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# Microbial Production of Polyhydroxyalkanoates by Bacteria Isolated from Oil Wastes

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## Abstract

A Gram-positive coccus-shaped bacterium capable of synthesizing higher relative molecular weight ($M_r$) polyhydroxybutyrate (PHB) was isolated from sesame oil and identified as *Staphylococcus epidermidis* (by Microbial ID, Inc., Newark, NJ). The experiment was conducted by shake flask fermentation culture using media containing fructose. Cell growth up to a dry mass of 2.5 g/L and PHB accumulation up to 15.02% of cell dry wt was observed. Apart from using single carbohydrate as a sole carbon source, various industrial food wastes including sesame oil, ice cream, malt, and soya wastes were investigated as nutrients for *S. epidermidis* to reduce the cost of the carbon source. As a result, we found that by using malt wastes as nutrient for cell growth, PHB accumulation of *S. epidermidis* was much better than using other wastes as nutrient source. The final dried cell mass and PHB production using malt wastes were 1.76 g/L and 6.93% polymer/cells (grams/gram), and 3.5 g/L and 3.31% polymer/cells (grams/gram) in shake flask culture and in fermentor culture, respectively. The bacterial polymer was characterized by $^{1}$H-nuclear magnetic resonance (NMR), $^{13}$C-NMR, Fourier transform infrared, and differential scanning calorimetry. The results show that with different industrial food wastes as carbon and energy sources, the same biopolymer (PHB) was obtained. However, the use of sesame oil as the carbon source resulted in the accumulation of PHB with a higher melting point than that produced from other food wastes as carbon sources by this organism under similar experimental conditions.

**Index Entries:** *Staphylococcus epidermidis*; polyhydroxybutyrate; food wastes.

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## Introduction

Microbial polyesters play an important role within the group of biodegradable plastics in the environment and solid wastes management. A number of these materials are already available on the market *(1,2)*. For this reason, the demand for these materials for commercial usage will intensify research and development in this field.

Polyhydroxybutyrate (PHB), which is a member of the microbial and biodegradable thermoplastics family polyhydroxyalkanoate (PHA), can be synthesized biologically by numerous microorganisms as a carbon and energy storage material under unbalanced nutrient conditions, such as nitrogen, phosphorus, oxygen, or trace element deficiency. Some reports indicate that PHB as a carbon and energy reserve is related to polyphosphates, which act as phosphorous reserve. PHB has been implicated as an energy source in the symbiotic nitrogen fixation process of some bacteria strains *(3)*. PHBs can occur in two different forms in the bacteria cell. In most microorganisms, they occur as inclusion bodies and are deposited in granules in the cytoplasm. PHBs can also be found in the cytoplasmic membrane from some species *(4)*. Since the isolation of PHB from *Bacillus megaterium* in 1925, this plastic-like polymer has been extensively investigated in both biological and polymer sciences *(5–9)*. Because of its inherent chiral, optically active, biodegradable, biocompatible characteristics and other desirable properties, such as high degree of crystallinity (≅80%), high molecular weight (up to $5 \times 10^5$), high melting point (~175°C), moisture resistance, and piezoelectricity, there are numerous applications of PHA to the technical processes of injecting molding, reinforcing organic fillers, spinning into fiber, and forming into films with excellent gas barrier properties. In addition, their total biodegradability with $CO_2$ and $H_2O$ as products also indicates biocompatibility in the area of medical applications. The products can be used for surgical suture, wound dressing, drug containers, and disposable items such as diapers.

Although the physicochemical properties of PHB, including Young's modulus (3.5 GPa) and tensile strength (40 MPa), are similar to those of polypropylene or poly(ethylene terephtalate) *(10)*, and it is also the most widespread and the best-characterized member of the PHAs, PHB has not been widely commercially exploited because of its high price compared with traditional thermoplastics. Therefore, the use of various industrial food wastes was recently investigated in the production of PHB *(11,12)*. On the other hand, some drawbacks of PHB, such as its crystallinity after solvent extraction, resulting in a rather brittle material protected from biodegradation by its highly crystalline state, and its thermal instability at temperatures close to its melting point, also need to be improved.

A long list of bacterial strains capable of producing PHB has been reported *(5)*. These include Gram-positive and Gram-negative species and cyanobacteria. However, there are few reports on the production of PHB from *Staphylococcus epidermidis*. As with most microbial products, the search

for new producer organisms is a continuous process, necessitated by the desire for higher product yield, more efficient utilization and conversion of specific raw materials, tolerance to environmental conditions, and novel end products. The present study focused on the isolation of bacterial strains, screening the strains for PHB-producing ability and its polymer properties using different industrial food wastes as nutrient sources.

## Materials and Methods

### *Bacterial Isolation and Culture*

The strain *S. epidermidis* was isolated from sesame oil samples of the sesame oil production plant. The bacterial strains were obtained by dilution streaking of agar plates with sesame oil as carbon source containing the following: 10 g/L of sesame oil, 5 g/L of $Na_2H_2PO_4 \cdot 12H_2O$, 2 g/L of $(NH_4)_2SO_4$, 0.4 g/L of $MgSO_4 \cdot 7H_2O$, 0.1% Tween-80. Cultures for PHA accumulation were grown at 35°C at 200 rpm for 48–96 h in 500-mL Erlenmeyer flasks containing medium with 1% (w/v) sesame oil as carbon source. Other mineral media were prepared according to ref. *13*, and adjusted to pH 7.0. Polymer synthesis was carried out through a one-stage cultivation in which cell growth and polymer synthesis occur simultaneously. Reference strain, *Alcaligenes latus* DSM 1124, was obtained from the Department of Biological Science and Biotechnology, Tsinghua University, Beijing.

### *Treatment of Food Wastes Media*

Dry milled malt and semisolid soya wastes were digested with 0.5 *N* HCl at 100°C for 10 h and then centrifuged. Afterward, the centrifuged filtrate was neutralized with NaOH. Liquid ice cream and confectionery wastes were boiled for 1 h to solubilize the undissolved solid first, and then centrifuged, followed by filtration. The clarified liquid was then brought to pH 7.0 with solid NaOH. Before inoculation, the prepared food wastes media solution was autoclaved at 121°C for 20 min. Treated food wastes were analyzed by total organic carbon (TOC) and total Kjeldahl nitrogen (TKN). The C:N ratios of malt, soya, ice cream, and confectionary wastes were 7, 8, 4, and 48, respectively.

### *Fermentation*

Cell culture and polymer accumulation were carried out in the two different kinds of fermentation apparatus. One fermentation was done in 500-mL Erlenmeyer flasks in an orbital shaker (Forma Scientific Model 4518 Table Top Incubator Orbital Shaker, Forma) at 35°C, 200 rpm, and pH 7.0 for 48 h. Culture media for PHB accumulation were included 4% carbohydrate from different kinds of commercial carbohydrate and various kinds of food wastes, and the same concentration of other media as already described. The other fermentation was performed in a computer-controlled

Bioengineering Fermentor (Bioengineering, Wald, Switzerland) with the conditions set as follows: 10% dissolved oxygen (DO), 30°C, pH 7.0, and 500 rpm.

## Polymer Extraction and Purification

The harvested cells were centrifugated, washed twice with deionized $H_2O$, and washed with a small volume of acetone. The polymer was isolated from lyophilized cells by Soxhlet extraction in chloroform according to the method of Cromwick et al. *(14)*. Then the polymers were precipitated by slowly adding methanol. Afterward, extracted polymer was redissolved in chloroform and purified by reprecipitation with hexane several times *(5)*.

## Polymer Analysis

### Gas Chromatography

Esterification of biopolymer with a mixture solution by mixing 97% (v/v) methanol with 3% (v/v) sulfuric acid was prepared first. Then, polymer analysis was performed on a Hewlett Packard 5890A Gas Chromatograph using a 25m Ultra 2 (crosslinked 5% Ph. Me. Silicone) capillary column with a 0.33-μm thick film. One microliter of sample was injected according to a method described by Jan et al. *(15)*.

### $^1$H- and $^{13}$C-Nuclear Magnetic Resonance

The identity of the individual monomer units was confirmed by proton and carbon nuclear magnetic resonance (NMR). $^1$H- and $^{13}$C-NMR spectra were recorded at room temperature in $CDCl_3$ on a Bruker Model AVANCE DPX 400 spectrometer (Bruker, Switzerland) (400 MHz for proton, $^1$H; 101 MHz for carbon, $^{13}$C) in the pulse Fourier transform (FT) mode. Tetramethylsilane was used as an internal reference *(16)*.

### Fourier Transform Infrared Spectrometer

Fourier transform infrared (FTIR) spectra were recorded on a Bruker VECTOR22 FTIR spectrometer. Samples were prepared by dissolving the polymer in chloroform, which was layered on an NaCl plate. After the chloroform was evaporated, the polymer film was subjected to IR analysis *(17)*.

### Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) analysis was performed to study thermal behavior, on a Perkin Elmer 1020 Series DSC7 Thermal Analysis System (Perkin Elmer, Germany) under a nitrogen flow of 30 mL/min. Samples 3 to 4 mg were encapsulated in aluminum pans and heated from the DSC to 100–200°C at a rate of 10°C/min. The melting temperature ($T_m$) and enthalpy of fusion ($\Delta H_m$) were determined from the DSC endotherm. The $T_m$ was taken as the peak temperature *(18)*.

### Viscosity Measurement

The relative molecular weight ($M_r$) was measured through the intrinsic viscosity of polymer dissolved in chloroform determined by using an

Table 1
Cell Growth of *S. epidermidis* in Media with Several Different Commercial Carbohydrates as Sole Carbon Source

| Carbon source (4 g/100 mL) | Cell density (g/L) |
|---|---|
| Sucrose | 2.40 |
| Fructose | 2.50 |
| Mannose | 1.82 |
| Xylose | 1.30 |
| Arabinose | 1.60 |
| Galactose | 2.10 |
| Lactose | 1.50 |
| Maltose | 1.70 |

Ubbelohde type capillary viscometer immersed in a thermostatic bath at 30(±0.01)°C. A series of five consistent flow times were made, and then the $M_r$ was obtained from the calculated instrinsic viscosities and the Mark-Houwink equation, using the constants $K = 1.18 \times 10^{-4}$ and $a = 0.78$, taken from Akita et al. *(19)*.

## Results and Discussion

The simplest first-line screening program for PHA-producing bacteria is the use of Sudan black staining. However, the *S. epidermidis* strain we used is very small (about 0.5–1.5 μm in diameter). Thus, neither Sudan black staining nor Nile blue staining would give a definite indication in the screening of PHA in this strain of *S. epidermidis*. Fortunately, the growth of this bacterial strain on solid or broth media would be pigmented (yellowish), and the more deep yellow cell pigment was found, the more biopolymer extracted from cells was obtained. Consequently, the alteration of cell color can be a good indicator for screening of PHA content in cells while fermentation is in progress. The production in this PHA-producing strain was verified by the analysis of esterificated lyophilized cells with gas chromatography (GC) directly.

For the first part of the experiment, the yields of cell in fermentation with shake flask culture using the commercial carbohydrates sucrose, fructose, mannose, xylose, arabinose, galactose, lactose, and maltose as a sole carbon source (4 g/100 mL) are shown in Table 1. After 48 h of fermentation, each 5-mL sample was analyzed for cell dry weight. The results indicated that higher cell densities were obtained from sucrose, fructose, and galactose used as a sole carbon source. Cell growth up to a dry mass of 2.5 g/L and PHB accumulation up to 15.02% of cell dry weight were obtained when using media based on fructose with shake flask culture. Final biomass and

polymer concentrations were 4.13 and 0.24 g/L dry wt, respectively, or 5.81% of the biomass dry weight was polymer when using sucrose as the carbon source in fermentation with fermentor culture.

In the second part of the experiment, a specific culture of the *S. epidermidis* was selected to ferment several types of food wastes by shake flask and fermentor culture in PHB biosynthesis. These various industrial food wastes were sesame oil, ice cream, malt, and soya wastes. Tables 2 and 3 give the yields of biopolymer and cell growth by the *S. epidermidis* using different food wastes with different fermentation culture. It can be seen that using malt wastes as nutrient for cell growth and polymer accumulation of the *S. epidermidis* gave better results than other wastes as nutrient sources, regardless of the shake flask or fermentor culture. The final dried cell mass and polymer production were 1.76 g/L and 6.93% polymer/cells (grams/gram), and 3.5 g/L and 3.31% polymer/cells (grams/gram) from malt wastes in shake flask culture and fermentor culture, respectively. The data compared with those of prior experiments performed in our laboratory, using different food wastes by *A. latus* as carbon source, indicated similar results: higher cell mass and higher polymer content were obtained when using malt wastes as nutrients. Hence, malt wastes was considered the best media to induce bacteria to grow and produce PHB.

The composition of the polymer extracted from the isolated *S. epidermidis* was determined by GC, $^{1}$H- and $^{13}$C-NMR, FTIR, and DSC analysis (*see* Figs. 1–3 and Tables 4 and 5). From the GC spectrum (Fig. 1), peaks of standard PHB and benzoic acid (internal standard) were seen at retention times of 3 and 7 min, respectively, which were identical to the retention time of extracted polymer. The $^{1}$H-NMR spectrum (Fig. 1) showed the presence of three groups of signals characteristic of the homopolymer PHB, and the $^{13}$C-NMR spectrum (Fig. 2) showed that there are four absorption bands at 19.75, 40.77, 67.60, and 169.13 indicating the presence of four different carbons. The FTIR spectrum (Fig. 3) showed that there was a remarkable C = O peak at wave number 1738. All these results indicate that with reference to the standard PHB, the polymer produced by this *S. epidermidis* was identified as PHB (GC and NMR spectra of standard PHB; *see* ref. *12*). Regardless of the type of food wastes used as carbon source, only one type of polymer was produced by this microorganism.

Tables 4 and 5 give the results of the thermal analyses of biopolymer produced by the *S. epidermidis*. The results indicate that the melting point ($T_m$) of the polymer produced from sesame oil as carbon source was 187.85°C, the highest temperature among those using ice cream, malt, and soya wastes as nutrients. The results of enthapy ($\Delta H_m$), which is related to crystallinity, indicate that polymer produced from single carbohydrate had higher crystallinity than that produced from food wastes. By comparing two kinds of fermentation culture, we also found that higher crystallinity was obtained in fermentor culture than in shake flask culture. For the fermentor culture, the conditions of DO, temperature, and pH were controlled.

Table 2
Production of Biopolymer by *S. epidermidis* Using Different Food Wastes as Nutrients in Fermentation with Fermentor Culture

| Carbon source | Culture mode | Polymer | Cell density (g/L) | Polymer density (g/L) | Polymer content (%) | Cell productivity (g/[L·h]) | PHB productivity (g/[L·h]) |
|---|---|---|---|---|---|---|---|
| Sucrose | Fermentor | PHB | 4.13 | 0.240 | 5.81 | 0.067 | 0.00333 |
| Sesame oil | Fermentor | PHB | 2.50 | 0.067 | 2.68 | 0.035 | 0.00093 |
| Malt wastes | Fermentor | PHB | 3.50 | 0.116 | 3.31 | 0.049 | 0.00161 |
| Soya wastes | Fermentor | PHB | 3.20 | 0.058 | 1.81 | 0.044 | 0.00081 |

Table 3
Production of Biopolymer by *S. epidermidis* Using Different Food Wastes as Nutrients in Fermentation with Shake Flask Culture

| Carbon source | Culture mode | Polymer | Cell density (g/L) | Polymer density (g/L) | Polymer content (%) | Cell productivity (g/[L·h]) | PHB productivity (g/[L·h]) |
|---|---|---|---|---|---|---|---|
| Fructose | Shaking flasks | PHB | 2.50 | 0.380 | 15.20 | 0.052 | 0.00792 |
| Ice cream wastes | Shaking flasks | PHB | 1.00 | 0.015 | 1.50 | 0.021 | 0.00031 |
| Malt wastes | Shaking flasks | PHB | 1.76 | 0.122 | 6.93 | 0.037 | 0.00254 |
| Soya wastes | Shaking flasks | PHB | 1.25 | 0.043 | 3.46 | 0.026 | 0.00090 |

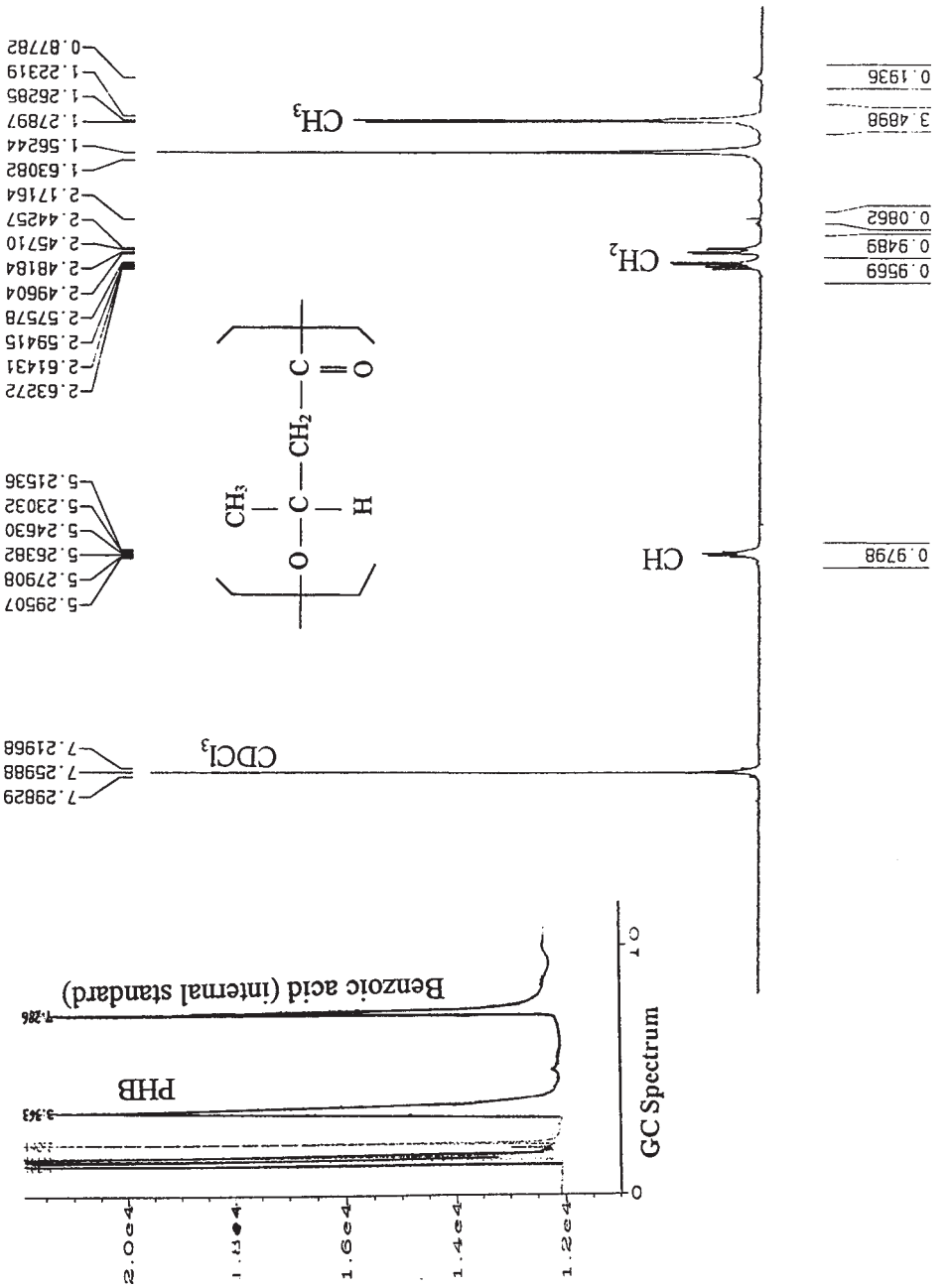


Fig. 1. GC and $^1$H-NMR spectrum of biopolymer produced by *S. epidermidis* using food wastes as nutrients.

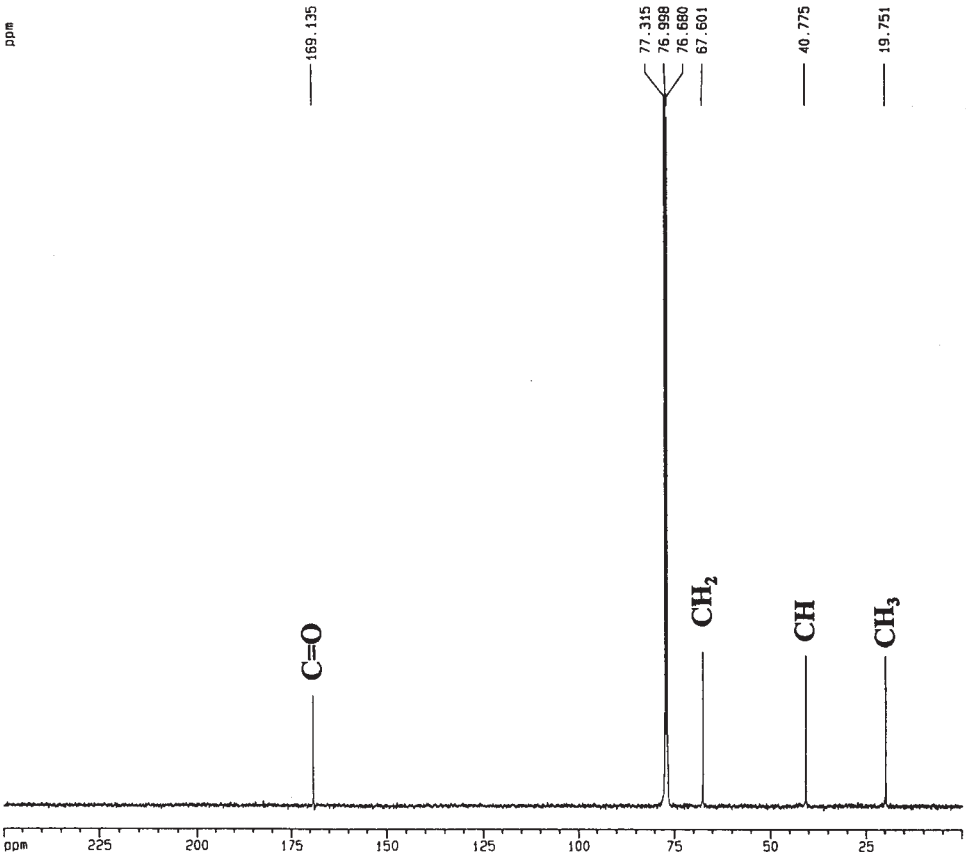


Fig. 2. $^{13}$C-NMR spectrum of biopolymer produced by *S. epidermidis* using food wastes as nutrients.

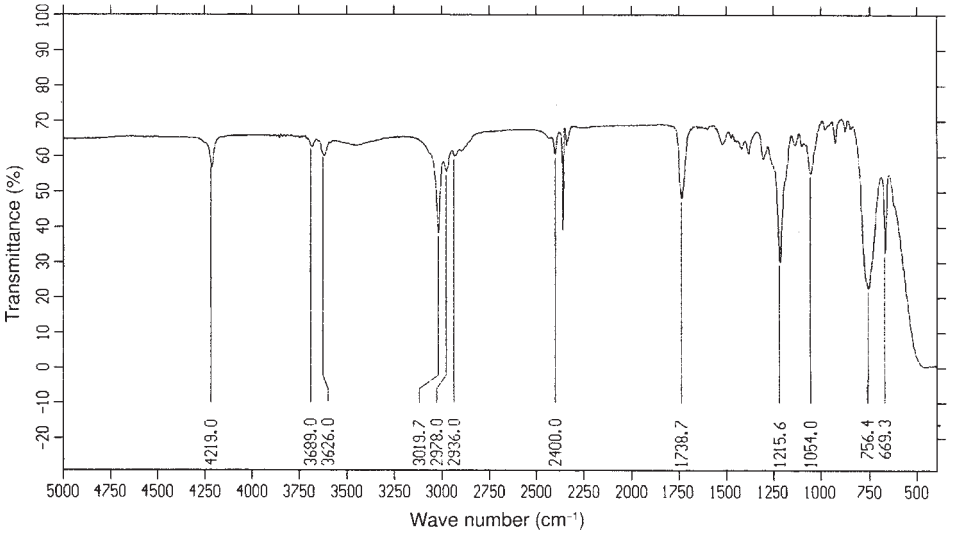


Fig. 3. FTIR spectrum of biopolymer produced by *S. epidermidis* using sucrose as carbon source.

Table 4
Thermal Analysis of Biopolymer Produced by *S. epidermidis*
Using Different Food Wastes as Nutrients in Fermentation with Fermentor Culture

| Sample | | | Onset temperature (°C) | Melting temperature ($T_m$ [°C]) | Heat of fusion ($\Delta H_m$ [J/g]) |
|---|---|---|---|---|---|
| Bacterium strain | Substrate | Culture mode | | | |
| *S. epidermidis* | Sucrose | Fermentor | 165.20 | 183.24 | 89.79 |
| | Sesame oil | Fermentor | 177.49 | 187.85 | 82.18 |
| | Glucose + malt wastes | Fermentor | 165.54 | 180.30 | 74.09 |
| | Glucose + soya wastes | Fermentor | 169.32 | 179.76 | 59.94 |

Table 5
Thermal Analysis of Biopolymer Produced by *S. epidermidis*
Using Different Food Wastes in Fermentation with Shake Flask Culture

| Sample | | | Onset temperature (°C) | Melting temperature ($T_m$ [°C]) | Heat of fusion ($\Delta H_m$ [J/g]) |
|---|---|---|---|---|---|
| Bacterium strain | Substrate | Culture mode | | | |
| *S. epidermidis* | Sucrose | Shaking flasks | 171.57 | 181.09 | 97.69 |
| | Fructose | Shaking flasks | 150.43 | 167.55 | 75.08 |
| | Ice cream wastes | Shaking flasks | 159.87 | 173.56 | 40.51 |
| | Malt wastes | Shaking flasks | 176.39 | 186.06 | 70.60 |
| | Soya wastes | Shaking flasks | 162.27 | 173.74 | 55.39 |

Table 6
Comparison of $M_r$ of Biopolymers Produced by *A. latus* and *S. epidermidis*

| Sample | | | | | Viscosity measurement | | | |
|---|---|---|---|---|---|---|---|---|
| Bacterium strain | Substrate | Culture mode | Concentration (%) | Flow time ($t$ [s]) | $\eta_r$ | $\eta_{sp}$ | [η] | $M_r$ ~ molecular weight |
| *A. latus* DSM 1124 | Sucrose | Fermentor | 2.0 | 94.70 | 2.3154 | 1.3154 | 0.4878 | 43,287 |
| *S. epidermidis* | Sucrose | Fermentor | 0.3 | 160.42 | 3.9222 | 2.9222 | 5.8795 | 1,052,889 |

However, in the shake flask culture, all conditions except pH were controlled, and, therefore, crystallinity was considered to be affected by pH values.

In addition, the most interesting finding is the difference in physical properties between the polymers produced by the *S. epidermidis* and *A. latus* strains. After extraction with chloroform, the extracted liquid was condensed by rotary evaporation. Then methanol was added slowly to precipitate the polymer. The polymer produced by *A. latus* had a powder-like appearance after adding methanol, whereas the polymer produced by *S. epidermidis* exhibited a film- or silklike appearance. We also found that the polymer produced by *S. epidermidis* was much softer and more flexible than that produced by *A. latus*. $M_r$ would be considered an important factor, so we attempted to measure the $M_r$ with intrinsic viscosity. The results (*see* Table 6) show that the $M_r$ of the polymer produced by *S. epidermidis* was 24-fold greater than the polymer produced by *A. latus*. From the results, we found that many physicochemical properties of the polymer are quite related to its $M_r$.

## Conclusion

The *S. epidermidis* strain was isolated from sesame oil and considered to be able to utilize oil without fatty acid extraction as carbon source. Consequently, the *S. epidermidis* could produce bioplastics and degrade the food oil as a nutrient at the same time. Thus, they may help solve the problem of the production of food oil wastes in the food industry while producing environmental friendly bioplastics.

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