Use of a Fungal Lipase for Enhancement of Aroma in Black Tea

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Lipases produced extracellularly by bacteria and fungi are being widely used in the food industry for flavour development. During tea processing, the possibility of enhancing the flavour of tea by the exogenous addition of lipase has not been reported. It was therefore considered worthwhile to amend tea leaves with microbial lipases and observe the changes in the flavour profile of the finished product. Among a few fungal lipases screened, the enzyme secreted by *Rhizomucor miehei* increased the formation of desired flavour compounds. Hence, studies were initiated to achieve enhanced production of this industrially useful enzyme. Under solid-state fermentation *R.M. miehei* produces an extracellular lipase in copious amounts on a simple solid substrate within 96 h, which is active at lower temperatures and at near-neutral pH. These attributes make this enzyme suitable for use during the manufacturing of tea, where reactions are carried out at temperatures of 15 to 20˚C and pH range of 5 to 7. In the present study, exogenous addition of lipase resulted in an increase in flavour volatiles, which contribute to the aroma of tea.

Keywords: lipase for tea processing, lipase for aroma enhancement, *Rhizomucor miehei*, extracellular lipase, solid-state fermentation

In recent years research on microbial lipase production has been intensified due to the potential use of the enzyme to hydrolise fats and synthesis of specific esters with desirable flavour properties. Modification of the existing enzymes to yield forms that can be used under conditions that are harsher than the present or that have altered activities or substrate specificity is a vital aspect that is gaining ground. Such manipulations are feasible with lipases since they can be obtained in large amounts from industrial fermentors (Torossion & Bell, 1991) and display a host of substrate selectivities.

Industrial enzymes have been increasingly used for an expanding variety of processes over the last few decades. Enzymes like proteases, lipases and amylases are currently widely used in detergents and the food technology industry. The hydrolysis of water-soluble carboxylic acid esters by many lipases is slow and the catalytic ability varies. The substrate specificity of lipases is often crucial to their applications for analytical and industrial purposes. While enzymes with a broad substrate range are suitable for certain applications, those lipases that are very selective in their substrate requirement are preferred for catalyzing certain other reactions. Microbial lipases are synthesized both intracellularly and extracellularly and their relative proportion is influenced by the culture conditions. Fungi are a valuable source of lipase as these organisms produce the enzyme extracellularly. Solid-state fermentation (SSF) where productivity is high offers a better alternative to submerged fermentation. In addition, the amount of lipase produced is dependent on several environmental factors such as cultivation temperature, pH, nitrogen and carbon sources, concentration of inorganic salts, and the availability of moisture and oxygen.

Lipases (EC 3.1.1.3) are enzymes widely distributed among animals, plants and microorganisms that catalyze the hydrolysis of glycerol-ester bonds at the fat-water interface. However, microbial lipases are the main source for industries (Hou & Johnston, 1992) as they can be obtained at a rapid pace in fairly large amounts and at low cost. Fungal lipases, predominantly *Pencillium* lipase (Eitenmiller et al., 1970) are used to hydrolyse fats and synthesise specific esters with desirable flavour properties to enhance the flavour (Arnold et al., 1975) of dairy products such as butter and cheese. Such exogenous application of lipases for development of aroma during tea processing has not been examined so far, and it was therefore considered worthwhile to study this aspect.

Tea that is available commercially is made from the apical two leaves and a bud of the plant *Camella sinensis* (L) O. Kuntze, cultivated in hilly terrains of countries like India, China, Japan and Sri Lanka. The conversion of fresh tea leaf to finished tea depends on the effect of oxidative and hydrolytic enzymes present endogenously in the green leaf. A number of biochemical changes take place before the green leaf is converted to the black tea of commerce. Processing of tea leaves can be done either through the orthodox process or the Cut-Tear-Curl (CTC) method. The orthodox method, though very elaborate, produces tea of high quality that is light and aromatic. The main feature of the CTC process is that it is much simpler but results in teas with more cuppage and lesser aroma. For these reasons, the CTC teas are more economical than the orthodox ones. If the flavour of CTC teas can be enhanced to the level of orthodox teas, it would be a favourable compromise between quality and economy. In the present work, conditions for SSF and extracellular secretion of lipase by the fungus *Rhizomucor miehei* have been optimised and its application to enhance aroma during tea processing has been attempted. It has been emphasised that the aroma of tea is
due to the volatile flavour compounds formed mainly from the conversion of unsaturated lipids (Sajio & Takeo, 1972) to long chain aldehydes and alcohols by lipases.

Materials and Methods

Organism and growth conditions Rhizomucor miehei was obtained from the Institute of Microbial Technology, Chandigarh, India, and maintained on potato-dextrose agar slants at 5°C. Olive oil agar medium containing Rhodamine B as the dye was used for screening for lipase activity (Hou & Johnston, 1992) in a few fungi also: Candida sp., Aspergillus sp., Rhizopus sp. and Penicillium sp., which were obtained from a local collection.

The basal medium for lipase production contained (g/l): NaNO₃, 5; MgSO₄·7H₂O, 0.5; KCl, 0.5; KH₂PO₄, 1; Na₂HPO₄, 1; ZnSO₄·7H₂O, 0.01; MnSO₄·0.01; sucrose, 5; and 1% olive oil (v/v). The nitrogen and carbon sources along with phosphate salts and cations in the culture medium were altered for optimization experiments. For enzyme induction, an aqueous spore suspension (1 ml) containing 10⁸ spores was inoculated into 100 ml of growth medium, incubated for 72 h. and enzyme activity was assayed every 24 h.

Solid-state fermentation For solid-state fermentation, the fungus was inoculated onto wheat/rice bran medium in perforated trays with developed standardised conditions (amended with sucrose, urea and olive oil), and induced for maximum lipase production. After 5-days of growth, the enzyme was leached from the wheat bran by agitation and repeated extractions with three volumes of distilled water. The extracted culture filtrate was then centrifuged at 13,000×g at 4°C for 40 min and the resultant supernatant used as a source of enzyme for industrial application.

Enzyme assay Lipase was assayed titrimetrically and by colorimetric methods. Titrimetrically, 5 ml of olive oil emulsified in 4 ml of sodium phosphate buffer (0.1 M, pH 7) was preincubated at 30°C and 1 ml of the enzyme extract added. The reaction was terminated with acetone after 20 min incubation at 30°C and titrated against 0.01 N NaOH, with a control containing one ml of the heat inactivated enzyme. One unit of lipase activity represents the amount of enzyme that releases one mole of free fatty acid in 1 min/ml of culture filtrate. In the colorimetric method (Kwon & Rhee, 1986) using olive oil as substrate, the absorption in the culture medium was altered for optimization experiments. For enzyme induction, an aqueous spore suspension (1 ml) containing 10⁸ spores was inoculated into 100 ml of growth medium, incubated for 72 h. and enzyme activity was assayed every 24 h.

Characterisation of the enzyme Lipase activity was measured at various pHs using phosphate buffer at 37°C to determine the optimal pH of the enzyme. Temperature optimum was obtained from an assay of activity at different temperatures at pH 7.0 with olive oil as substrate.

The enzyme was preincubated with buffers of different pH for 30 min at 30°C and incubated at different temperatures for 30 min at pH 7 to study its stability to pH and temperature changes.

The culture filtrate was incubated with detergents (TWEEN 80 and SDS) at a concentration of 1 mg/ml each at 30°C for 30 min, after which an aliquot (0.2 ml) was withdrawn and assayed for lipase activity. SDS-PAGE was performed on 10% gels (Leamli, 1970).

Manufacture of black tea Tea processing essentially consists of partial removal of moisture (withering), disruption of cellular integrity of the shoots (rolling), thereby mixing endogenous enzymes with substrates resulting in the initiation of a series of biochemical reactions (fermentation), and finally the formation of pigmented hot water soluble compounds (drying) characteristic of black tea.

Analysis of volatile flavour compounds (VFC) VFCs were extracted by steam distillation, collected in ether and analyzed on a Nucon gas chromatograph equipped with a flame ionization detector and separated on a glass column (2 m x 3 mm i.d.) packed with 5% Carbowax 20M on Chromosorb G (80–100 mesh) using nitrogen gas as carrier at a flow rate of 30 ml/min (Hara & Kubota, 1976). The proportion of each compound was analysed and the quantity of the compounds was expressed as a ratio of the area of the peaks of the compounds to that of the internal standard peak. Extraction was by the simultaneous distillation and extraction method using water/ diethyl ether with ethyl decanoate as internal standard. The ether mixture was concentrated and the concentrate subjected to gas chromatography analyses under conditions using peak identification and comparison of retention time (Yamanishi et al., 1966) with authentic samples (4 µg/g tea), and was confirmed by GC-MS.

Chemicals Linalool, geraniol and ethyl decanoate, as well as olive oil, urea, Tween 80, SDS and other chemicals for SDS-PAGE were purchased from Sigma Chemical Co., USA. All other chemicals used were of analytical grade.

Results and Discussion

Fungi are normally known to grow rapidly on simple substrates and to secrete enzymes that make them very attractive for industrial applications. Since microbial lipases are widely diversified in their enzymatic properties and substrate specificities, there is a need for screening and selecting the appropriate enzyme for a particular application. While all the fungal strains screened: Candida sp., Aspergillus sp., Rhizopus sp., R.M. miehei and Penicillium sp., produced halo zones on Rhodamine B agar plates, indicating lipase activity, only the enzyme from R.M. miehei was effective in generating the desired aroma in tea. The enzyme from R.M. miehei was extracellular and this would be an added advantage for use during tea processing, as large-scale production of the enzyme, though of lesser purity, is needed. Therefore, significant parameters in the production of lipase were optimized in Erlemeyer flasks, and these conditions were employed for SSF.

The best nitrogen source was found to be urea (Fig. 1). R.M. miehei produced maximum lipase when supplemented with sucrose (Fig. 1) compared to other carbon sources. R.M. miehei utilized glucose and fructose very poorly for lipase production, which is similar to the behaviour of A. niger and Pseudomonas (Narasaki et al., 1968). This could be due to the catabolite repression by glucose that leads to a delay in utilisation of olive oil for lipase production. In A. niger, urea (0.5%) favours lipase production but in conjunction with meat extract (Kamini et al., 1997). The best source of phosphate KH₂PO₄ at 0.1% supported lipase production in R.M. miehei, while the optimum level of Mg²⁺, Ca²⁺ and potassium was 0.05%. There was no significant difference in lipase activity upon supplementation with Zn²⁺, Mn²⁺ and Fe²⁺ at 10 mg/l.

The lipase from R.M. miehei showed optimum activity at 30°C (Fig. 2A) and displayed high activity (67%) even at 10°C. This unique feature of this lipase, not common among other microbial
enzymes, would be ideal for tea processing where reactions are carried out at temperatures below 15°C. At temperatures higher than its optimum, the enzyme showed fairly high activity, being 93% at 40°C and 70% at 50°C, respectively; at 60°C, activity dropped down to 28%. Lee and Lee (1989) reported a decrease in activity at temperatures as low as 30 and 40°C for *A. niger* lipase. The lipase from *R.M. miehei* showed good stability at temperatures between 10 and 50°C with stability decreasing only at temperatures above 50°C. Compared to the enzyme from *R.M. miehei*, *R. delemar* lipase appears to be more tolerant to temperature as it has been reported to be stable even at 60°C (Iwai & Tsujisaki, 1974).

The *R.M. miehei* lipase was active over a wide range of pH with optimum activity at 7 (Fig. 2B). Activity decreased drastically when the pH was increased in the alkaline range above 8. Between pH values of 4 and 6, activity was fairly high, being nearly 81 to 94% of that at pH 7, a property suited to the conditions prevailing during fermentation of tea leaves. Iwai and Tsujisaka (1974) reported an optimum pH of 5.6 for activity of *Rhizopus delemar* lipase and stability in the range of pH 3 to 8. While the lipase from *Aspergillus niger* (Pal et al., 1978) and *Calvatia gigantea* (Christokopoulos et al., 1992) show optimum activity at pH 7, the optimum for the enzymes from *Penicillium roqueforti* (Eitenmiller et al., 1970) and *Rhizopus oryzae* (Salleh et al., 1993) are 8 and 5.0, respectively. The lipase from *R.M. miehei* was stable over a range of pH between 2 and 10 (Fig. 2B), with very high residual activity at pH 6 to 8. Such variations in pH and temperature tolerance and shifts in the optima for activity decide the usefulness of a particular enzyme for a specific industrial application.

The protein profile of the acetone precipitate obtained from the culture filtrate of *R.M. miehei* showed protein bands lying in the molecular weight range of 22 to 41 KDa (Fig. 3). The molecular weight of the fungal lipases *M. javanicus* (Ishihra et al., 1975), *M. lipolyticus* (Nagaoka & Yamada, 1973) and *Geotrichum candidum* (Jacobson et al., 1989) has been shown to lie in the range of 21 to 61 KDa.

With the optimised conditions, the production of lipase by
R. M. miehei was carried out under SSF using wheat/rice bran as substrate and 1.0% olive oil, urea and peptone as inducers. In the absence of these inducers the culture grew well but produced a lower amount of extracellular lipase. Wheat bran was better than rice bran and maximum lipase production of 37 U/g wheat bran could be obtained. It was observed that 70–80% humidity and aeration at 1 l/min were additional parameters required for the production of lipase on a solid substrate. Olive oil was the most effective in stimulating the formation of lipase compared to production of lipase on a solid substrate. Olive oil was the most effective in stimulating the formation of lipase compared to groundnut or castor oil. However, gingelly and coconut oils proved to be better inducers than castor oil. Tsujisaka et al. (1973) and Hang and Woodams (1990) have reported that fungi show more lipase activity when grown on wheat bran with olive oil (0.3–2%), while Rao et al. (1993) observed further increment in presence of urea.

The improvement of flavour in the teas treated with lipase was examined with the control where water was sprayed instead of the enzyme as depicted in Table 1. The lipase from R. M. miehei was added at the end of the fermentation stage of tea manufacture at a concentration of 100 units per kg of leaves. Taking into consideration the effect of the enzyme application on flavour val-

ance, other major volatile flavour constituents like methyl salicy-
late, phenyl acetaldehyde, linalool and geraniol have also been analyzed and it was observed that the enzyme in no way ham-
pered the production of these volatiles.

During tea manufacture, the transformation of the fresh green leaf to black tea of commerce depends on the oxidative and hydrolytic enzymes in the leaves. The hydrolysis of the structural components (pectic, cellulosic and lipolic) of the cell wall, membranes and the middle lamellae, is carried out mainly by the endogenous enzymes pectinases, cellulases and lipases. Due to mechanical damage of the tea leaves in the process of rolling, proper mixing of oxidative enzymes and substrates takes place, which is otherwise prevented by compartmentalization and suppression of enzymic and non-enzymic mechanisms. The substrates interact with respective enzymes to generate the flavour precursors, which are important for the generation of flavour components in tea. Several lipid soluble components are invariably precursors of flavour development and are transported as acid derivatives or glycosylated compounds. These compounds undergo transformation during rolling and fermentation to give the typical flavour notes of tea. Flavour precursors of linalool, geraniol, benzyl alcohol and methyl salicylate are also present as glycosides and are transported to appropriate sites. The products of glycoside hydrolysis (Takeo, 1981) during fermentation in tea manufacture are linalool, linalool oxides, geraniols and geranic acid. The rate of enzymatic hydrolysis affects the production of these compounds. The major volatile constituents of organoleptic importance are (E)-2-hexenal, n-butyraldehyde, (Z)-3-hexenol, 1-octen-3-ol, linalool, methyl salicylate and geraniol.

Table 1. Content of volatile flavour compounds in finished tea.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Lipase treated</th>
</tr>
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<tbody>
<tr>
<td>(E)-2-Hexenal</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>(Z)-3-Hexenol</td>
<td>0.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Linalool</td>
<td>1.3</td>
<td>3.9</td>
</tr>
<tr>
<td>Phenyl acetaldehyde</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Methyl salicylate</td>
<td>0.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Geraniol</td>
<td>0.3</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 2. Organoleptic evaluation of finished teas.

<table>
<thead>
<tr>
<th>Colour</th>
<th>Strength</th>
<th>Quality (Aroma)</th>
<th>Briskness</th>
<th>Taster’s Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49</td>
<td>50</td>
<td>45</td>
<td>50</td>
</tr>
<tr>
<td>Treated</td>
<td>51</td>
<td>53</td>
<td>52</td>
<td>52</td>
</tr>
</tbody>
</table>

Marks for liquor characteristics are the averages of all tasting reports, each to a standard scale of 60.

An enhancement in the aroma of lipase-treated tea was also observed by tea tasters during organoleptic evaluation. Flavour impressions are due to the interactions of active constituents with sense of taste and smell according to the professional tea tasters, and on evaluation of the various lipase-treated tea samples by the taste panel a good agreement was found between the sensory ranking and quality indices derived from chromatographic stud-
ies. The tasters’ score is based on the appearance of the liquor, infused leaf, colour, strength, briskness, quality and flavour. The results (Table 2) were tallied and confirmed by organoleptic evaluation of the teas.

The degradation of lipids accomplished by application of this lipolytic enzyme showed rapid reaction rates even at low temperatures and under mild acidic conditions, and resulted in a significant increase in the flavour volatiles of the tea. Comparing the relative concentrations of the volatile flavour compounds (Table 1), it is evident that the content of all VFCs is higher in lipase treated tea with (E)-2-hexenal showing an inverse relationship. (Z)-3-hexenol and linalool, the key volatiles responsible for the flavour of high quality teas showed a significant increase. Although this study was restricted to some of the major volatile flavour compounds, it is important to note that even compounds present in minute quantities contributed to the overall flavour profile of tea. The endogenous enzymes were allowed to carry out the reaction until the fermentation step of the processing, and the addition of the exogenous lipolytic enzyme to a great extent facilitated moving of the same reaction to the finishing point. The enzymatic system of R.M. miehei can thus be used to split the lipids containing fatty acids with a high degree of unsaturation as well as their further conversion to volatile flavour compounds contributing to the flavour of tea.

References