Journal of Applied Polymer Science

Microbial Mediated Dimerization of Fattyacids of Sunflower Oil: An Effective Role of Lipase and Biosurfactant

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ABSTRACT: This study emphasizes microbial mediated transformation of sunflower oil to an adhesive product and characterization in detail. Marine bacterial isolates *Bacillus* (MTCC 5514), when grown in mineral medium, releases both hydrolytic enzymes and surface-active components during the log phase of growth. When this species was grown in the presence of sunflower oil at an optimized concentration of 5% (w/v) under room temperature, enzymatic hydrolysis of oil proceeds with the release of fatty acids and glycerol. Further, on increasing the incubation period, the presence of surface-active components, lipase and glycerol, influence the dimerization of the fatty acids, which further, transformed to a polymerized product sunflower oil-based adhesive product with adhesive nature. Liquid chromatography-mass spectrometry (electrospray ionization) analysis further authenticates the presence of dimeracids. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40555.

KEYWORDS: adhesives; biopolymers and renewable polymers; biosurfactant; lipase; marine *Bacillus sp*; polycondensation

Received 18 November 2013; accepted 3 February 2014
DOI: 10.1002/app.40555

INTRODUCTION

Triglycerides and their constituents are the major energy sources for human life and are also used for the synthesis of other valuable products. Triglycerides upon lipolysis generate free fatty acids and these free fatty acids act as a precursor in numerous polymerization reactions. According to Cowan[1] and Berman and Loeb,[2] heating of fatty acids in the presence of radical, oxidation and using cationic sources, respectively, produces dimer fatty acids. For commercial preparation of dimers, fatty acids are thermally transformed at 230°C for 4–8 h using clay (montmorillonite) as catalysts.[2–4] Tall oil fatty acids containing both oleic and linoleic acids are frequently used for such dimerization processes.

Furthermore, the current scenario on bioprocesses for product development involves microbes and enzymatic catalysis, which favors less pollutants generation. Among the enzymes, the role of proteases is well reported both at basic as well as applied level. Lipase, an important enzyme in the field of detergents, showed extensive applications due to its hydrolytic potential of fats/oils. In addition, enzyme lipase is involved in the condensation reaction; as a result most of the biodegradable polymers are prepared from lipase mediated polymerization processes.[5]

With regard to microorganisms, aerobic microorganisms, in general, when exposed to triglycerides in aqueous medium, the prevalent interfacial tension restricts the growth.[6] However, in some cases, release of lipase and surface-active agents reduce the interfacial tension and subsequently promote the growth. Numbers of reports are available on production and utilization of lipase and surface-active agents from microbial sources. In addition to assisting the growth of these organisms, these agents are also involved in the biotransformation of substrates when the environment is conducive, which is supported by our earlier findings.[7–9] Recently, we have identified a bioadhesive product upon biotransformation of soybean oil using marine *Bacillus sp*. MTCC 5513 ETW 1.[8] Interestingly, this biotransformation occurred only with soybean oil and not with other oils such as sunflower oil. Although both soybean and sunflower oil contain unsaturated fatty acids, the question on why the transformation does not take place in sunflower oil, remains unanswered. Thus, we probe further to look for other marine organisms and other vegetable oils that can undergo biotransformation and finally found a marine organism that can transform sunflower oil to an adhesive product. In this study, we describe hypothetically and instrumentally the process of transformation of sunflower oil to an adhesive glue.

In recent times, synthesis of polymers using vegetable oils exhibit appreciable properties at reduced costs and number of patents are in the public domain, in which vegetable oil as starting material.[10] Research on polymerization of oleic acid and evaluation of suitable catalysts and process conditions are from the past 20 years and the major choice for the catalyst is diisocyanates, a toxic chemical.[11] Thus, in order to obviate the use of toxic components, enzyme mediated dimerization processes

have been suggested,[12] in which commercial enzymes have been used which provides products with molecularly pure and ordered structures. However, process optimization and the conditions to keep and maintain the activity of the enzymes are the major limiting factors for the implementation of these processes and the feasibility of the processes is uncertain at commercial level.

Thus, with reference to the demand and the unanswered questions on transformation of vegetable oils by the suitable microbes and microbial products, this study has been taken up to evaluate microbial mediated *in situ* dimerization of unsaturated fatty acids released upon enzymatic hydrolysis of sunflower oil followed by characterization of the dimerized polymer product designated as Sunflower oil-Based Adhesive Product (SUBAP). Although, the study has been initiated with all the available vegetable oils, viz., soybean, sunflower, coconut, seasame, mustard, and peanut, the said microbial-mediated transformation was realized only with soybean and sunflower oils. Furthermore, the selection of microbial species also play an immense role as evidenced from the results and observation with soybean oil during 2010.[8] Hence, this study has been attempted with sunflower oil and exemplifies the observations.

EXPERIMENTAL

Microorganism

A marine bacterial isolate *Bacillus sp.* MTCC 5514 isolated from marine sediments, identified, characterized, and submitted to culture bank (IMTECH) and deposited the 16s rDNA sequence to NCBI sequence deposit with the accession number HM145910.

Substrates

Refined sunflower oil, obtained directly from the manufacturers (Kaleesuwari Refinery) was used as a substrate. The fatty acid composition of the oil (wt %) was as follows; palmitic acid (7%); stearic acid (5%); oleic acid (19%); linoleic acid (68%); linolenic acid (1%); and the acid value were in the range of 0.9–1.1%.[13]

Experimental Setup

The growth medium used in this study contains (per liter distilled water); 1 g NH_4NO_3; 2.55 g NaH_2PO_4; 0.5 g $MgSO_4{\cdot}7H_2O$; 0.1 g $CaCl_2{\cdot}H_2O$; 0.02 g $MnSO_4{\cdot}H_2O$; 1 g peptone; 0.5 g glucose; pH 7.8. All the ingredients were mixed and autoclaved at 121°C for 15 min. Prior to inoculation (1×10^6 cells/mL), sunflower oil at different concentration of 0, 1.0, 2.5,5.0,7.5 and 10% (w/v) was added to sterile medium and incubated at 37°C at 200 rpm for 168–240 h individually. Growth medium alone, medium with oil alone and medium with cells alone were the respective blank (control) samples used in this study. Growth of the organism was measured as absorbance at 600 nm in UV-visible spectrophotometer. The interference of oil during optical density measurements was corrected using respective blank sample.

Physical Observations Made During Incubation

Followed by inoculation and incubation, physical observations were made at 12-h intervals. Biochemical and instrumental analyses were carried out at 24-h intervals. Optical microscopy analysis was also made at scheduled time periods. A slow transformation of oil to thread-like structures and then to semisolid product was observed till the completion of the experimental period. The completely transformed product obtained after 240 h of incubation was collected after decanting the medium and stored at room temperature for further characterization as summarized in the following paragraphs.

Characterization of External Medium during the Growth of the Organism

Followed by growth profile analysis, quantification of glycerol in the external medium was made according to Bondioli and Bella[14] by spectrophotometric method using periodate and acetyl acetone. Quantification of lipase activity was made according to Ota and Yamada[15] using emulsion of polyvinyl alcohol and olive oil. pH of the medium was determined using Elico pH meter.

Surface tension analysis of the medium was carried out by plate method according to Nitschke et al.[16] In brief, the trough containing enough volume of the sample (cell free broth) was placed over the platform of tensiometer. To this, a plate (0.2-mm thick cover slip) was immersed in such a way and the force given to pull the plate was measured using GBX-3S tensiometer. Plain water was used for calibration.

Free fatty acids and amino acid[17] content [Thin layer chromatography (TLC)] of the cell-free broth were made at 24-h intervals for the period of 240 h using chloroform : methanol: acetic acid (65 : 24 : 1) and butanol : acetic acid : water (4 : 1 : 1), respectively, and the Rf values were compared with the standards. All the said experimental analyses were carried out in triplicates.

Characterization of the Product

Physical Characterization. Nature, color, solubility, and pH were the parameters analyzed for the product. Solubility of the product was assessed using water, dimethyl sulfoxide, acetonitrile, acetone, methanol, ethanol, ethyl acetate, chloroform, tetrahydrofuran, isoproponal, diethylether, carbon tetrachloride, and hexane. pH of the product was measured using pH paper (Merck, India). Bound water content was measured using Karl Fischer titration method.

Yield. Followed by collection, the product was washed with water and the mass of the product measured using a weighing balance and yield was calculated accordingly.

Instrumental Analyses

The semisolid product further subjected to Fourier transform infrared spectroscopy (FT-IR), thermo gravimetric analysis (TGA), differential scanning calorimetry (DSC), and liquid chromatography-mass spectrometry-electrospray ionization (LC-MS-ESI) analyses as described below. With respect to FT-IR spectroscopy analysis, the resultant product was directly mixed with potassium bromide and the spectrum was obtained after 500 scans in the wavelength range of 4000–400 cm^{-1} using spectrum one Perkin–Elmer model. With respect to TGA, about 5–10 mg of the semisolid product was taken in a platinum TGA pan. Gravimetric analysis was made under inert nitrogen atmosphere (40–60 mL/min), from 30 to 800°C using a temperature

gradient of 10°C/min. Scans were routinely recorded as duplicates using TGA Q50 (V20.6 Build 31). Similarly, DSC analysis was made using DSC Q200 (V23.10 Build 79) calorimetric measurements were made under inert nitrogen atmosphere (50 mL/min), from 0 to 300°C using a temperature gradient of 10°C/min. To confirm the formation of dimers, mass analysis was performed using ion trap LC-MS (Bruker HCT plus) instrument. In brief, the final product as well as the intermediate product in the form of thread-like structures were dissolved individually in methanol [High performance LC (HPLC) grade] and were injected into the Agilent XDB C8 column (150 × 4.6 mm ID) with the mobile phase of acetonitrile : water (87 : 13 % v/v) at the flow rate of 1.0 mL/min to the ESI source of the mass spectrometer. The capillary was operated at 350°C, and the electrospray voltage was set to 4.5 kV. High purity nitrogen was used as the sheath and auxiliary gases, which were set to 50 and 9 psi. Scans were obtained from m/z 100–1000 up to 15 min and all the scans were in positive ion mode.

Test for Adhesiveness

The final semisolid product obtained from the experimental section was tested for its adhesiveness property initially by finger string tests according to the test method followed[18] and further authenticated by following ASTM standard (ASTM F2255-03). In the first step, the product was placed in between the thumb and index finger, the strings formed and the length of strings was measured when the fingers are released at a time. This test was performed for the product obtained immediately after decanting the medium as well as after 48 h of removal. During ASTM procedure, testing machine INSTRON W3369 was used. In brief, two polypropylene plates of size 75 × 20 mm were taken and the lap ends (30 mm) of both the plates were made rough using salt paper and joined using the product (bioadhesive) as shown below (Figure 1) and compressed.

Excess adhesive was removed and the specimens were kept at room temperature without any disturbance for the period of 2 h. The specimens were then subjected to shear test using INSTRON W3369 using a crosshead speed of 10 mm per minute. The maximum load at which the joint bonding was broken was calculated as adhesive strength and was expressed in kilo Pascal (kPa). Dermobond (Ethicon, India) and gelatin-resorcinol-formaldehyde were used as standards for comparisons. Both these adhesives were functioning as tissue adhesives and/or sealants.

RESULTS AND DISCUSSION

In this study, an attempt was made on biotransformation of sunflower oil to adhesive product mediated through microbial product. The organism chosen was *Bacillus* MTCC 5514, which showed appreciable growth at 37°C in growth medium under shaking conditions. When triglycerides in the form of sunflower oil was given at different concentrations from 1 to 10% (wt %), we observed, oil concentration above 5% significantly affected the growth [Figure 2(a)]. Compared to 1.0 and 2.5%, only at 5.0% concentration visible changes in the oil phase were exemplified and this could be due to, (i) the supplied triglycerides may be at lower concentration (in the case of 1 and 2.5%) for the organism to use for their growth or (ii) the higher concentrations (7.5 and 10%) may prevent the cells to multiply because of the formation of thick film, which restrict the oxygen availability in the medium and ultimately reduces the growth.

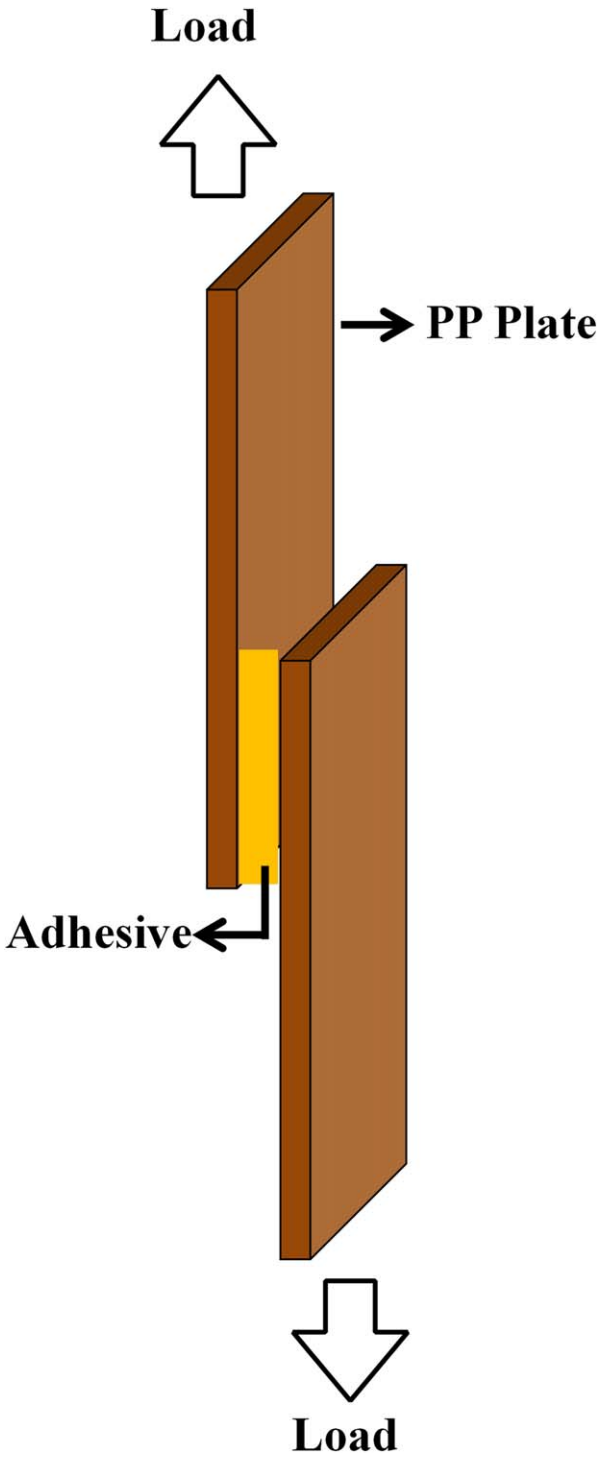


Figure 1. Schematic diagram of lap shear test method followed for the product SUBAP. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

With reference to the hydrolysis of applied sunflower oil during the growth of the organism, Figure 2(b) demonstrates (i) change in pH of the medium, (ii) lipase activity, (iii) release of

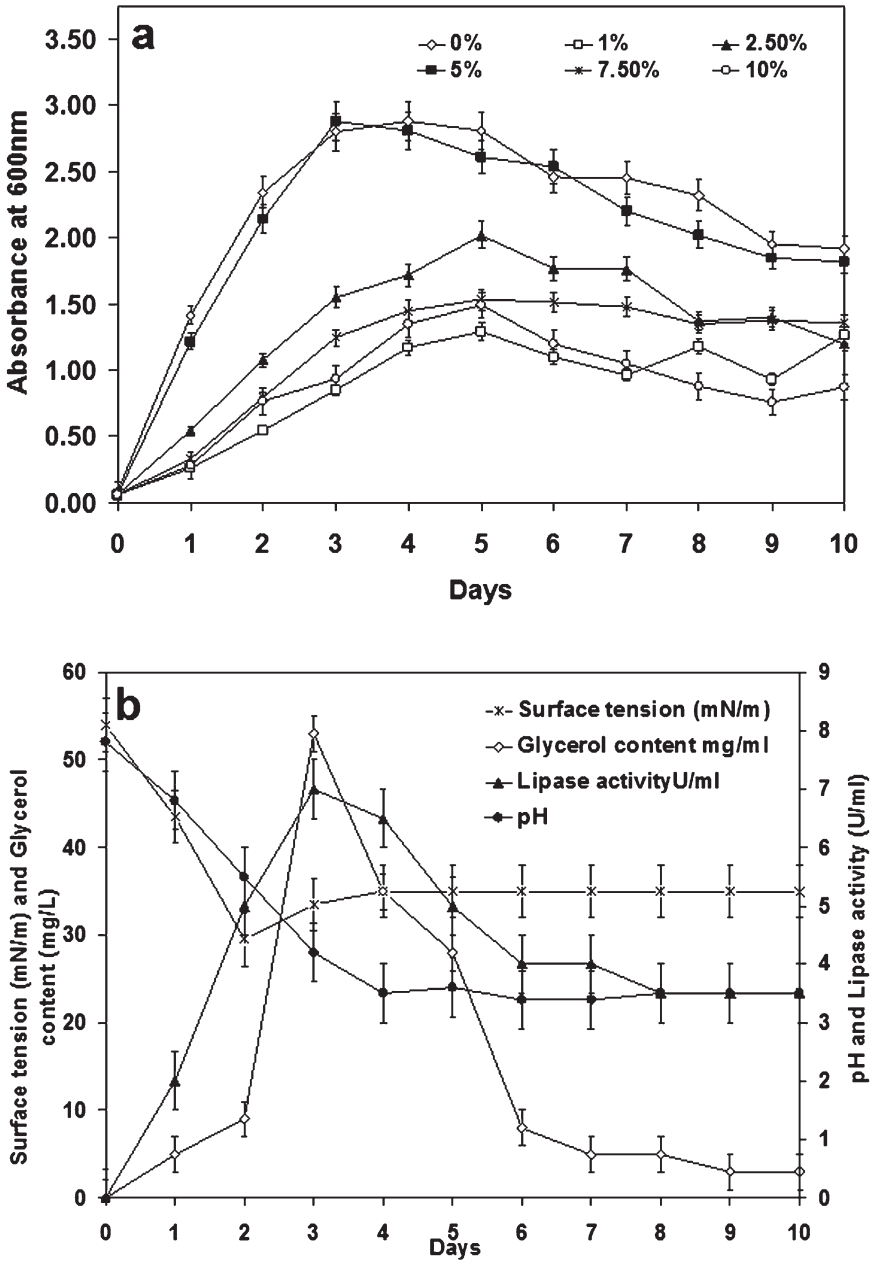


Figure 2. (a) Growth profile of *Bacillus* MTCC 5514 in the presence of different concentrations [0, 1.0, 2.5, 5.0, 7.5, and 10% (w/v)] of sunflower oil. (b) pH, lipase activity (U/mL), glycerol content (mg/mL), and surface tension (mN/m) of the cell-free broth analyzed during the growth of *Bacillus* MTCC 5514 with 5.0% concentration of sunflower oil at different incubation period.

glycerol and (iv) the surface activity measurements made at different days of incubation. pH of the medium slowly decreased from 7.2 ± 0.4 to pH of 3.5 during hydrolysis. Lipase activity showed an initial increase (the maximum of 7 ± 2 U/mL) up to 72 h and then declined to 3.5 ± 0.5 U/mL at 168 h. Similarly, glycerol content showed an increase up to 120 h and the final concentration was measured as 5.0 ± 2.0 mg/mL on the final day of the experiments. With reference to surface activity measurements, the cell-free broth reduced the surface tension of water from 70 ± 2 to 32 ± 4 mN/m. Analysis of free fatty acids (TLC) demonstrated the presence of oleic, linoleic and linolenic acids in the cell free broth.

All the above summarized results confirmed the hydrolysis of oil during the growth of the organism within 72 h of incubation. On further extending the incubation period, we observed a thread-like structures as shown in Figure 3(a) after Day 4. Transformation of these thread-like structures into a semisolid adhesive product named as "SUBAP" observed from Day 8 onward and complete transformation was observed on Day 10. Followed by complete transformation, the product (SUBAP) was stuck on the walls of the flask and difficult to remove. Followed by the removal, adhesiveness of the product was tested as described in the experimental section and as shown in Figure 3(b).

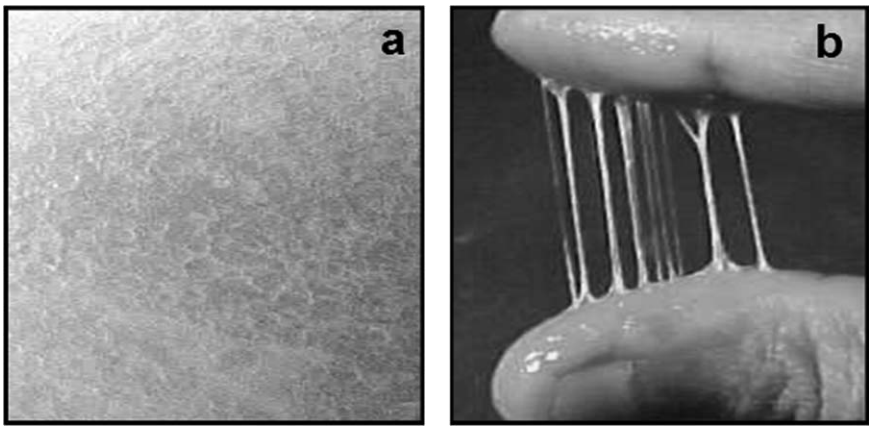


Figure 3. (a) *In situ* transformation of thread-like structure of hydrolyzed fatty acids. (b) Finger test on adhesive product.

Interestingly, we observed lengthy strings (more than 8 cm) while tested immediately after the removal from the flask. However, after 48 h of removal, the length of the strings reduced (>2 cm). These observations suggested the product has high elasticity when it was fresh and after 48 h, the elasticity reduces with increase in adhesiveness. Further to confirm the adhesiveness, plate shear test (lap shear test) was made according to ASTM procedures using INSTRON and the shear strength was calculated as 300–400 kPa. The experiments were repeated for more than five times and the final adhesive strength was observed in the same range as mentioned in the previous lines.

Followed by the adhesiveness tests, the product SUBAP was subjected to various physical and chemical characterizations as described in experimental section. The physical features revealed, SUBAP was a creamy white in color and did not have any irritating odor and solubilized completely with ethyl acetate and methanol. With respect to viscosity, six to seven fold increases in viscosity (measured using Ostwald viscometer after completely solubilizing with ethyl acetate) was realized for the product SUBAP compared to the substrate sunflower oil. Further, liquefaction of SUBAP was observed at 100°C, solidification under freezing and during thawing it immediately regains its original form under room temperature. Further, the pH of the product SUBAP was determined as 6.8 and about 7–7.5% of bound water content was estimated. No significant difference in weight (yield) of the final product and the substrate was observed (Table I).

Figure 4(a) illustrates the FT-IR spectrum of the product with peaks at 3458 cm^{-1} corresponding to a weak broad characteristics band for carboxylic acid; at 2928 and 2856 cm^{-1} responsible for very strong alkyl absorptions; at 1746 cm^{-1} corresponds to a strong band of the ester with a shoulder; at 1704 cm^{-1} for free carboxyl groups and peaks at 1476 and 1375 corresponding to methyl stretch; and peaks at 725–719 cm^{-1} corresponding to —CH rocking or dimer acids. The presence of dimers in SUBAP was further authenticated with the reports of Bajpai and Khare,[19] where these authors observed dimer formation in oleic and linoleic acid through FT-IR analysis and suggested dimerization may be proceeded either at unsaturation position or at carboxylic acids. However, it needs further explorations. Similarly, Harry-O'kuru et al.[20] observed formation of estolides/dimers of oleic acid in the presence of perchloric acid under agitation and Hayes and Kleiman[21] reported enzyme catalyzed, especially lipase-catalyzed estolides. Yoshida et al.[22] summarizes, dimer acid is ultimately limited by the availability of C18 and approximately 80–85% of dimer acid comes from tall oil fatty acids and the balance is based on oleic acid feed stock.

Table I. Characteristics of the Substrate (Sunflower Oil) and Product SUBAP Obtained on *In Situ* Transformation of Sunflower Oil

S. No	Characteristics	Substrate (Sunflower oil)	Product (SUBAP)
1	Color	Pale yellow	Creamy white
2	Odor	Slight odor	Odorless
3	Appearance	Clear, oily liquid	Opaque, thick gummy mass
4	Viscosity (cps)	19.21×10^{-3}	128.67×10^{-3}
5	Weight (yield; g)	5	5
6	Solubility	Completely soluble in ethyl acetate	Completely soluble in ethyl acetate
7	Freezing at −20°C	No change	Solidify
8	Thawing	No change	Semi solid mass
9	Heating at 100°C	Boiling	Liquefy
10	Bound water (%)	0	7–7.5

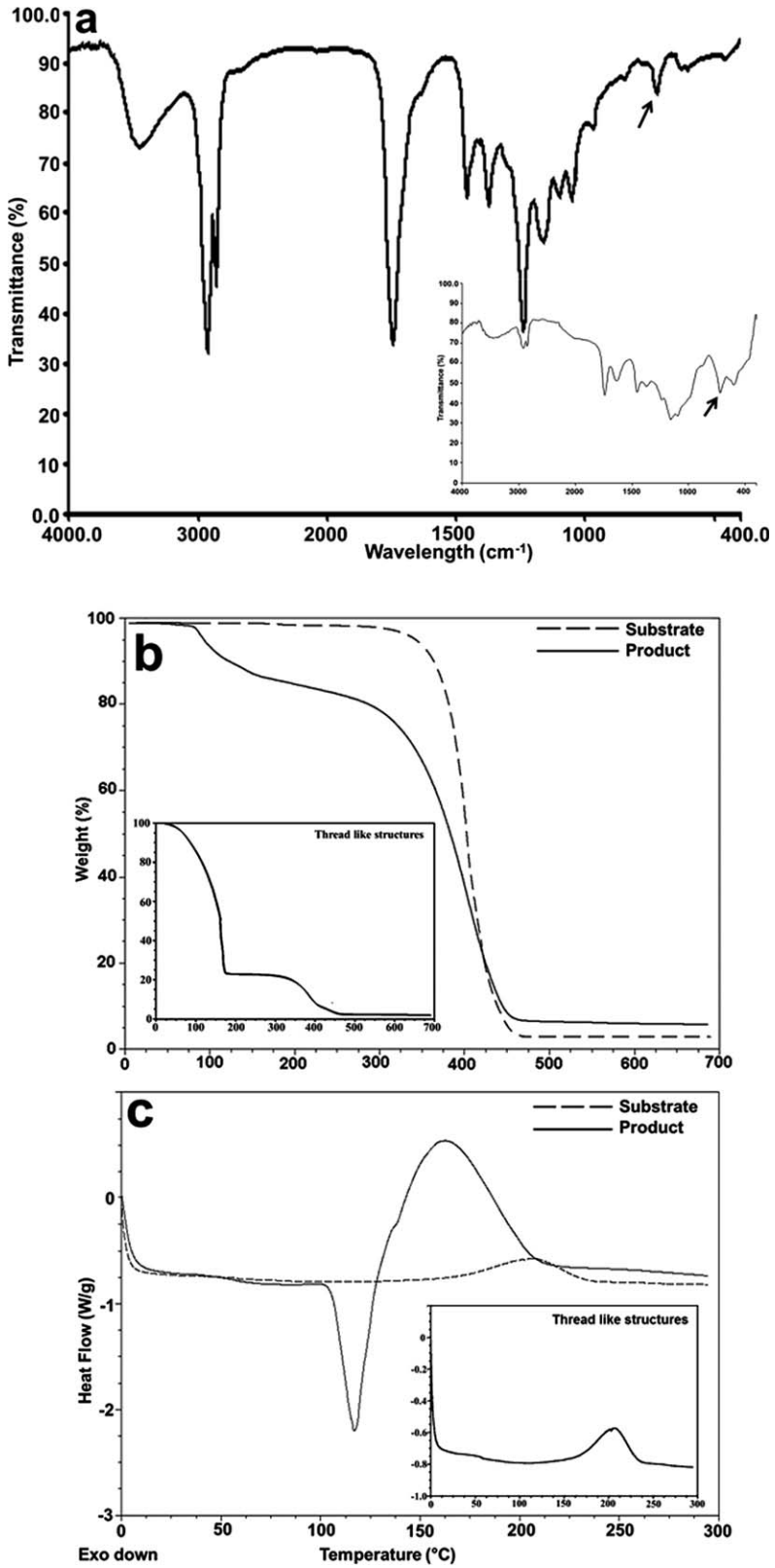


Figure 4. (a) FT-IR spectrum of the adhesive product—SUBAP Inserted figure: IR spectrum of the thread-like structures (Black arrow indicates the presence of the peak responsible for the dimers); (b) TGA of the substrate and the adhesive product (SUBAP). Inserted figure: TGA of the thread-like structures; and (c) DSC analysis of the substrate and the adhesive product (SUBAP). Inserted figure: DSC analysis of the thread-like structures.

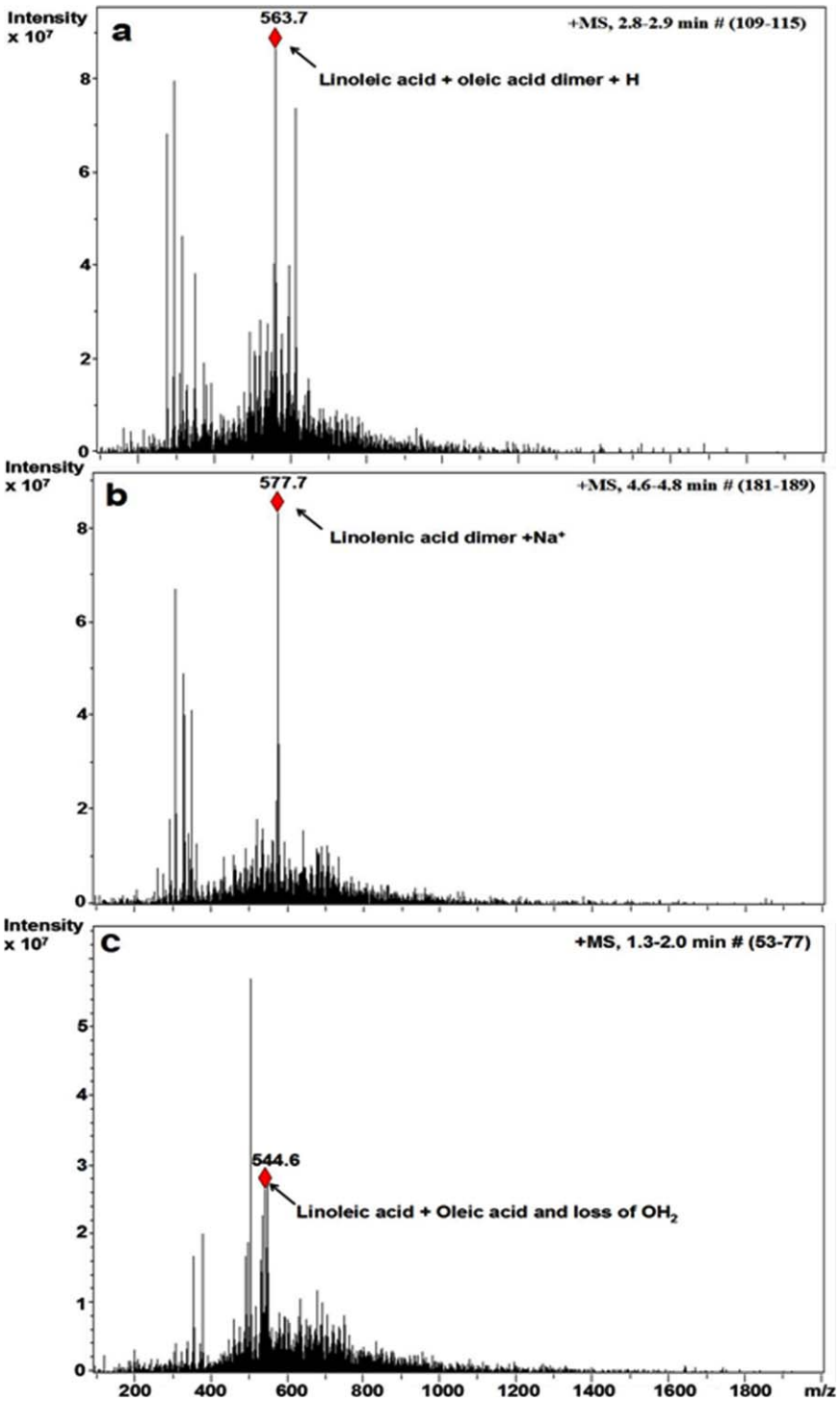
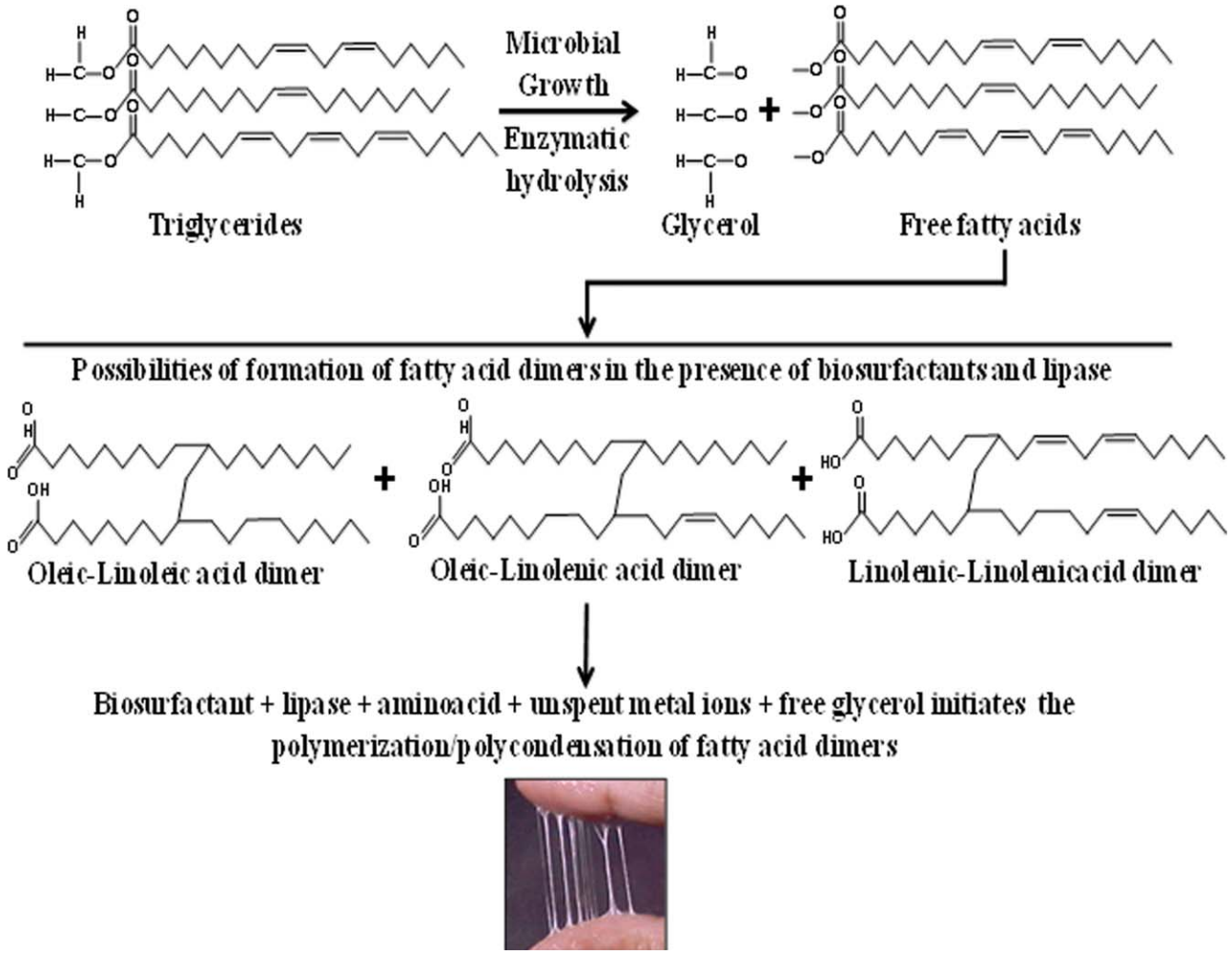


Figure 5. (a–c). LC-MS (ESI) spectra of fatty acid dimers present in the product (SUBAP) (a and b) and thread-like structures (c). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TGA [Figure 4(b)] of SUBPA revealed, the substrate sunflower oil showed high stability up to 328°C and further increase in temperature decomposition starts with a reduction in the mass percentage weight at 477°C. However, TGA of the adhesive product SUBAP showed initial reduction in mass percentage of 1.5 at 98.5°C due to the presence of bound water and the decomposition observed at 288°C with a mass reduction of 18.5% could be due to the presence of a meager percentage of other free biomolecules. And, at 463°C only 5% mass was remaining. With respect to the TGA of thread-like structures, initial mass reduction was observed at 175°C and stable till 350°C. After 450°C, the residual mass was only 3%. [Figure 4(b)].



Scheme 1. Schematic explanation on the biotransformation of sunflower oil to adhesive product mediated through marine *Bacillus* MTCC 5514. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4(c) depicts DSC thermogram of the sunflower oil and the product SUBAP. With respect to substrate sunflower oil and thread-like structures [Figure 4(c)], both did not show any transition, but the product SUBAP showed an initial crystallization at 116°C and further increase in temperature melting with decomposition starts at 161°C. This could be due to the increase in length of the fatty acid units after polymerization.[23] These observations were similar to the observations made by a number of researchers on polymers.[24,25]

Figure 5(a–c) shows the LC-MS-ESI analysis of the SUBAP and the intermediate thread-like structures, respectively. Mass analysis coincides with the hypothesis (Scheme 1) elucidated by the authors and authenticates the presence of dimers in the product. Dimers may be formed by the addition of a proton or by the loss of water of two individual fatty acid molecules as shown in Figure 5. The possible dimers were identified as linoleic acid–oleic acid dimers (m/z 563.7); linolenic acid dimers (m/z 577.7); and linoleic acid–oleic acid dimers (m/z 544.6).

The above summarized results imply, without any physical or chemical agents, triglycerides are transformed into a semisolid adhesive product mediated through microbial products. Among the microbial products, the surface-active components and the enzyme lipase play a major role as the number of literatures suggested the condensation and the polymerization reactions[26] and the intramolecular network are effective in the presence of enzyme and surface-active agents. Markevich et al.[27] detailed the role of surfactant in increasing the adhesive nature of the product, where they used synthetic surfactants.

Furthermore, formation of thread-like structures during the growth of the organism may be considered as the dimers of fatty acids of triglycerides oligomers and these dimers were the precursors for the product formation as evidenced from the mass analysis. FT-IR analysis of the thread-like structures and the product showed a band at 725–700 cm^{-1} [Figure 4(a)].

Thus, followed by dimerization, the reaction may be further proceeded and provided the product with adhesiveness. The product formation may take place via the dimerization of oleic acid + oleic acid; or oleic acid + linoleic acid; or linolenic acid- + linolenic acid as shown in Scheme 1.

Table II. Comparisons on the Process and Product Obtained from Two Different Vegetable Oils

S. No	Description	Substrates	
		Soybean oil	Sunflower oil
1	Microorganisms used	*Bacillus sp.* (MTCC 5513)	*Bacillus sp.* (MTCC 5514)
2	Growth medium used	Zobell marine broth	Mineral medium
3	Lipase activity (U/mL)	7 ± 2	7 ± 2
4	Biosurfactant activity (mN/m)	30 ± 4	32 ± 4
5	Quantity of oil used (%)	10	5
6	Yield of the product (g)	21 ± 2	5 ± 0.5
7	Shear Strength (kPa)	150–200	300–400
8	Bound water content (%)	12–15	7–7.5
9	Name of the product	FAbBP	SUBAP

It has been stated that, dimerization followed by product formation could also be effected by the presence of (i) biosurfactant; (ii) free amino acids; (iii) unspent metal ions; (iv) free glycerol; and (v) monomers of triglycerides in the medium. With regard to dimerization in aqueous medium, Lyons,[28] Wheeler and Godfrey[29] and Isbell[30] reported less percentage of water increases the yield of dimers. However, in this study, dimerization was evidenced in the aqueous medium.

The results of the study provided the answer for the question on why there was an inconsistency in the biotransformation of vegetable oil in the presence of microbes. For example, we found biotransformation of soybean oil with the bacterial isolate *Bacillus* MTCC 5513-ETW 1[8] and no transformation observed with sunflower oil. Nevertheless, in this study, biotransformation of sunflower oil was with *Bacillus* MTCC 5514. The results of both the studies reveal, other than the percentage difference in the fatty acid constituents and the nature of the vegetable oils, nature of microbial species and the surface-active agents produced, play a major role in the biotransformation.

Comparative Assessment

Table II depicts the comparisons on the microbial-mediated process and the product obtained from two different vegetable oils. It has been understood that the individual fatty acids of vegetable oils and the process of dimerization has impact on the adhesive strength of the biopolymer material as discussed in the previous paragraphs.

CONCLUSIONS

This study emphasizes that the release of hydrolytic enzymes (lipase) during the growth of the organisms hydrolyze the triglycerides of sunflower oil. The fatty acids thus released dimerized in the presence of surface-active agents and other metal ions (as evidenced from FT-IR and ESI-MS analysis), which on further increase in incubation period results with the polymerized product with appreciable adhesive property as tested. The observations made in this study infers that effective dimerization of fatty acids and the resultant adhesive product without incorporating any external harmful agents may be considered as the green method of preparation of adhesive product with immense applications.

ACKNOWLEDGMENTS

All the authors acknowledge the Department of Biotechnology, Ministry of Science and Technology, New Delhi, India for their financial support in the form of project (BT/PR8204/ AAQ/03/ 302/2006). One of the authors thanks the Council of Scientific and Industrial Research (CSIR), New Delhi, India for financial assistance in the form of Senior Research Fellowship (SRF).

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