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SHORT COMMUNICATION

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Fermentation of soybean oil deodorizer distillate with *Candida tropicalis* to concentrate phytosterols and to produce sterols-rich yeast cells

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Abstract Phytosterols have been recovered from the deodorizer distillate produced in the final deodorization step of vegetable oil refining by various processes. The deodorizer distillate contains mainly free fatty acids (FFAs), phytosterols, and tocopherols. The presence of FFAs hinders recovery of phytosterols. In this study, fermentation of soybean oil deodorizer distillate (SODD) with *Candida tropicalis* 1253 was carried out. FFAs were utilized as carbon source and converted into cellular components as the yeast cells grew. Phytosterols concentration in SODD increased from 15.2 to 28.43 % after fermentation. No significant loss of phytosterols was observed during the process. Microbial fermentation of SODD is a potential approach to concentrate phytosterols before the recovery of phytosterols from SODD. During SODD fermentation, sterols-rich yeast cells were produced and the content of total sterols was as high as 6.96 %, but its major sterol was not ergosterol, which is the major sterol encountered in *Saccharomyces cerevisiae*. Except ergosterol, other sterols synthesized in the cells need to be identified.

Keywords Phytosterols · Deodorizer distillate · Fermentation · Yeast · Free fatty acids

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Introduction

Phytosterols, or plant sterols, are triterpenes similar to cholesterol, both in structure, given the four-ring steroid nucleus, the 3β-hydroxyl group, and often a 5,6-double bond. Free phytosterols are insoluble in water, and poorly soluble in most foods (fats and oils) [18]. Phytosterols are known to have a cholesterol-lowering effect. The mechanism played by phytosterols is based on the ability of plant sterol esters to reduce the intestinal absorption of diet and biliary cholesterol. Moreover, plant sterols possess anti-inflammatory and anti-atherogenicity activity and may possess anti-cancer and anti-oxidative activities [23]. Phytosterol and its derivatives are widely applied in food, pharmaceutical, and cosmetic industries due to their special biological activity, physical, and chemical properties [15, 20].

One of the major sources of phytosterols is vegetable oil deodorizer distillate. Soybean oil deodorizer distillate (SODD), for instance, contains about 3–15 % phytosterols [5]. More than 100 types of phytosterols have been reported in plant species, but the more abundant are stigmasterol, campesterol, and β-sitosterol [3]. Vegetable oil deodorizer distillate is a complex mixture due to the great number of its components. It is composed of free fatty acids (FFAs), sterols, tocopherols, sterol esters, hydrocarbons, breakdown products of fatty acids, aldehydes, ketones, and acyl glycerol [16]. FFAs constitute 25–75 % of vegetable oil deodorizer distillate depending on the type of raw material and the conditions of the oil refining process [11]. High levels of FFAs hinder the recovery of phytosterols and other high-value products. Normally, the removal of FFAs is the first step for recovery of phytosterols from vegetable oil deodorizer distillate. Several processes have been developed to manufacture phytosterol concentrates.

These include saponification [12], vacuum distillation [7], methyl esterification of FFAs catalyzed by acid or enzyme, followed by vacuum distillation or molecular distillation [17, 22, 25], enzymatic hydrolysis, followed by neutralization and washing [6]. Most of the processes above require toxicologically and ecologically unsafe solvents or metal-containing catalysts, which are furthermore expensive and require regeneration.

Free fatty acids and glycerides can be utilized as a carbon source by yeast such as *Candida utilis* and *Candida tropicalis* [24]. In the present study, SODD was fermented with *Candida tropicalis* 1253. FFAs were utilized and converted into cellular components as the yeast cells grew. After microbial fermentation of SODD, the fermented broth was centrifugated and a phytosterol-rich residue of SODD was obtained. Surprisingly, it was found that the harvested cells of *C. tropicalis* 1253 contained a large amount of sterols. Removal of FFAs from SODD with microbial fermentation was a more environmentally friendly and economical approach compared to the chemical and enzymatic processes.

Materials and methods

Materials

SODD was provided by Shijiazhuang Yihai oil Co., Ltd. (China), which contained 15.2 % phytosterols and 66.13 % FFAs. Stigmasterol was purchased from Xian BlueSky Biological Co., Ltd. (China). Other chemicals, such as phosphoric acid and ethanol, were all analytic reagents.

Microorganisms and culture conditions

The yeast *C. tropicalis* 1253 used in this study was obtained from China Center of Industrial Culture Collection. Stock cultures were maintained on slants of YM agar (malt extract 3 g/l; glucose 10 g/l; yeast extract 3 g/l; peptone 5 g/l; agar 20 g/l) at 4 °C and were transferred monthly.

Starter cultures were grown in YM broth at 30 °C on a rotary shaker at 200 rpm for 12 h. The cultures were then used to inoculate 100 ml GY medium (glucose 20 g/l; yeast extract 10 g/l; peptone 5 g/l) at a final concentration of 5 % (v/v), and were incubated at 30 °C in a shaker at 200 rpm for 24 h.

Fermentation of SODD

SODD fermentation medium (SFM) was composed of (g/l): SODD 50.0, yeast extract 1.0, $MgSO_4$ 0.1, K_2HPO_4 0.2, KH_2PO_4 0.2, and pH of the medium was about 7.0. SFM was sterilized at 115 °C for 15 min. There was an oil layer on the surface of SFM because SODD was insoluble in water. The cultures of *C. tropicalis* 1253 were inoculated into 100 ml SFM at a final concentration of 10 % (v/v), and were incubated at 30 °C in a shaker at 200 rpm. During fermentation of SODD, the cell growth, the total sterols of yeast cells, phytosterols and residual FFAs in SODD were monitored periodically. The reported data were the average of three independent experiments.

Recovery of SODD after fermentation

When fermentations of SODD with *C. tropicalis* 1253 were finished, the fermented broths were centrifugated at 4,000 rpm for 15 min. The oil layer (residual SODD) was removed and used for analysis.

Observation of phytosterol release from SODD during fermentation

During the SODD fermentation, the fermented broths were sampled periodically and centrifugated at 4,000 rpm for 15 min. After removal of the oil layer, the concentration of phytosterol in the supernatant was determined.

Analytical methods

Quantification of phytosterol in SODD

The sulfate-phosphate-ferric method (SPF) was commonly used for determination of cholesterol content in serum. Lin et al. [14] used the SPF method to determine the total phytosterol content in corn flour or soybean flour, and demonstrated that it was valid and accurate. To facilitate determination of phytosterol content in SODD, the SPF method was used in this study and phytosterol content as stigmasterol was calculated.

SPF chromogenic reagent was prepared as follows: 2.5 g $FeCl_3{\cdot}6H_2O$ was dissolved in 25 ml of phosphoric acid, then 10 ml was dissolved in 100 ml of sulfuric acid before the experiment. The final 4 ml of assay system containing 2 ml stigmasterol dissolved in ethanol and 2 ml SPF chromogenic reagent were shaken to homogeneity. The OD value of reaction solution was determined by a spectrophotometer (SP-756; Shanghai Spectrum Instrument Limited Company, China) at 550 nm. Ethanol was used as the control. OD550 values measured by SPF method and stigmasterol concentrations had a very good linear relationship ($Y = 93.097X + 2.179$, $R^2 = 0.9995$).

Determination of FFAs

FFAs in SODD as oleic acid were determined by the AOCS official method [1].

Determination of yeast cell growth

The cell growth of *C. tropicalis* 1253 was monitored by dry weight of yeast biomass. The dry weight of biomass was obtained by centrifuging culture samples (5 ml) for 10 min at 5,000 rpm, washing the cells twice with distilled water, and drying the solids to a constant weight at 100–105 °C.

Determination of total sterols in the yeast cells

The yeast cells were harvested by centrifuging the fermentation broth at 4,000 rpm for 15 min. After washing and drying, the total sterols in the yeast cells were determined by SPF.

Determination of ergosterol in the yeast cells

The 0.4 g wet yeast biomass (about 0.05 g dry weight) was suspended in 32 ml of ethanol–KOH solution (20 % KOH: 95 % ethanol = 5:3, v/v) in a 250-ml flask. The flask was covered with a film and saponified in a water bath at 90–95 °C for 3 h. The flask was cooled to room temperature, 50 ml of petroleum ether was added, and the mixture was mixed on a vortex mixer for 15 min. After setting for 2 h, 5 ml of ether phase was taken and was dried under air. Then, 10 ml of ethanol was added to dissolve the sample for HPLC analysis. The ergosterol content was determined by HPLC (Angilent 1100; ZORBAX Eclipse XDB-C18 column, 4.6 × 150 mm, 5 μm; mobile phase: methanol, flow rate 1.0 ml/min using UV detector at 284 nm). Ergosterol was purchased from Sigma Company as standard.

Results

Fermentation of SODD with *C. tropicalis* 1253 to concentrate phytosterols

In our past work, four strains of yeast, *C. utilis* 1769, *C. tropicalis* 1253, *Saccharomycopsis fibuligera*, and *Geotrichum candidum*, were tested their growth on SODD agar, and it was found that *C. tropicalis* 1253 grew best. The cells of *C. tropicalis* can also be used as animal feed. So in this study *C. tropicalis* 1253 was chosen to ferment SODD.

As shown in Fig. 1, during the first fermentation phase (0–12 h), the cells grew faster and its dry weight increased quickly from 1.47 to 5.67 g/l, but FFAs concentration in residual SODD hardly decreased and FFAs were not consumed, which means that the carbon source consumed by the cell growth was not FFAs. The possible reason was that yeast extract in SFM was a better carbon source for *C. tropicalis* 1253 than FFAs, although it was used as nitrogen source in SFM. The phytosterol concentration in residual SODD did not obviously increase. During the second fermentation phase (12–36 h), the cell growth slowed, which means that there was a lag phase of the cell growth. However, the FFA concentration in the SODD decreased. This result might indicate that yeast extract in SFM had been exhausted and FFAs began to be used as a carbon source, and the cells had an adaptation period for growth. Phytosterol concentration of residual SODD also increased as FFAs were consumed. During the third fermentation phase (36–96 h), the cells of yeast grew rapidly again and had a secondary growth. At the same time, FFAs were consumed and phytosterol concentration of residual SODD increased gradually. The cell growth rate was more than the rate of FFA consumption, which might mean that other components of SODD such as glycerides were also consumed when the yeast cells grew. At the last fermentation phase (96–108 h), the cell growth nearly stopped. FFA concentration decreased slightly.

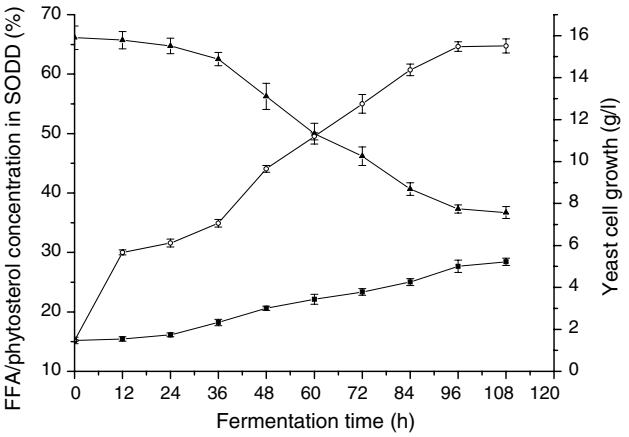

Fig. 1 SODD fermentation with *C. tropicalis* 1253. *Open circle* yeast cell growth. *Filled triangle* FFA concentration. *Filled square* phytosterol concentration

When SODD fermentation with *C. tropicalis* 1253 was over, the residual SODD contained 36.72 % FFAs and 28.42 % phytosterols.

Release of phytosterol from SODD during fermentation

Phytosterols are mixed together with FFAs, tocopherols, sterol esters, hydrocarbons, and other compounds in SODD. As shown in Fig. 2, phytosterol concentrations in the fermented broths increased as the fermentation time increased. This result indicated that some phytosterols were released from SODD into the fermented broth as FFAs were consumed during fermentation. However, the total amount of released phytosterols was very low and the phytosterol loss was only 0.036 %.

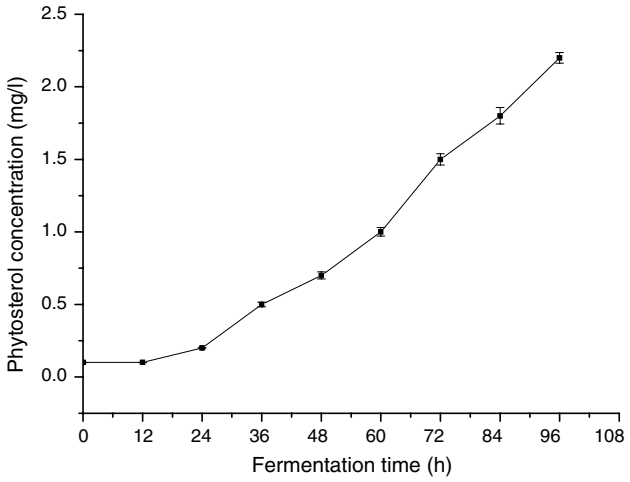

Fig. 2 Release of phytosterols form SODD during fermentation

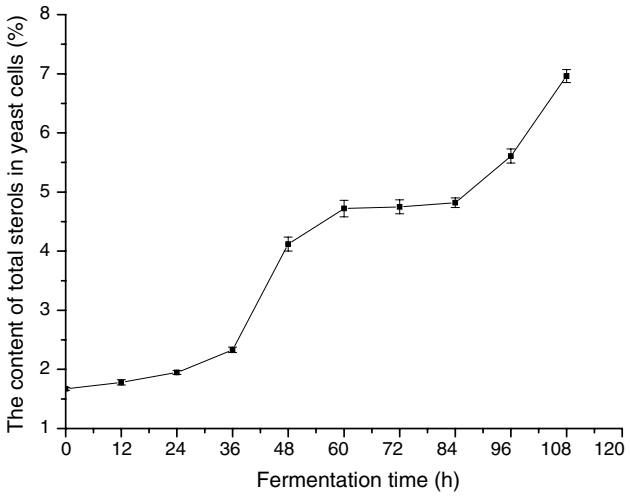

Fig. 3 Observation of total sterols in yeast cells during fermentation

Observation of total sterols in yeast cells during fermentation

During SODD fermentation with *C. tropicalis* 1253, total sterols in the yeast cells were monitored and the results are shown in Fig. 3. The SODD fermentation process can be divided into four stages according to Fig. 3. During the first fermentation phase (0–36 h), total sterols in the cells increased very slowly after the starter cultures grown in YM broth were inoculate into SFM. During the second fermentation phase (36–60 h), the cells started to synthesize sterols quickly, and the content of total sterols increased from 2.33 to 4.72 %. During the third fermentation phase (60–84 h), the content of total sterols slowly increased. At the last fermentation phase (84–108 h), the cells synthesized swiftly again sterols and the content of total sterols increased rapidly from 4.82 to 6.96 %.

After SODD fermentation, the content of ergosterol in the yeast cells was determined by HPLC, and the cells only contained 0.36 % ergosterol. This result indicated that *C. tropicalis* 1253 could synthesize a large amount of sterols, but ergosterol was not the major sterol.

Discussion

Vegetable oil deodorizer distillate such as SODD is one of the main raw materials for production of phytosterols and one of its main components is FFAs. FFAs at high concentrations are an obstacle to the extraction of phytosterols from SODD. Various pretreatment processes are used to eliminate FFAs and to manufacture of phytosterol and tocopherol concentrates. Commercially, the most important is esterification of FFAs using chemical or enzymatic means, followed by molecular distillation [22]. In most cases, methanol is used to esterify with FFAs, producing fatty acid methyl esters that can be used as biodiesel. Methyl esterification of FFAs was carried out at high temperature, such as 180 °C, using sulfuric acid or tin oxide as a catalyst [9]. Esterification of FFAs with methanol could be achieved under the catalytic action of lipase [8, 25]. Enzymatic esterification of FFAs has some advantages over chemical esterification such as an efficient catalytic ability, no need to remove large amounts of reagents and industrial waste, and use of lower temperatures and ambient pressure. However, a bottleneck in enzymatic esterification of FFA for industrial application is the high cost of lipase and its short operational life [21]. In this work, SODD was fermented with *C. tropicalis* 1253, and FFAs in SODD were utilized as a carbon source and converted into cellular components as the yeast cells grew. Phytosterol concentration in SODD increased from 15.2 to 28.43 % after fermentation. Phytosterols in SODD were successfully concentrated. During SODD fermentation, some phytosterols were released from SODD into the fermented broth, but released phytosterols were very low and phytosterol loss could be ignored. SODD also contained tocopherols. Changes of tocopherols were not evaluated in this study, but tocopherols in SODD would also be concentrated after the fermentation. Pretreatment of SODD with microbial fermentation for concentration of phytosterols was shown to be a technically feasible process. This approach did not require expensive lipase and toxic methanol compared with the enzymatic method. The concentrate of phytosterols and tocopherols (the oil layer) was easy to separate from the cells of yeast after the fermentation. Furthermore, an added bonus of this approach was that the yeast cells obtained were sterol-rich and might be used to extract very valuable

sterols. Therefore, it is a potential alternative to chemical and enzymatic pretreatments. Nevertheless, the cells of *C. tropicalis* 1253 grew more slowly when FFAs were used as a carbon source than when glucose was. The theoretical maximum phytosterol concentration in SODD after microbial fermentation is 44.87 %. Fermentation technology of SODD is necessary to be optimized and improved further.

The SPF principle for determination of cholesterol is that cholesterol reacts with SPF chromogenic reagent and generates an amaranth compound. In our other work, validity of the SPF method was verified by using cholesterol, stigmasterol, campesterol, β-sitosterol, and ergosterol, respectively. It was found that they all could react with SPF chromogenic reagent and generate stable amaranth compounds. OD550 values measured by the SPF method and those sterol concentrations had a very good linear relationship. So the SPF method was used to determine the content of total sterols both in SODD and in the yeast cells in this work.

Sterols are essential structural components of eukaryotic cell membranes. Yeasts of the genus *Saccharomyces* are particularly rich in sterols. The level of the sterol components has generally been determined to range from 0.03 to 4.6 % of the cell dry weight [2]. *Saccharomyces cerevisiae* is the most intensely studied yeast with respect to the biosynthesis and function of sterols. Ergosterol, an important pharmaceutical intermediate, is identified as the major sterol and can make up over 90 % of the total sterols [10, 19]. In this work, surprisingly, it was found that *C. tropicalis* 1253 could synthesize a large amount of sterols, and the content of total sterols was as high as 6.96 %. Yeast cells do not take up free sterols from the environment when cultivated in the presence of oxygen, i.e., under aerobic conditions [13]. Why did *C. tropicalis* 1253 produce such high sterol components? When *C. tropicalis* 1253 was cultivated in YM broth in which glucose was used as carbon source, the cells contained only 1.76 % total sterols. However, the content of total sterols was 6.96 % when fermented in SFM in which FFAs was carbon source. In order to further explore the reason for this, another experiment was conducted to determine the effect of different carbon sources on total sterols in the yeast cells. When glucose, fructose, and fructose were used as the carbon source to cultivate *C. tropicalis* 1253, the total sterol contents were very low, less than 1.8 %. Nevertheless, when *C. tropicalis* 1253 were grown with oleic acid, the total sterol content was 6.1 %, which was very similar to that of SODD. These results suggested that when FFAs were used as a carbon source, *C. tropicalis* 1253 synthesize a large amount of sterols. Boll et al. [4] also found that a supplement of unsaturated fatty acids (linolenic acid) could accelerate sterol biosynthesis of yeast. In *C. tropicalis* 1253, ergosterol was not the major sterol, which was different from the case of *S. cerevisiae*. Other sterols synthesized in *C. tropicalis* 1253 were not identified in this study.

Conclusions

Fermentation of SODD with *Candida tropicalis* 1253 was carried out in this work. FFAs in SODD were converted into cellular components as the yeast cells grew. Phytosterols in SODD were successfully concentrated after fermentation. No significant loss of phytosterols was observed during the process. Microbial fermentation of SODD is a potential approach to pretreat SODD before the recovery of phytosterols from SODD. After SODD fermentation, sterol-rich yeast cells were obtained and the content of total sterols was as high as 6.96 %, but its major sterol was not ergosterol, which is the major sterol encountered in *S. cerevisiae*. The other sterols synthesized in the cells need to be further identified.

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References

1. American Oil Chemists' Society (1997) Official methods and recommended practices, 5th edn. AOCS Press, Champaign
2. Arnezeder C, Hampel WA (1990) Influence of growth rate on the accumulation of ergosterol in yeast cells. Biotechnol Lett 12:277–282
3. Berger A, Jones PJH, Abumweis SS (2004) Plant sterols: factors affecting their efficacy and safety as function food ingredients. Lipids Health Dis 3:5–23
4. Boll M, Löwel M, Berndt J (1980) Effect of unsaturated fatty acids on sterol biosynthesis in yeast. BBA Lipids Lipid Metab 620:429–439
5. Carmona MA, Jiménez C, Jiménez-Sanchidrián C, Peña F, Ruiz JR (2010) Isolation of sterols from sunflower oil deodorizer distillate. J Food Eng 101(2):210–213
6. Chu BS, Quek SY, Baharin BS (2003) Optimisation of enzymatic hydrolysis for concentration of vitamin E in palm fatty acid distillate. Food Chem 80(3):295–302
7. Czuppon T, Kemeny Z, Kovari E, Recseg K (2003) Process for recovery of plant sterols from by-product of vegetable oil refining. WO2004000979
8. Facioli NL, Barrera-Arellano D (2001) Optimisation of enzymatic esterification of soybean oil deodoriser distillate. J Sci Food Agric 81:1193–1198
9. Fizet C (1996) Process for tocopherols and sterols from natural sources. US5487817
10. Gao H, Tan TW (2003) Fed-batch fermentation for ergosterol production. Process Biochem 39:345–350
11. Gunawan S, Novy S, Kasim NS, Ju YH (2008) Separation and purification of squalene from soybean oil deodorizer distillate. Sep Purif Technol 60(2):128–135
12. Hattori Y, Horio M, Kono J (2001) Process for producing phytosterols by saponification in an alcohol/water solvent. WO0132681
13. Jacquier N, Schneiter R (2012) Mechanisms of sterol uptake and transport in yeast. J Steroid Biochem 129:70–78

14. Lin Y, Song X, Fu J, Lin J, Qu Y (2009) Microbial transformation of phytosterol in corn flour and soybean flour to 4-androstene-3,17-dione by *Fusarium moniliforme* Shield. Bioresour Technol 100(5):1864–1867
15. Ling WH, Jones PJH (1995) Dietary phytosterols: a review of metabolism, benefits and side effects. Life Sci 57(3):195–206
16. Martins PF, Ito VM, Batistella CB, Maciel MRW (2006) Free fatty acid separation from vegetable oil deodorizer distillate using molecular distillation process. Sep Purif Technol 48(1):78–84
17. Ramamurthi S, McCurdy AR (1993) Enzymatic pretreatment of deodorizer distillate for concentration of sterols and tocopherols. J Am Oil Chem Soc 70(3):287–295
18. Robles-Manuel S, Barrault J, Valange S (2011) Selective synthesis of phytosterol esters from natural sterols and fatty methyl esters over Mg-containing solid catalysts. C R Chimie 14(7):656–662
19. Thomas KC, Hynes SH, Ingledew WM (1998) Initiation of anaerobic growth of *Saccharomyces cerevisiae* by amino acids or nucleic acids bases: ergosterol and unsaturated fatty acids cannot replace oxygen in minimal media. J Ind Microbiol Biotechnol 21:247–253
20. Thomas HJ, Li SC, John CG (2002) Phytosterol content in American ginseng seed oil. J Agric Food Chem 50(4):744–750
21. Wang L, Du W, Liu D, Li L, Dai N (2006) Lipase-catalyzed biodiesel production from soybean oil deodorizer distillate with absorbent present in *tert*-butanol system. J Mol Catal B Enzym 43:29–32
22. Wollmann G, Schwarzer J, Gutsche B (2005) Processes for producing sterols from fatty acid production residues. US6956125
23. Yang H, Yan F, Wu D, Huo M, Li J, Cao Y, Jiang Y (2010) Recovery of phytosterols from waste residue of soybean oil deodorizer distillate. Bioresour Technol 101(5):1471–1476
24. Zheng S, Yang M, Yang Z (2005) Biomass production of yeast isolate from salad oil manufacturing wastewater. Bioresour Technol 96(10):1183–1187
25. Zhou WW, Qin DH, Qian JQ (2009) Optimisation of enzymatic pretreatment of soybean oil deodoriser distillate for concentration of tocopherols. Int J Food Sci Tech 44(7):1429–1437