should be studied in more details.

3.3. SDS-PAGE, Native-PAGE and reverse zymography

To distinguish the influence of amylolytic enzymes on hydrocarbon biodegradation from the others in the crude enzyme solution, SDS-PAGE and Native PAGE were performed and the process was followed by reverse zymography. The obtained results have been illustrated in Fig. 5. In Fig. 5-a, the first column at the left part indicates the protein marker band covers the range of 10–175 KDa.Columns 1 and 2 are related to SDS-PAGE of *B. subtilis* TB1 purified crude enzyme displaying all of the protein bands present in the system.

Fig. 5-b represents the reverse zymography of the obtained Native-PAGE on the starch agar plate (Vandooren et al., 2013). The protein bands showing amylolytic activity were recognized by the observed decrease in the intensity of the blue color of KI-I_2 solution as a result of the hydrolytic enzymes action on the starch plate which has been marked by a black circle in the molecular weight

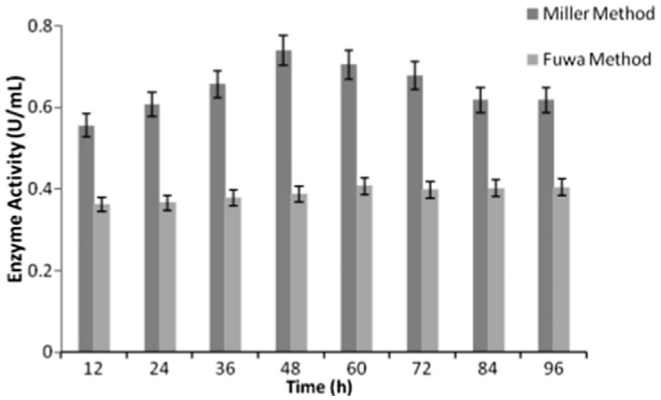

Fig. 3. Amylase activity based on Fuwa and Miller methods in crude enzyme of *B. subtilis* TB1 (U/mL).

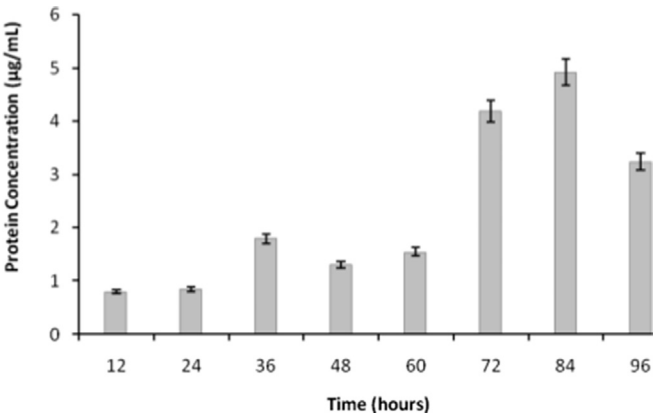

Fig. 4. Protein concentration in *B. subtilis* TB1 crude enzyme (µg/mL).

materials. In this case, during the first 48 h C_{10} peak disappeared and the other n-paraffins peaks slightly decreased. After 72 h, no hydrocarbons were detected in the system as all the n-paraffins related peaks disappeared.

These observations help us to claim that the amylase contribution in hydrocarbons biodegradation is not just limited to a synergetic effect with other related enzymes and it can cause biodegradation independently. In fact, the biodegradation of hydrocarbons in a system containing bacteria and starch would be influenced by both specific (e.g. oxygenase) and nonspecific (i.e. amylase) enzymes produced by the bacteria. However, this process can not be identified as "cometabolism" because the utilized bacteria can consume both starch and hydrocarbons as carbon and energy source. On the other hand, the cell free enzyme solution produced through cultivating bacteria in the starch broth was capable of converting hydrocarbons to some intermediate compounds. We tried to examine if the degraded hydrocarbons have been converted to CO_2 or other gaseous compounds in the system. To this end, the top gas phase of a sealed flask containing n-paraffins and alpha-amylase Liquozyme SC DS in the aqueous phase after 72 h was analyzed by GC (data not shown). The results were the same as the blank sample which meant the hydrocarbons had not been converted to CO_2 by amylase. The identification of the chemical nature of these intermediates needs more research.

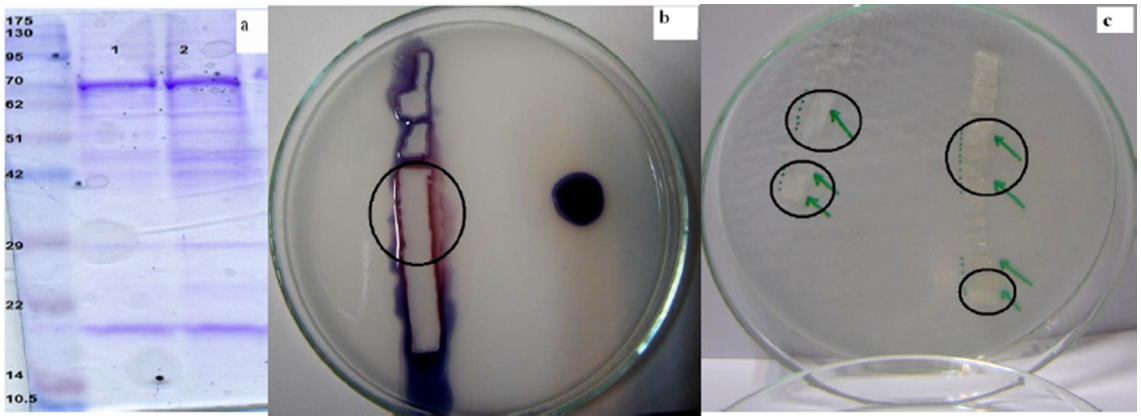

Fig. 5. (a) Determination of *B. subtilis* molecular weight by SDS-PAGE, (b) reverse zymography of *B. subtilis* crude enzyme on native-PAGE and starch agar and (c) reverse zymography of *B. subtilis* crude enzyme on native-PAGE and BH- agar (n-paraffin as carbon source) plate.

range of 42–70 KDa. The KI-I_2 solution was added to a circular area in the other side of the plate and no color changes was detected meaning that the starch had not hydrolyzed at the rest of the plate with no enzyme.

In Fig. 5-c the effective enzymes on n-paraffin degradation were determined in two areas and identified as proteins with estimated molecular weight of 10–22 KDa and 42–70 KDa which have been marked by black circles. Evidently, the enzymes with molecular weight within the range of 42–70 KDa are in common in both plates which means that amylolytic enzymes can affect n-paraffins. In addition, the higher efficiency of hydrocarbons biodegradation in the starch added system can be explained by the synergistic effect of the two different kinds of enzyme existing in *B. subtilis* crude enzyme solution.

3.4. Enzymatic hydrocarbon biodegradation

The n-paraffins biodegradation by the act of bacterial amylase solution was investigated. The results of GC analysis have been shown in Fig. 6. Apparently, after 36 h of incubation the GC peak areas of n-paraffins in the medium containing crude enzyme solution demonstrate the omission of C_{10} and C_{11} and diminution of C_{12}–C_{14}. The complete removal of n-paraffins after 3 days was a more fascinating result. Forasmuchas the dominant *B. subtilis* enzymes in starch broth are hydrolysis enzymes, the impact of a commercial alpha-amylase (Liquozyme SC DS) was also examined. The results have been provided in Fig. 4s of the supplementary

3.5. In silico docking analysis

In the molecular docking studies, the interaction of alpha-amylase (PDB ID: 1BAG) and maltopentaose (the specific substrate) as well as n-alkanes like decane, dodecane, thridecane, tetradecane, pentadecane and hexadecane (nonspecific ligands) at the same binding cavity were separately investigated using Molegro Virtual docker (MVD 5.5, 2012). The universal thermodynamic hypothesis predicts that a negative higher binding energy demonstrates a greater binding compared to higher energy amount. The obtained results were in the adequate range so that the negative amounts of MolDock score and total energy reported in Table 1 confirmed the possibility of binding of alpha-amylase to the studied n-alkanes which was in agreement with the experimental observations in the previous sections.

The utilized n-paraffin mixture in the experiments composed of several n-alkanes. To sort the affinity of amylase to n-alkanes with different molecular weigths, three dimensional structures of

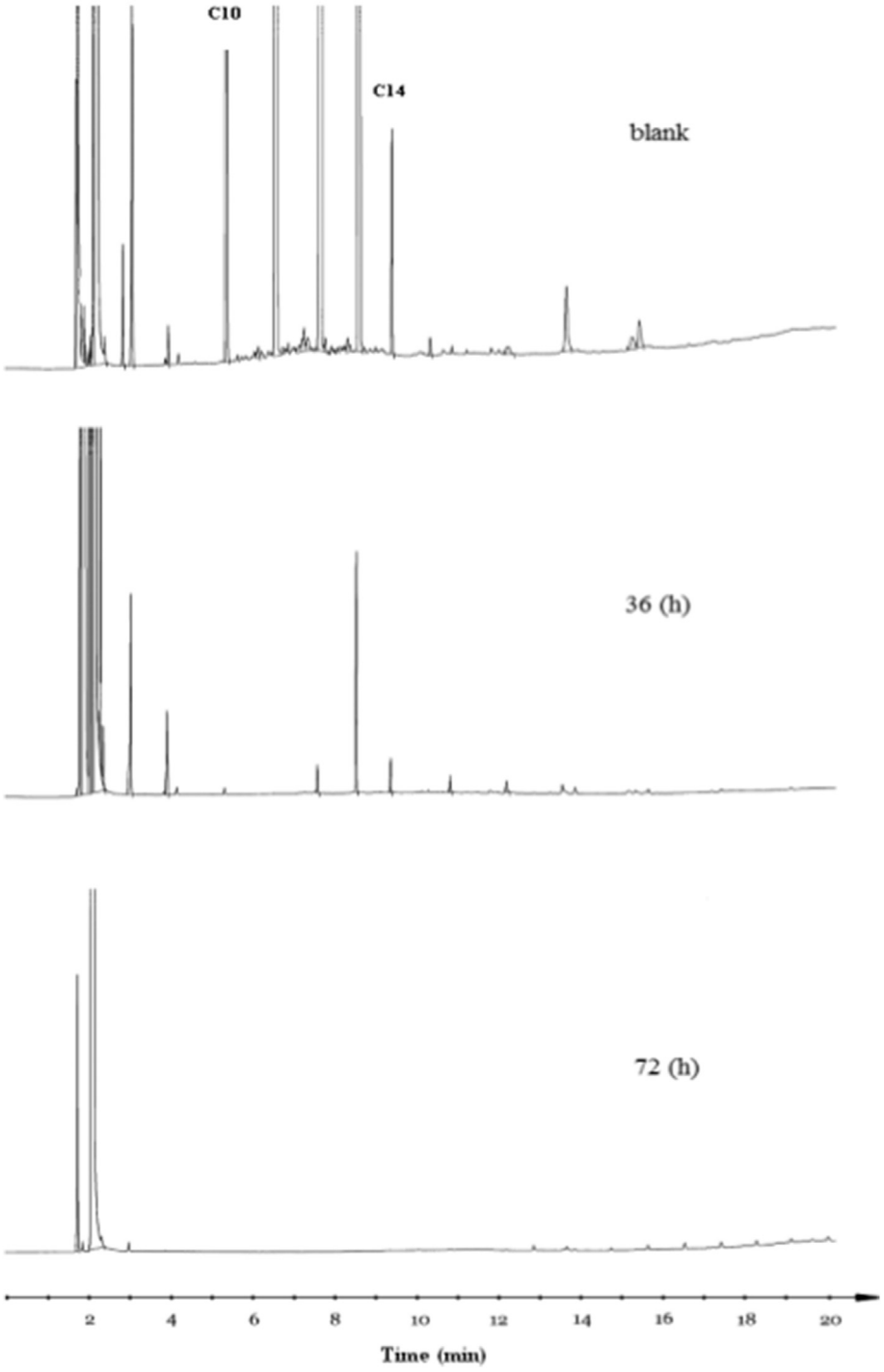

Fig. 6. Time course of n-paraffin biodegradation with the presence of *B. subtilis* TB1 crude enzyme, C10–C14 indicates the peaks of n-alkanes carbon number.

C_{10}–C_{16} were simultaneously imported to Molegro work space and in a competitive condition; alpha-amylase binding to them was investigated. The reported results in the last two columns of Table 1 (min and max of the total energy) show the longer the n-alkane chain, the larger total binding energy. In fact, the longer alkanes can produce greater hydrophobic interactions and have a higher molecular flexibility. It should be noted that the greater binding energy of amylase to alkanes with higher molecular weight is not in conflict with the obtained experimental results that shows the biodegradation of lighter hydrocarbons is faster. In fact, the kinetics of enzymatic biodegradation can be influenced by several factors such as steric hindrance or ligand inhibitory effects which require further investigations.

4. Conclusion

The influence of starch addition to biodegradation of n-paraffins by *B. subtilis* TB1 was investigated. The results indicated that starch added samples had a higher biodegradation efficiency and biomass density. The impact of bacterial enzymes was studied to find a

Table 1
Molecular interaction analysis of Alpha-amylase (1BAG) with specific and nonspecific ligands (alkanes).

Ligand	MolDock score[a]	Total energy[a]	External ligand interaction[a]	Internal ligand interaction[a]	Max total energy[a]	Min total energy[a]
Maltopentaose	−241.74	−155.86	−255.4	+69.54	–	–
n-Decane	−71.44	−71.44	−72.13	+0.68	−72	−68
n-Undecane	−75.44	−75.44	−77.8	+2.38	−76	−73
n-Dodecane	−80.9	−80.9	−76.36	−4.53	−80	−78
n-Thridecane	−86.98	−86.98	−85.70	−1.27	−85	−83
n-Tetradecane	−91.48	−91.48	−90.14	−1.33	−94	−83
n-pentadecane	−99.49	−99.49	−100.24	+0.74	−95	−90
n-Hexadecane	−99.79	−99.79	−98.69	−1.1	−101	−96

[a] The energy units are Kcal/mol.

reasonable explanation for this observation. It was revealed that the produced bacterial amylase can degrade the alkanes present in the system after 72 h and the same effect was obtained by a commercial amylase sample. In-silico docking of amylase with different molecular weight n-alkanes, it was shown that high binding energies actually support the experimental results. Therefore, it was concluded that alpha-amylase could have a biocatalytic activity to degrade n-alkanes.

Acknowledgments

The present study was supported by the University of Isfahan. The authors thank Rose Polymer Company for its assistance during carrying out the research.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.02.120.

References

Al-Hadhrami, M.N., Lappin-Scott, H.M., Fisher, P.J., 1996. Effects of the addition of organic carbon sources on bacterial respiration and n-alkane biodegradation of omani crude oil. Mar. Pollut. Bull. 32 (4), 351–357.
Ammala, A., Bateman, S., Dean, K., Petinakis, E., Sangwan, P., Wong, S., Yuan, Q., Yu, L., Patrick, C., Leong, K.H., 2011. An overview of degradable and biodegradable polyolefins. Prog. Polym. Sci. 36, 1015–1049.
Barin, R., Talebi, M., Biria, D., Beheshti, M., 2014. Fast bioremediation of petroleum-contaminated soils by a consortium of biosurfactant/bioemulsifier producing bacteria. Int. J. Environ. Sci. Technol. 1–10.
Bertoloini, C.A., 2010. Starches Characterization, Properties, and Applications. CRC Press, Taylor and Francis group.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Bushnell, L., Haas, H., 1941. The utilization of certain hydrocarbons by microorganisms. J. Bacteriol. 41, 653.
Das, N., Chandran, P., 2011. Microbial degradation of petroleum hydrocarbon contaminants. Biotechnol. Res. Int. http://dx.doi.org/10.4061/2011/941810. Article ID 941810.
Fujimoto, Z., Takase, K., Doui, N., Momma, M., Matsumoto, T., Mizuno, H., 1998. Crystal structure of a catalytic-site mutant α-amylase from *Bacillus subtilis* complexed with maltopentaose. J. Mol. Biol. 277, 393–407.
Fuwa, H., 1954. A new method for microdetermination of amylase activity by the use of amylose as the substrate. J. Biochem. 41, 583–603.
Huang, L., Xie, J., Lv, B.Y., Shi, X.F., Li, G.Q., Liang, F.L., Lian, J.Y., 2013. Optimization of nutrient component for diesel oil degradation by *Acinetobacter beijerinckii* ZRS. Mar. Pollut. Bull. 76 (1), 325–332.
Hadibarata, T., Kristanti, R.A., 2012. Fate and cometabolic degradation of benzo [*a*] pyrene by white-rot fungus *Armillaria* sp. F022. Bioresour. Technol. 107, 314–318.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Lan, G., Fan, Q., Liu, Y., Liu, Y., Liu, Y., Yin, X., Luo, M., 2015. Effects of the addition of waste cooking oil on heavy crude oil biodegradation and microbial enhanced oil recovery using *Pseudomonas* sp. SWP-4. Biochem. Eng. J. 103, 219–226.
Lee, S.W., Keeney, D.R., Lim, D.H., Dispirito, A.A., Semrau, J.D., 2006. Mixed pollutant degradation by methylosinus trichosporium OB3B expressing either soluble or particulate methane monooxygenase: can the tortoise beat the hare? Appl. Environ. Microbiol. 72 (12), 7503–7509.
Li, Z.P., Rosen, M.J., 1981. Two-phase mixed indicator titration method for determination of anionic surfactants. Anal. Chem. 53 (9), 1516–1519.
Manchenco Genandy, P., 2003. Handbook of Detection of Enzymes on Electrophoretic Gels, second ed. CRC press.
Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.
Mishra, S., Behera, N., 2008. Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. Afr. J. Biotechnol. 7.
Montagnolli, R.N., Lopes, P.R.M., Bidoia, E.D., 2015. Assessing *Bacillus subtilis* biosurfactant effects on the biodegradation of petroleum products. Environ. Monit. Assess. 187 (1), 1–17.
Mukherjee, A.K., Das, K., 2005. Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. FEMS Microbiol. Ecol. 54 (3), 479–489.
Nakamura, E.M., Cordi, L., Almeida, G.S.G., Duran, N., Mei, L.H.I., 2005. Study and development of LDPE/starch partially biodegradable compounds. J. Mater. Process. Technol. 162–163, 236–241.
Nzila, A., 2013. Update on the cometabolism of organic pollutants by bacteria. Environ. Pollut. 178, 474–482.
Rehm, H.J., Reed, G., 2008. Biotechnology Environmental Processes II, second ed., 11b
Rittmann, B.E., McCarty, P.L., 2001. Environmental Biotechnology Principles and Applications. McGraw-Hill, New York.
Suja, F., Rahim, F., Taha, M.R., Hambali, N., Rizal Razali, M., Khalid, A., Hamzah, A., 2014. Effects of local microbial bioaugmentation and biostimulation on the bioremediation of total petroleum hydrocarbons (TPH) in crude oil contaminated soil based on laboratory and field observations. Int. Biodeterior. Biodegrad. 90, 115–122.
Thomsen, R., Christensen, M.H., 2006. MolDock: a new technique for high-accuracy molecular docking. J. Med. Chem. 49, 3315–3321.
Tomei, M.C., Daugulis, A.J., 2013. Ex situ bioremediation of contaminated soils: an overview of conventional and innovative technologies. Crit. Rev. Environ. Sci. Technol. 43 (20), 2107–2139.
Vandooren, J., Geurts, N., Martens, E., Van den Steen, P.E., Opdenakker, G., 2013. Zymography methods for visualizing hydrolytic enzymes. Nat. Methods 10, 211–222.
Wentzel, A., Ellingsen, T.E., Kotlar, H.K., Zotechev, S.B., Holst, M.T., 2007. Bacterial metabolism of long-chain n-alkanes. Appl. Microbiol. Biotechnol. 76, 1209–1221.
Xiao, Z., Storms, R., Tsang, A., 2006. A quantitative starch–iodine method for measuring alpha-amylase and glucoamylase activities. Anal. Biochem. 351, 146–148.
Zuchowska, D., Hlavata, D., Steller, R., Adamiak, W., Meissner, W., 1999. Physical structure of polyolefin-starch blends after ageing. Polym. Degrad. Stab. 64, 339–346.