# Screening of Basidiomycete Yeast with Oil Production

Ayumi Tanimura, Masako Takashima, Sugita Takashi, Jun Ogawa, and Jun Shima

***Abstract*—**The lipid-accumulating ability of 500 yeast strains isolated in Japan was evaluated. Primary screening revealed that 31 strains were potential lipid producers, from which 12 strains were cultivated in a medium containing 3% glucose. It was found that JCM 24511 accumulated the highest lipid content, up to 61.53%, while JCM 24512 grew the fastest. These strains were tentatively identified as *Cryptococcus* sp. and *Cryptococcus musci*, respectively. The maximum lipid concentration of 1.49 g/L was achieved by JCM 24512. Similarly, JCM 24511 also achieved a high lipid production of 1.37 g/L. High lipid productivity is the most important characteristic of oleaginous yeasts from the viewpoint of practical production. Among the strains tested here, JCM 24512 had the best lipid productivity, 0.37 g/L/day. The results show that the isolated yeasts could be promising candidates for biodiesel production.

***Keywords*—***Cryptococcus musci*, *Cryptococcus podzolicus*, Fatty acids, Lipid productivity, Oleaginous yeast

## I. Introduction

BIOLIPIDS - including triacylglycerol - produced by oleaginous yeasts are among the most important raw materials for biodiesel production [1]. The quality of biodiesel depends upon the fatty acid composition of the biolipids [2]. In general, biolipids produced by oleaginous yeasts are suitable feedstock for biodiesel, because the fatty acid composition satisfies important criteria i.e., chain length and saturation degree. However, the fatty acid composition of biolipids is strain-specific, and it is therefore important to select oleaginous yeast strains to ascertain their suitability for biodiesel production.

The other advantage of oleaginous yeasts is their ability to produce lipids from non-utilized biomass, including lignocellulosic biomass [3], [4]. It is known that many oleaginous yeasts, such as *Rhodosporidium toruloides*, *Cryptococcus curvatus* and *Lipomyces starkeyi*, accumulate lipids in more than 20% of their dry yeast cells [5] - [7].

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To evaluate the lipid-accumulating ability of oleaginous microorganisms, lipid content (% of dry cell weight) is the most commonly used parameter [1], [8]. There are other derived values, such as the lipid concentration (g-lipid/L), lipid coefficient (lipid yield on glucose consumption; g-lipid/g-glucose), biomass concentration (g-cell/L) and lipid productivity (specific rate of lipid production; g-lipid/L/h or g-lipid/L/day). However, lipid productivity is currently receiving much attention, since the selection of rapidly growing and accumulating strains is fundamental to the success of practical biodiesel production [9], [10]. Based on this background, we undertook the selection of oleaginous yeasts that can rapidly accumulate lipids—that is, yeasts capable of producing high levels of lipids with a suitable fatty acid composition in typical culture media.

Recently, Takashima et al. reported the taxonomic diversity of yeasts in Japan within subtropical and cool temperature areas [11]. It can be considered that the yeast strains collected in Japan also exhibit functional diversity, including lipid-producing ability. In the present study, therefore, we used the yeast collection established by Takashima et al. to perform our comprehensive evaluation of the lipid-accumulating ability of yeast strains.

Our results showed that several yeast strains classified as basidiomycetes, including *Cryptococcus* sp. and unclassified strains, accumulated biolipids with higher productivity than the previously reported oleaginous yeasts.

## II. Materials and Methods

### A. Yeast Strains

Yeast strains collected and taxonomically identified by Takashima et al. [11] were used as the main screening resource. Yeast strains isolated from the campus of Kyoto University (Kyoto, Japan) were also assessed. As control strains, L. starkeyi NBRC 10381 and R. toruloides NBRC 0559 were obtained from the National Institute of Technology and Evaluation (NITE) Biological Resource Center.

### B. Media

Synthetic defined (SD) medium (0.17% yeast nitrogen w/o ammonium sulphate and amino acids [Difco], 0.5% ammonium sulphate and 3% glucose) was used for the primary comprehensive screening of biolipid-accumulating ability. SS2 medium (3% glucose, 0.5% ammonium sulphate, 0.05%

http://dx.doi.org/10.15242/IICBE.C0214055

magnesium sulphate, 0.01% sodium chloride, 0.01% calcium chloride and 0.01% yeast extract [Difco]) was used for the secondary screening of oleaginous yeasts with high lipid productivity.

### *C. Measurement of intracellular fatty acids*

Total intracellular lipids were estimated as total fatty acids. The fatty acids of the yeast strains were extracted from the lyophilized cells using a hydrochloric acid-catalysed direct methylation [12]. The resultant fatty acid methyl esters (FAMEs) were analysed using a gas chromatograph (GC-2010 Plus; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID). A DB-23 capillary column (30 m × 0.25 mm ID and 0.25 μm film thickness) (Agilent Technologies, Palo Alto, CA, USA) was used. The column temperature was programmed to start at 50 °C for 2 min and then increased by 10 °C/min up to 180 °C, where it remained for 5 min. The temperature was then increased at a rate of 5 °C/min to 240 °C, and held for 3 min. Helium was the carrier gas, which was pumped at 1.0 mL/min, and nitrogen was used as the make-up gas. The injector temperature was 250 °C and the detector temperature was 300 °C, with a split ratio of 50:1. The identification of major peaks was performed based on the retention time using controls obtained from Sigma-Aldrich (St. Louis, MO, USA). The fatty acid concentrations were determined using a standard curve generated by a series of external standards.

### *D. Comprehensive evaluation of the biolipid-accumulating ability of strains in the yeast collection*

Yeast strains were suspended in 6 mL of SD medium in a test tube, to a cell optical density at 600 nm ($OD_{600}$) of 0.2, then cultured at 30 °C with reciprocal shaking for 3 days at 150 opm. Cells from 3 mL of culture broth were harvested by centrifugation (15,000 rpm for 10 min) and washed twice with distilled water. Intracellular total lipids were determined after lyophilizing the wet cells (primary screening).

### *E. Selection of oleaginous yeasts which accumulate intracellular lipids at a high level*

The yeast strains screened by the primary screening were cultivated in Erlenmeyer flasks containing 100 mL of SS2 medium at 27 °C on a rotary shaker at 150 rpm. The cell dosage were the same as described above. Cells from 3 mL of culture broth were harvested by centrifugation (15,000 rpm for 10 min) after 1, 2, 3, and 4 days of cultivation, and washed twice with distilled water. The intracellular total lipids, fatty acid composition and cell mass were determined after lyophilizing the wet cells (secondary screening). All experiments were performed in triplicate.

## III. Results and Discussion

### *A. Comprehensive evaluation of the biolipid-accumulating ability of strains in the yeast collection*

During primary screening, a comprehensive evaluation of the lipid-accumulating ability of yeast strains (a total of 500 strains) was carried out. Approximately 6% of the tested strains accumulated total lipids to more than 0.8 g/L of the medium volume. The yeast strains examined in this study are listed in Table I. As expected, the yeast strains which accumulated to more than 0.8 g/L belonged to the basidiomycetes and were used for further analysis.

TABLE I
CHARACTERISTICS OF THE 12 SELECTED OLEAGINOUS YEASTS

| JCM number | Species | Phylogenetically close to: | Source |
|---|---|---|---|
| JCM 24502 | *Cryptococcus* sp. | *Cryptococcus podzoricus* | Soil, Iriomote Island |
| JCM 24503 | *Cryptococcus ramirezgomezianus* | | Soil, Rishiri Island |
| JCM 24504 | *Cryptococcus podzoricus* | | Soil, Rishiri Island |
| JCM 24505 | *Cryptococcus podzoricus* | | Soil, Rishiri Island |
| JCM 24506 | *Cryptococcus* sp. | *Cryptococcus podzoricus* | Soil, Iriomote Island |
| JCM 24507 | *Cryptococcus* sp. | *Cryptococcus podzoricus* | Soil, Iriomote Island |
| JCM 24508 | *Cryptococcus podzoricus* | | Soil, Rishiri Island |
| JCM 24509 | *Cryptococcus* sp. | *Cryptococcus podzoricus* | Soil, Iriomote Island |
| JCM 24510 | *Cryptococcus podzoricus* | | Soil, Rishiri Island |
| JCM 24511 | *Cryptococcus* sp. | *Cryptococcus podzoricus* | Soil, Iriomote Island |
| JCM 24512 | *Cryptococcus musci* | | Soil, Rishiri Island |
| JCM 24513 | *Rhodotorula* sp. | *Occultifur externus* | Soil, Rishiri Island |

### *B. Screening of oleaginous yeasts which can accumulate biolipids at a higher level*

In this experiment, the control strains used were *L. starkeyi* NBRC 10381 and *R. toruloides* NBRC 0559, which are known as typical oleaginous yeasts.

The lipid-accumulating ability of the yeast strains was expressed as lipid content after 4 days of cultivation (Fig. 1). From the viewpoint of lipid content, many of the yeasts showed higher lipid-producing ability under the experimental conditions used in this study, compared with those of the control strains. In particular, JCM 24511 showed the highest lipid content at 61.53 ± 2.25%.

We next examined the fatty acid composition of the lipids produced by the yeast strains after 4 days (Table II), as it is known to influence the quality of biodiesel produced from biolipids. No huge differences in fatty acid composition were

observed between the 12 strains and control strains. In most strains, the major fatty acids were the C18 species; for example, oleic acid (18:1) ranged from 55.43% to 72.95%; stearic acid (18:0) from 6.11% to 25.50% and linoleic acid (18:2) from 3.43% to 27.44%. The percentages of fatty acids remained at a constant level during cultivation. A suitable fatty acid composition for biodiesel production is palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids [13]. The 12 selected strains contained these fatty acids at a high ratio, ranging from 92.77% to 97.17%. It can be considered that the fatty acids produced by the selected strains were suitable for biodiesel production.

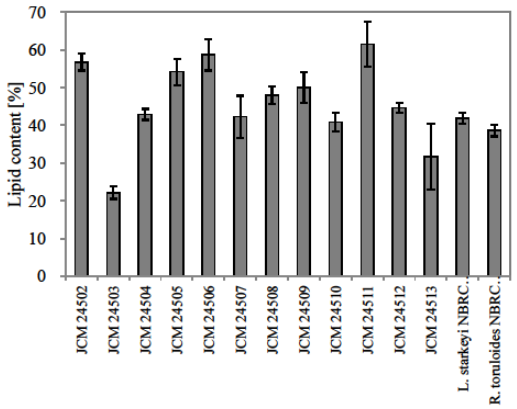


Fig. 1 Comparison of lipid contents of the 12 selected oleaginous yeast strains and 2 control strains after a 4-day culture

TABLE II
FATTY ACID COMPOSITION OF THE 12 SELECTED OLEAGINOUS YEAST STRAINS AND 2 CONTROL STRAINS AFTER A 4-DAY CULTURE

| | C14:0 myristic | C16:0 palmitic | C16:1 palmitoleic | C18:0 stearic | C18:1 oleic | C18:2 linoleic | C18:3 linolenic | Other fatty acids |
|---|---|---|---|---|---|---|---|---|
| JCM 24502 | 0.38 ±0.03 | 3.00 ±0.13 | 0.13 ±0.02 | 19.18 ±0.43 | 69.02 ±0.28 | 4.28 ±0.23 | 0.45 ±0.03 | 3.57 ±0.14 |
| JCM 24503 | 1.02 ±0.10 | 2.92 ±0.15 | 0.67 ±0.06 | 9.74 ±0.91 | 55.43 ±1.80 | 27.44 ±2.28 | 0.70 ±0.11 | 2.10 ±0.06 |
| JCM 24504 | 0.37 ±0.03 | 2.64 ±0.06 | 0.14 ±0.04 | 14.07 ±1.48 | 72.95 ±1.25 | 5.38 ±0.31 | 0.82 ±0.04 | 3.71 ±0.23 |
| JCM 24505 | 0.26 ±0.02 | 2.39 ±0.23 | 0.09 ±0.02 | 16.52 ±1.41 | 71.68 ±1.42 | 4.06 ±0.15 | 0.57 ±0.01 | 4.42 ±0.31 |
| JCM 24506 | 0.40 ±0.01 | 2.86 ±0.04 | 0.09 ±0.01 | 22.34 ±1.06 | 66.29 ±1.06 | 3.43 ±0.30 | 0.38 ±0.04 | 4.21 ±0.08 |
| JCM 24507 | 0.39 ±0.02 | 2.99 ±0.08 | 0.11 ±0.01 | 21.48 ±0.54 | 65.98 ±0.57 | 5.29 ±0.14 | 0.40 ±0.01 | 3.36 ±0.08 |
| JCM 24508 | 0.36 ±0.02 | 2.87 ±0.06 | 0.14 ±0.01 | 14.53 ±0.17 | 70.48 ±1.34 | 7.44 ±0.98 | 0.74 ±0.12 | 3.74 ±0.18 |
| JCM 24509 | 0.29 ±0.02 | 2.63 ±0.22 | 0.11 ±0.08 | 25.50 ±1.21 | 62.57 ±0.60 | 4.24 ±0.69 | 0.44 ±0.09 | 4.29 ±0.38 |
| JCM 24510 | 0.15 ±0.02 | 1.64 ±0.13 | 0.05 ±0.04 | 23.70 ±0.43 | 62.84 ±1.17 | 3.92 ±0.40 | 0.67 ±0.05 | 7.07 ±0.60 |
| JCM 24511 | 0.33 ±0.03 | 2.75 ±0.06 | 0.09 ±0.01 | 21.21 ±0.90 | 67.37 ±1.25 | 3.91 ±0.39 | 0.41 ±0.05 | 4.12 ±0.09 |
| JCM 24512 | 0.82 ±0.13 | 2.95 ±0.05 | 0.22 ±0.02 | 20.03 ±0.99 | 63.09 ±0.43 | 9.98 ±1.41 | 0.31 ±0.03 | 2.72 ±0.28 |
| JCM 24513 | 0.95 ±0.10 | 3.51 ±0.14 | 0.57 ±0.07 | 6.11 ±0.28 | 68.95 ±0.35 | 18.56 ±0.15 | 0.05 ±0.04 | 1.48 ±0.09 |
| *L. starkeyi* NBRC 10381 | 0.61 ±0.01 | 4.34 ±0.14 | 4.70 ±0.17 | 6.03 ±0.43 | 74.08 ±0.29 | 6.75 ±0.60 | 0.71 ±0.07 | 2.78 ±0.05 |
| *R. toruloides* NBRC 0559 | 1.31 ±0.07 | 1.86 ±0.10 | 0.43 ±0.06 | 17.60 ±0.53 | 64.21 ±0.54 | 9.11 ±1.24 | 1.64 ±0.11 | 3.85 ±0.12 |

The lipid productivity (specific rate of lipid production) and biomass concentration of selected and control strains after 4 days of culturing are shown in Table III. Every selected strain showed higher values, compared with the control strains, for both parameters.

TABLE III
LIPID PRODUCTIVITY (LIPID CONCENTRATION PER DAY) AND BIOMASS CONCENTRATION OF THE 12 SELECTED OLEAGINOUS YEAST STRAINS AND 2 CONTROL STRAINS AFTER A 4-DAY CULTURE

| | Lipid productivity [g/L/day] | Biomass concentration [g/L] |
|---|---|---|
| JCM 24502 | 0.35 ±0.05 | 2.44 ±0.36 |
| JCM 24503 | 0.14 ±0.02 | 2.46 ±0.05 |
| JCM 24504 | 0.21 ±0.03 | 1.98 ±0.54 |
| JCM 24505 | 0.30 ±0.11 | 2.18 ±0.64 |
| JCM 24506 | 0.29 ±0.07 | 2.00 ±0.43 |
| JCM 24507 | 0.26 ±0.04 | 2.43 ±0.30 |
| JCM 24508 | 0.31 ±0.08 | 2.52 ±0.29 |
| JCM 24509 | 0.30 ±0.02 | 2.16 ±0.12 |
| JCM 24510 | 0.16 ±0.05 | 1.59 ±0.15 |
| JCM 24511 | 0.34 ±0.04 | 2.23 ±0.29 |
| JCM 24512 | 0.37 ±0.15 | 3.27 ±0.35 |
| JCM 24513 | 0.22 ±0.03 | 2.76 ±0.48 |
| *L. starkeyi* NBRC 10381 | 0.14 ±0.02 | 1.39 ±0.25 |
| *R. toruloides* NBRC 0559 | 0.12 ±0.01 | 1.25 ±0.06 |

JCM 24512 showed the highest biomass concentration of 3.27 ± 0.35 g/L and the highest lipid productivity of 0.37 ± 0.15 g/L/day, while its lipid content and lipid coefficient were relatively low. These parameters are related to each other, and it can be concluded that the lipid content of each cell of JCM 24512 was dispersed into small fat droplets, but lipid productivity was high due to the large cell mass. It is considered that the most important parameter is lipid productivity. High lipid productivity increases the yield per harvest volume and decreases the production cost. Therefore, it was suggested that JCM 24512 could play a key role in the economic production of biodiesel. Similarly, JCM 24502 and JCM 24511 also achieved high lipid productivity, which were close to those of *C. podzolicus*. Among the 12 selected strains, 9 strains belonged to or were close relatives of *C. podzolicus*.

### *C. Kinetic analysis of yeast strains with high lipid productivity*

To examine the lipid-accumulating rate of yeast strains with

high lipid productivity, a kinetic analysis of three yeast strains (JCM 24502, JCM 24511 and JCM 24512) was performed, and the results were compared with those for the control strains *L .starkeyi* NBRC 10381 and *R. toruloides* NBRC 0559. The time courses of the lipid concentration and biomass concentration of the three yeast strains and control strains are shown in Figure 2.

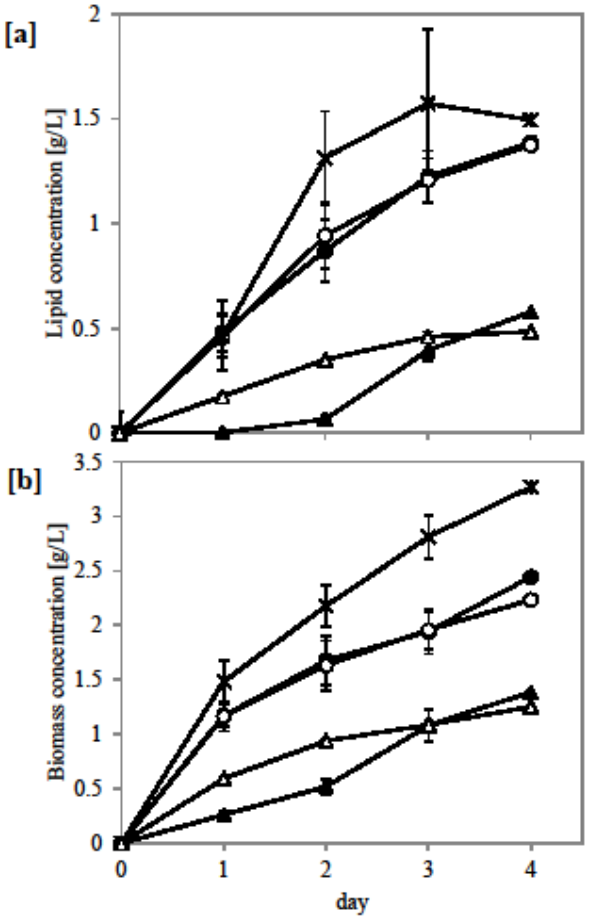


Fig.2 Time course of lipid concentrations (a) and biomass concentrations (b) of JCM 24502 (●), JCM 24511 (○), JCM 24512 (*), L. starkeyi (▲) and R. toruloides (Δ).

JCM 24512 gave the highest rate of increase of lipid concentration and biomass. It should be noted that the lipid concentration of JCM 24512 reached the maximum value of 1.57 ± 0.38 g/L at day 3 of culture, and then slightly decreased, while the biomass kept increasing until day 4. This may suggest that it was related to factors influencing the exhaustion of essential elements. As can be seen, however, the lipid productivity of the first two days of JCM 24512 was remarkable (0.66 ± 0.14 g/L/day). This result confirmed that JCM 24512 can accumulate lipid rapidly and in high yield. Thus this strain could potentially shorten fermentation times and reduce production costs.

## IV. Conclusion

The results indicate that the yeast strains isolated from Japan have great potential for biodiesel production. JCM 24502 and JCM 24511, tentatively identified as *Cryptococcus* sp., reached lipid contents of 56.77±2.80% and 61.53±2.25%, respectively. Based on these studies, it was concluded that the most promising strain was *Cryptococcus musci* JCM 24512, with a lipid content of 44.7±15.04% and lipid productivity of 0.37±0.15 g/L/day; indeed, during the first 2 days of cultivation this strain achieved a lipid productivity of 0.66±0.14 g/L/day. This finding was important, since high lipid productivity is crucial for any species used in biodiesel production.

## Acknowledgment

This work was partly supported by the Institute for Fermentation, Osaka (IFO) and the Advanced Low Carbon Technology Research and Development Program of Japan (ALCA).

## References

[1] Meng, X., Yang, J.M., Xu, X., Zhang, L., Nie, Q.J., Xian, M., "Biodiesel production from oleaginous microorganisms," *Renew. Energ.*, 34(1), 1-5.2009.

[2] Knothe, G., Steidley, K.R., "Kinematic viscosity of fatty acid methyl esters: Prediction, calculated viscosity contribution of esters with unavailable data, and carbon-oxygen equivalents," *Fuel*, 90(11), 3217-3224. 2011. http://dx.doi.org/10.1016/j.fuel.2011.06.016

[3] Gong, Z.W., Shen, H.W., Wang, Q., Yang, X.B., Xie, H.B., Zhao, Z.B.K., "Efficient conversion of biomass into lipids by using the simultaneous saccharification and enhanced lipid production process," *Biotechnol. Biofuels*, 6, 36. 2013. http://dx.doi.org/10.1186/1754-6834-6-36

[4] Liang, Y., Tang, T., Siddaramu, T., Choudhary, R., Umagiliyage, A.L., "Lipid production from sweet sorghum bagasse through yeast fermentation," *Renew. Energ*., 40(1), 130-136. 2012. http://dx.doi.org/10.1016/j.renene.2011.09.035

[5] Thiru, M., Sankh, S., Rangaswamy, V., "Process for biodiesel production from *Cryptococcus curvatus*," *Bioresour. Technol*., 102(22), 10436-10440. 2011. http://dx.doi.org/10.1016/j.biortech.2011.08.102

[6] Wild, R., Patil, S., Popovic, M., Zappi, M., Dufreche, S., Bajpai, R., "Lipids from *Lipomyces starkeyi*," *Food Technol. Biotech*., 48(3), 329-335. 2010.

[7] Wu, S., Zhao, X., Shen, H., Wang, Q., Zhao, Z.K., "Microbial lipid production by *Rhodosporidium toruloides* under sulfate-limited conditions," *Bioresour. Technol*., 102(2), 1803-1807. 2011. http://dx.doi.org/10.1016/j.biortech.2010.09.033

[8] Song, M.M., Pei, H.Y., Hu, W.R., Ma, G.X., "Evaluation of the potential of 10 microalgal strains for biodiesel production," *Bioresour. Technol*., 141, 245-251 2013. http://dx.doi.org/10.1016/j.biortech.2013.02.024

[9] Griffiths, M.J., Harrison, S.T.L., "Lipid productivity as a key characteristic for choosing algal species for biodiesel production," *J. Appl. Phycol*., 21(5), 493-507. 2009. http://dx.doi.org/10.1007/s10811-008-9392-7

[10] Ordog, V., Stirk, W.A., Balint, P., Lovasz, C., Pulz, O., van Staden, J., "Lipid productivity and fatty acid composition in *Chlorella* and *Scenepdesmus* strains grown in nitrogen-stressed conditions," *J. Appl. Phycol*., 25(1), 233-243. 2013. http://dx.doi.org/10.1007/s10811-012-9857-6

[11] Takashima, M., Sugita, T., Van, B.H., Nakamura, M., Endoh, R., Ohkuma, M., "Taxonomic Richness of Yeasts in Japan within Subtropical and Cool Temperate Areas" *PLoS One*, 7(11). 2012. http://dx.doi.org/10.1371/journal.pone.0050784

[12] Ichihara, K., Fukubayashi, Y, "Preparation of fatty acid methyl esters for gas-liquid chromatography," *J. Lipid Res*., 51(3), 635-640. 2010. http://dx.doi.org/10.1194/jlr.D001065

[13] Knothe, G., "Improving biodiesel fuel properties by modifying fatty ester composition," *Energ. Environ. Sci*., 2(7), 759-766. 2009. http://dx.doi.org/10.1039/b903941d