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Biodegradation of animal fats and vegetable oils by *Rhodococcus erythropolis* PR4

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ABSTRACT

The alkane (pristane) degradation capacity of *Rhodococcus erythropolis* PR4 (NBRC 100887), isolated from marine environment, was previously observed. In this study, the ability of this strain for biodegradation of various animal fats, such as pig lards and poultry fats as well as butter, margarine and sunflower cooking oil was studied. Bioconversion of fats and oil was determined as methyl-ester (FAME) derivatives by GC–MS. *R. erythropolis* PR4 strain could utilize all substrates tested but the bioconversion rate and efficacies varied. The optimum pH for decomposition of pig lard and poultry fat was 8.5, respectively. Addition of carbonate to the media dramatically improved the efficiency of the process via stabilization of pH of the fermentation. Biotransformation of poultry fat was complete in four days and around 80% conversion was reached in the case of pig lard in media containing carbonate. The extracellular lipase activity of the *R. erythropolis* PR4 strain was also demonstrated by various techniques. The results suggest the *R. erythropolis* PR4 strain studied is a promising candidate in bioremediation/bioconversion of fat-containing wastes within a relatively short time.

1. Introduction

Among the organic fraction of industrial and municipal wastes, hydrophobic compounds cause outstanding problems. These hydrophobic pollutants, such as diesel oils, animal fats, oils and greases (FOG) are produced by the energy sector, food industry, restaurants, slaughter houses, kitchens (Wakelin and Forster, 1997; Lefebvre et al., 1998; Mari et al., 2003; Saatci et al., 2003). Fats cool down in the gutter, attach to other ingredients of sewage and form hard, soap-like deposits, that create blockages in sewage systems (He et al., 2011, 2012; 2013). In addition, the hydrophobic materials form a layer on the surface of water that blocks oxygenation (Becker et al., 1999; Chipasa and Mędrzycka, 2006; Čipinytė et al., 2009).

Hydrophobic materials are chemically diverse. Diesel oil is composed of both saturated and aromatic hydrocarbons. Many lipids consist of various fatty acids and a glycerol moiety. The fatty acid compositions of a given lipid strongly depend on its origin. Bitman (1976) demonstrated that pig lard contains oleic acid in a large percentage (47%), palmitic acid (25%) and also contains stearic acid (14%), linoleic acid (8%), palmitoleic acid (3%) and myristic acid (1%). Poultry fat contains fewer unsaturated fatty acids such as oleic acid (43%), but more linoleic acid (18%), palmitoleic acid (7%) and also contains palmitic acid (25%) stearic acid (4%) and myristic acid (1%). Butter, margarine and sunflower oil contain mainly glycerol-esters composed of saturated and unsaturated fatty acid in various ratios (USDA database, http://ndb.nal.usda.gov/ndb/nutrients/index). The different proportion of saturated and unsaturated fatty acids in the animal fats can influence how oils are utilized by microorganisms.

Few *Rhodococcus* species were described as promising hydrocarbon degraders (De Carvalho and Da Fonseca, 2005a, 2005b) which could solubilize and oxidize water insoluble hydrocarbons (Bell et al., 1998; De Carvalho and Da Fonseca, 2005b; Lee et al., 2010). Several bacteria isolated from the environment were documented to be effective for treatment of waste waters containing FOG (Wakelin and Forster, 1998; Keenan and Sabelnikov, 2000; Mongkolthanaruk and Dharmsthiti, 2002; Sugimori et al., 2002; Prasad and Manjunath, 2011; Affandi et al., 2014). Most of these microorganisms (pseudomonads, bacilli) were able to secrete extracellular lipases being essential for biodegradation of lipids

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(Pandey et al., 1999; Jaeger and Eggert, 2002; Serikovna et al., 2013). As an initial step of bioconversion, microbial lipases hydrolyze the ester bonds of glycerol-esters for removing the fatty acids from the glycerol moiety. Next, the fatty acids are converted into acetyl-CoA via β-oxidation pathway (Tan and Gill, 1985; Ruggieri et al., 2008). However, the microbial degradation of fats is slow due to the limited availability of these hydrophobic materials for microorganisms (Fukase et al., 1993; Lefebvre et al., 1998).

The genera *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Rhodococcus* can synthesize several types of surfactants (Banat et al., 2010). Some of these surfactants reduce the surface tension of the hydrophobic compounds and so-called micro-droplets (micelles) accessible for the microbes are formed (Fukase et al., 1993; Banat et al., 2010). Another way to access FOG is to modify the cell wall to be endowed by hydrophobic feature.

The use of Rhodococci (Bell et al., 1998) in a broad variety of biotechnological applications is rapidly and steadily increasing. *Rhodococcus erythropolis* PR4 (NBRC 100887) is a marine bacterium capable to degrade several alkanes (Komukai-Nakamura et al., 1996; Sekine et al., 2006) like pristane (Urai et al., 2007a, 2007b). *R. erythropolis* PR4 (shortly PR4) can produce extracellular polysaccharides that are responsible for emulsification of the hydrophobic substrates (Urai et al., 2007a, 2007b; Hamada et al., 2008). A comparative analysis by Hamada et al. (2008) presented that the PR4 cells possessed a highly hydrophobic cell surface. The genome of the strain has been completely sequenced (Sekine et al., 2006; GenBank ID: GCA_000010105.1) and a number of lipase genes were annotated: 4 codes for triacyl-glycerol lipases and one for monoacyl-glycerol lipase, the others were annotated as lipase.

Although, a number of studies were published about degradation of hydrocarbons by rhodococci, very little is known about the ability of this strain to decompose animal fats and similar wastes. There are a number of publications for FOG biodegradation using pure strains (Tan and Gill, 1985; Sugimori et al., 2002; Prasad and Manjunath, 2011; Affandi et al., 2014) or consortium (Wakelin and Forster, 1998; Keenan and Sabelnikov, 2000; Mongkolthanaruk and Dharmsthiti, 2002). In a recent review (Prasad and Manjunath, 2011), the fat degradation abilities of six lipase producing strains (none of them belonged to Actinobacteria) were compared and showed that the lipid content of the wastes could be practically removed within 12 days.

The objective of this work is to establish an efficient biological treatment for various hydrophobic wastes using a *Rhodococcus* strain having promising biotechnological potential.

2. Materials and methods

2.1. Chemicals, materials

Chemicals (chloroform, sulfuric acid, methanol) used for analytical methods were reagent grade and they were purchased from standard commercial suppliers (Reanal, VWR, Merck, Sigma–Aldrich).

Pig lard and poultry fats are shop traded products (Hungary). According to a nutrient database (USDA database, http://ndb.nal.usda.gov/ndb/nutrients/index), pig lard typically contains around 40% of saturated (palmitic acid, 25–27%, 12–14% stearic acid) and 60% of unsaturated (44–47% oleic acid, 8–10% linoleic acid, 3–4% palmitoleic acid). The typical fatty acid composition of poultry fat is: 29% saturated (palmitic acid, 22–25%, 4–6% stearic acid), 65% unsaturated (37–43% oleic acid, 16–20% linoleic acid, 6–8% palmitoleic acid) fatty acids and 6% other components. The fatty acids present in our samples were analyzed by GC–MS technique (see 2.4.2 section) and the major components (palmitic acid, stearic acid, oleic acid, linoleic acid and palmitoleic acid) could be identified. The peaks of oleic acid and linoleic acids notably overlapped therefore they could be qualitatively but not quantitatively separated. The ratio of the fatty acids in pig lard and poultry fat coincided with the data of the USDA database. Butter was a "Hungarian butter" produced by Alfold Tej Ltd and had 82% (m m^{-1}) of fat content. The ratio of saturated and unsaturated fatty acids in butter was around 2:1 (Alfold Tej Ltd). Rama margarine derived from Unilever Ltd contained 70 g fats in 100 g margarine (33 g with saturated, 37 g with unsaturated fatty acids) (Univer Ltd). Venusz sunflower oil was the product of Bunge Corp, Hungary. In this vegetable oil, the ratio of the fatty acids containing 0, 1 or multiple double bounds was approximately 1:2:5 (Bunge Corp). All substrates were autoclaved for 1 h before use.

2.2. Culture conditions

The PR4 strain (Komukai-Nakamura et al., 1996) was obtained from the Japanese culture collection (NBRC 100887, NITE). The cells were grown in 100 mL flasks containing either 20 mL of minimal salt medium (Kis et al., 2013) (shortly, MSM: 0.68 g L^{-1} KH_2PO_4; 0.87 g L^{-1} K_2HPO_4; 0.58 g L^{-1} NaCl; 0.125 g L^{-1} $MgSO_4 \times 7H_2O$; 0.044 g L^{-1} $CaCl_2 \times 2H_2O$; 0.0012 g L^{-1} NH_4NO_3; 0.014 g L^{-1} $FeSO_4$ complexed with EDTA; 2 mL of trace element solution (0.1 g L^{-1} $ZnSO_4 \times 7H_2O$; 0.03 g L^{-1} $MnCl_2 \times 7H_2O$; 0.3 g $L^{-1}H_3BO_4$; 0.2 g L^{-1} $CoCl_2 \times 6H_2O$; 0.01 g L^{-1} $CuCl_2 \times 2H_2O$; 0.02 g L^{-1} $NiCl_2 \times 6H_2O$; 0.03 g L^{-1} $NaMoO_4 \times 6H_2O$)) or modified minimal salt medium containing 10.08 g L^{-1} $NaHCO_3$ (CMSM). The concentration of each substrate (pig lard, poultry fat, butter, margarine and vegetable oil) was 1% (m v^{-1}) unless otherwise indicated. All samples were inoculated with 1% (v v^{-1}) of starter culture ($OD_{600} = 1.0$) grown in Luria Broth (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl). Before inoculation, the starter culture was centrifuged (13,000 rpm, 10 min, 4 °C) and washed twice in physiological salt solution to prevent the transfer of the organic compounds of LB into the fresh media. In the control samples, bacteria were omitted. In each case, the samples were incubated in a rotary shaker (150 rpm at 25 °C) for at least a week. Data from three independent experiments were collected and analyzed.

2.3. Lipase activity assays

The agar plate assay of Rajan et al. (2011) was used to visualize lipase activity. Tween 20 (Sigma–Aldrich) was an adequate substrate for simple and fast detection of microbial lipolytic activity on agar plate. White-like precipitate appears around the colony having lipase activity (Nagarajan et al., 2014).

2.4. Analytic methods

2.4.1. Respiration activity

Oxygen content of the headspace was measured by Agilent 6890 gas chromatograph equipped with a HP-MOLESIEVE column (30 m × 0.53 mm i.d. × 0.25 μm) and a thermal conductivity detector (TCD). The injector was kept at 150 °C, while the oven temperature was adjusted to 60 °C. The injector was in splitless mode and nitrogen was used as carrier gas.

For the carbon dioxide measurements, Shimadzu GC-2010 gas chromatograph equipped with a TCD and HP-PlotQ column (30 m × 0.53 i.d. × 0.25 μm) was used. The temperature of the injector and oven were 200 °C and 90 °C, respectively. Samples of 50 μL were injected via a split injection port at a split ratio of 0.5:1. Carrier gas was nitrogen at a flow rate of 63.8 mL/min.

2.4.2. Analytics of animal fats

In order to get methyl esters of poultry fat and pig lard, whole

cultures were extracted with 7 mL chloroform. The bottles were shaken for 2 h at 25 °C in a rotary shaker at 150 rpm. The organic phase was transferred into 10 mL Hypo-Vials then chloroform was evaporated using a Savant Speed-Vac Concentrator. A mixture composed of 90% methanol and 10% sulfuric acid solution (2 mL) was added to each sample. Hypo-Vials were sealed with PTFE septa and samples were incubated at 100 °C for 210 min and stirred in every half hour. After the samples cooled down to room temperature, 2 mL of deionized water and 1 mL of chloroform were added. In order to extract fatty acid methyl esters, each sample was stirred vigorously for 2 min. From the organic phase, 10 μL aliquot was transferred into a 0.3 mL interlock crimp top vial and diluted with 990 μL chloroform and stored at −80 °C till further use. In each independent experiment, standards of 10 mg, 50 mg, 100 mg, 150 mg and 200 mg pig lard and poultry fat were also prepared and methyl esters were created; the analyses were always calibrated.

An Agilent 6890 gas chromatograph equipped with HP-Ultra-2 column (25 m × 0.2 mm × 33 μm) and flame ionization detector (FID) was used for measuring methyl esters. 1 μL sample was injected with an Agilent 7683 autosampler. Inlet was set in split mode with a split ratio of 30:1. Carrier gas was H_2 (purity: 5.0). Initial oven temperature was 90 °C then the following heating profile was used: heating to 120 °C at a rate of 30 °C min^{-1}, then to 210 °C at 10 °C min^{-1}, 210 °C for 2 min, heating to 280 °C with a 50 °C min^{-1} heating rate. In order to identify the compounds corresponding to the peaks, the samples were split between FID and an Agilent 5975C mass spectrometer equipped with electron impact ionization cell and simple quadrupole ion analyzer. All relevant peak areas coming from the FID signal were summed and considered as fatty acid content of a sample. Fat concentrations were calculated from calibration curve. Peaks were identified from the mass spectra in the NIST08 Mass Spectral Library and the data obtained for saturated and unsaturated fatty acids were combined, respectively.

2.4.3. Thin layer chromatography

Thin layer chromatography was used for monitoring the lipid degradation. The cultures were grown (as above) for 0, 2, 4, 5 and 7 days and used for analyses. Cell free samples were used as negative controls. The samples were extracted with chloroform, the solvents were evaporated and the dried materials were suspended in hexane. TLC Silica gel 60 F_{254} (Merck: 1.05554.0001) was used for monitoring the degradation of animal fats (mobile phase: hexane:diethyl ether:acetic acid (70:28:2)) (Čipinytė et al., 2009). The spots were visualized with 1% $KMnO_4$.

2.5. Analysis of data

Each experiment carried out with 3 parallel samples was repeated using independent starters leading to 6 datasets. The yield of biodegradation was calculated by the following equation:

Bioconversion yield (%) = $[(Fats_{control} - Fats_{treatment}/Fat_{control}) \times 100]$. The fat concentrations were calculated according to the calibration mentioned above.

3. Results and discussion

3.1. Detection of the lipase activity

Lipase genes could be recognized in the genome of PR4 (Sekine et al., 2006), thus we assumed that the strain could utilize greasy hydrophobic wastes. The lipase activity of the cells was qualitatively assayed. The lipase hydrolyzes Tween or fatty acids of polyoxyethylene sorbitan into free fatty acids which eventually bind to calcium salt included in the agar plate (see Materials and methods). Thus, white crystal-salt like precipitates appear around the bacteria colony. Fig. 1 clearly shows a visible zone formed due to the intensive lipase activity of the PR4 strain.

3.2. Preliminary biodegradation experiments with animal fats, vegetable oil, butter and margarine

First, the PR4 strain was shown to use oxygen and evolve CO_2 when either pig lard or poultry fat was provided as sole carbon and energy source (Fig. 2). It can be seen, that both O_2 consumption (Fig. 2 A) and CO_2 evolution (Fig. 2 B) start immediately and O_2 is completely taken up within three days. The biodegradation capacity of the strain was also tested on other substrates, such as butter, margarine and vegetable oil (VO, sunflower oil). From the results illustrated in Fig. 2, it was concluded that the PR4 strain could utilize VO, butter and margarine. The oxygen utilization of this strain was fast in the case of VO, oxygen was completely consumed within 3–4 days. The time dependent oxygen concentration profile of margarine was similar to that of VO, but in the experiments with butter, the oxygen consumption was completed only after a week.

PR4 strain was able to utilize all substrates tested but the efficiencies and time dependence varied with the substrates (Fig. 2). In the next sections, we focus on the biodegradation of pig lard and poultry fat.

3.3. Monitoring the consumption of animal fats

In the following experiments, fat consumption was monitored by GC–MS and TLC analysis.

Fatty acids were analyzed as their methyl-ester (FAME) derivatives by GC–MS. Four large peaks were separated on the chromatogram in both cases. They were assigned to methylated palmitoleic acid, palmitic acid, oleic + linoleic acid and stearic acid. Small amount of methyl-myristic acid was also present in both samples. The ratio of the various fatty acids in pig lard and poultry fats corresponded to the data of USDA (see 2.1 section). In pig lard, four other minor FAME peaks could be detected, but their amount was unimportant.

In a preliminary study, the efficiency of fat biodegradation in flasks containing MSM (pH = 6.8) was monitored by GC–MS. After a week 18% (poultry fat) and 21% (pig lard) conversion yields were obtained. Since oxygen was not completely consumed, oxygen might not be the limiting factor. However, the pH of the media dropped to pH = 4.0 in 2 days (data not shown) which might

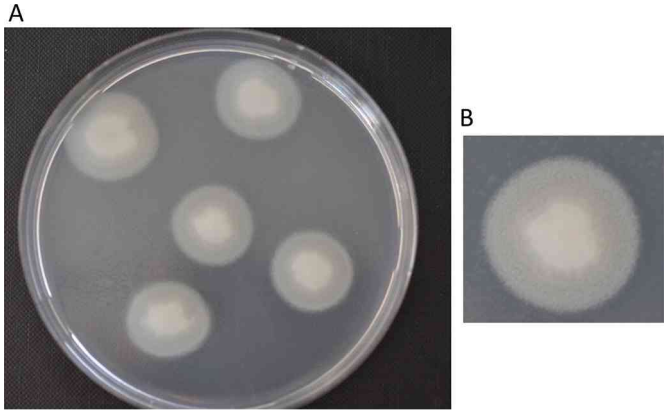

Fig. 1. Agar plate assay for lipase activity. The calcium salts of fatty acids appeared around the colonies of PR4 (A). One selected colony with calcium fatty acid salt ring is zoomed in (B).

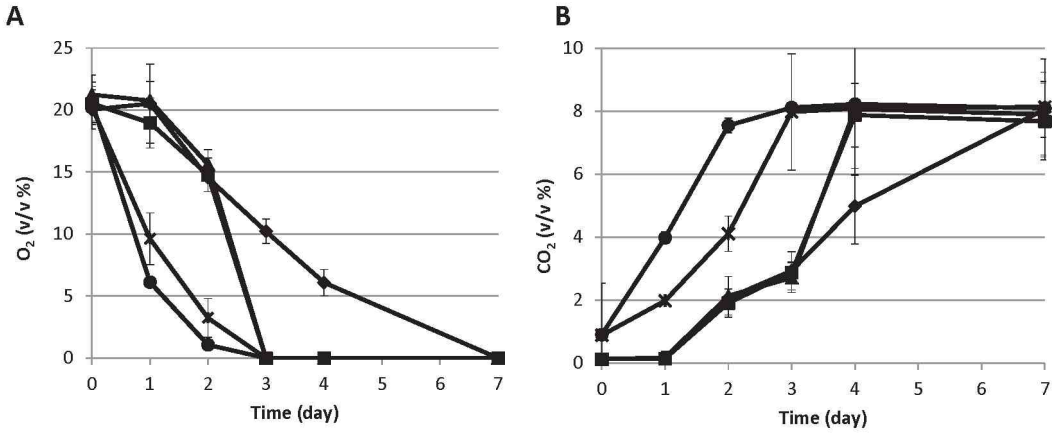

Fig. 2. O_2 consumptions and CO_2 emissions of strains grown on poultry fat, pig lard, butter, margarine and vegetable oil. Oxygen consumption (A), carbon dioxide evolution (B), poultry fat: circle, pig lard: star, butter: diamond, margarine: square and cooking vegetable oil: triangle.

potentially block the metabolism of the cells. Additionally, the intermediate products of fermentation might also diminish the activity of the cells. Replacing the "used" medium with fresh one after two days led to a slight increase in the degradation efficiency of poultry fat (29%), while the utilization of pig lard (10%) remained practically the same as before. Therefore, the low yield could not be explained by product inhibition.

Next, the effect of the initial pH of the media was tested. Varying the initial pH of the MSM between 6.0 and 9.0 showed that the PR4 strain could degrade fats with the highest conversion: the yield was better than 80% (pH = 8.5) for poultry fat and around 70% for pig lard (pH = 8.0) (Fig. 3 A, B). The pH also dropped in each case suggesting that a) there was an intensive acid production and b) the buffering capacity of the medium was not enough. From these data, pH seemed to be a critical parameter of decomposition which must be kept at or near to the optimal value. It might be solved by either controlling the pH during fermentation or by increasing the buffering capacity of the medium. Since, the pH cannot be easily controlled during *in situ* applications, the pH stabilization was aimed by supplementing the MSM with $NaHCO_3$.

3.4. Optimization of the fat biodegradation conditions

In order to increase the efficacy of the biodegradation of fats by *R. erythropolis* PR4, the previously used MSM was supplemented with various buffers at various concentrations. Finally, carbonate as a cheap material was chosen and the initial pH value of the fermentations was adjusted between 6.0 and 9.5.

Carbonate supplementation (CMSM media) dramatically enhanced the conversion yield using both types of fats (Fig. 3C, D). Conversion of fats was measured by fatty acids methyl esters with GC–MS (see Materials and methods 2.4.2.) Biodegradation of

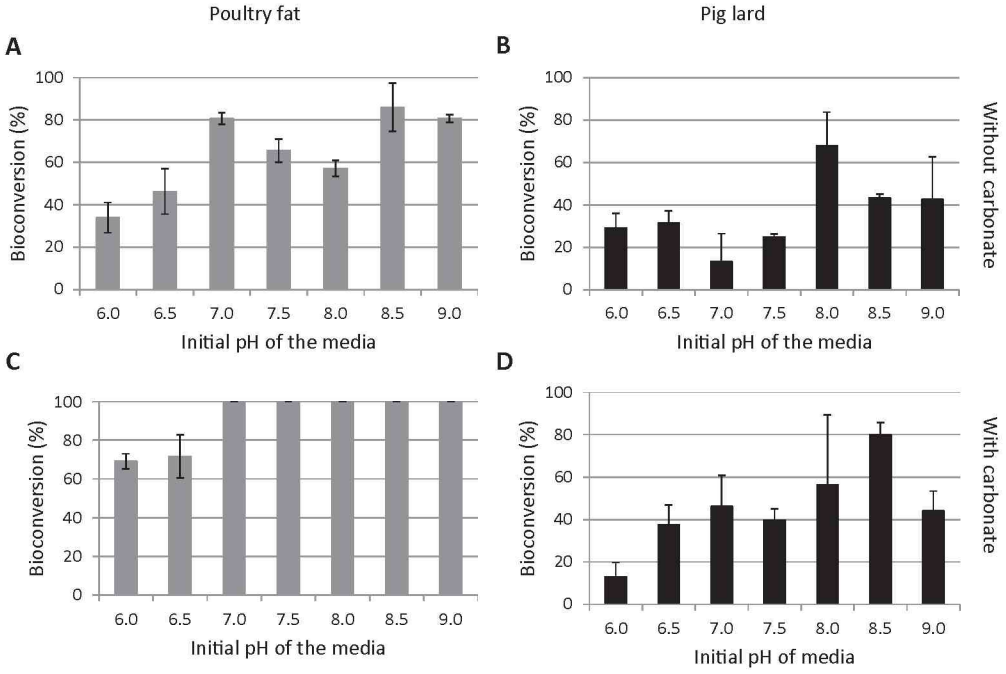

Fig. 3. Bioconversion (%) of poultry fat (A and C) and pig lard (B and D) at various initial pH of the MSM medium (A and B) and carbonate supplemented MSM medium (C and D) after a week. The fat concentration was measured by GC–MS (see Materials and methods).

poultry fat was complete at pH = 7.0 and above. Almost 80% of the initial pig lard content was eliminated from the cultures at pH = 8.5 in a week. The involvement of carbonate resulted in a quite good consumption of poultry fat (72%) at lower pH (pH = 6.0), as well. Additionally, the initial pH of the CMSM medium of the samples remained remarkably stable during the fermentations. These data suggested that addition of carbonates could dramatically improve the bioconversion of animal fats likely due to its pH stabilizing effect. The pH optimum for pig lard degradation was narrow with a relatively sharp maximum in the efficacy at pH = 8.5.

3.5. Time dependence of poultry fat and pig lard consumption

After establishing a good medium for degradation of fats in laboratory conditions, the temporal changes of the fat contents were monitored.

In the experiments above, the optimal pH range for biodegradation was pH = 7.0–9.0 in case of poultry fat, while highest yield was achieved at pH = 8.5 for pig lard. The consumption of these substrates was followed in time at these initial pH values in CMSM media (Fig. 4). Longer time was required for consumption of pig fat likely due to higher viscosity of pig lard. Nevertheless, it could be concluded that the PR4 strain could efficiently convert both types of fats within a reasonable time.

Mass spectrometric analysis was performed to follow the degradation of lipids with saturated and unsaturated fatty acids. The degradation curves illustrated in Fig. 5 were quite similar for fats containing either saturated and unsaturated fatty acids for both substrates (it is also true for the 5 individual fatty acid components identified). The degradation of poultry fat had a lag phase of around 36 h but the substrate was completely decomposed within four days. The conversion of pig lard started immediately, but was incomplete coinciding with the data of Fig. 4.

The biodegradation processes were followed by TLC analysis (data not shown). In the case of poultry fat, in consistency with the GC–MS analysis, the triglycerides disappeared from the medium and much less amount of fatty acids appeared as intermediates. Using pig lard, the amount of the triglycerides also decreased in time but the degradation was slower as compared to the picture obtained for poultry fats.

3.6. Comparison of biodegradation performance of our and other strains

There are a number of studies for degradation of lipids and/or removal of animal fats from various wastes (Wakelin and Forster, 1998; Keenan and Sabelnikov, 2000; Mongkolthanaruk and Dharmsthiti, 2002; Sugimori et al., 2002; Prasad and Manjunath, 2011; Affandi et al., 2014). However, none of them studied the capacity of any Rhodococcus strain for this activity. In a recent study, Prasad and Manjunath (2011) compared the potential of six individual strains (three Bacillus species, one Serratia, one Pseudomonas and one Staphylococcus strain) for degradation of fat-containing wastes such as vegetable oils and dairy wastes. In their experiments, a *Pseudomonas aeruginosa* and a consortium had the best performance for decomposition of lipids: almost complete removal of the contaminant could be achieved within a 12 days period utilizing fluid lipids in 25 g L^{-1} concentration.

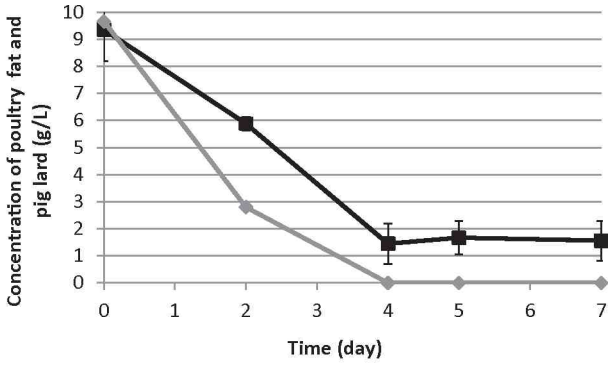

Fig. 4. Biodegradation kinetics of poultry fat and pig lard in CMSM. Poultry fat: gray and diamond; Pig lard: black and square. Methyl-ester derivatives of fatty acids were quantified by GC–MS (for details, see Materials and methods).

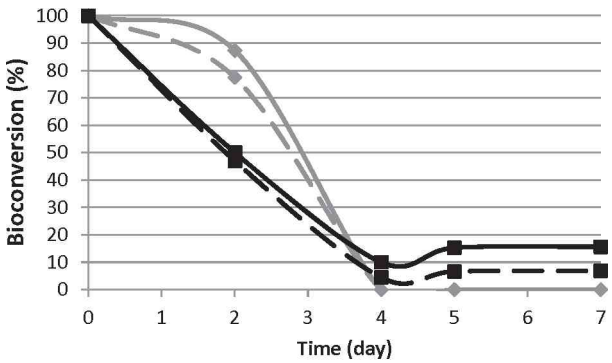

Fig. 5. Bioconversion (%) of saturated fatty acid (continuous line) and unsaturated fatty acid (broken line) in CMSM. Poultry fat: gray and diamond; Pig lard: black and square.

In our study, all substrates were used in 10 g L^{-1} concentration and vegetable oil seemed to be an "easy" substrate for *R. erythropolis* PR4. Moreover, efficient removal of the other lipids, such as poultry fat or pig lard could be achieved within 4–7 days after an optimization of the processes.

4. Conclusion

The *R. erythropolis* PR4 strain was successfully applied for degradation of various hydrophobic kitchen wastes including pig lard, poultry fats, margarine, butter and vegetable oil. The performance of our strain was compared to those published in the literature. Although, the systems are not fully comparable, these data are strong indicators that the PR4 strain is a promising candidate for time-intensive removal/refinery of fat-containing wastes either individually and/or in a properly designed consortium for bioremediation or other purposes.

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