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SHORT COMMUNICATION

Enzymatic synthesis of short chain citronellyl esters by a new lipase from *Rhizopus* sp

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Short chain citronellyl esters were synthesized by a new microbial lipase from *Rhizopus* sp strain isolated and lipase produced at UNICAMP, Brazil. Direct esterification and transesterification reactions have been performed to produce citronellyl acetate and butyrate in a free-solvent system and with n-hexane in reaction medium. Reaction mixture for direct esterification and transesterification was carried out at 45ºC in equimolar concentration of substrates: acid or ester and alcohol. Only citronellyl butyrate was synthesized by direct esterification with yields from 95 to 100% after 24 hrs. of reaction time, with or without n-hexane. Citronellyl acetate was synthesized by transesterification with ethyl acetate and cytronellol, with yield of 58% after 48 hrs. and 48% of conversion for reaction butyl acetate and cytronellol. The results suggest that the size of the aliphatic chain from acyl donor was importance to conversion rate. Acids with more than two carbons showed to be better substrate for *Rhizopus* sp lipase. Transesterification reaction showed different behaviour, ester substrate with four carbons was better than six carbon for citronellyl acetate synthesis.

Esters of short chain fatty acids and alcohols are known as flavour and fragrance compounds.

Used in the food, beverage, cosmetic and pharmaceutical industries. Currently, most of the flavour compounds are provided by traditional methods as chemical synthesis or extraction from natural sources. The great recent interest for "natural" products pushed the flavour industry to seek new methods to obtain flavour compounds naturally. The use of Biotechnology specifically direct biosynthesis by fermentation has been described as potential source of esters (Christen and Munguia, 1994). However, the esters concentration and productivities obtained using fermentation are rather low. A new possibility to produce natural esters was opened by enzyme catalysis carried out in non-aqueous organic solvents or in free-solvent reaction medium (Gubicza et al. 2000). Direct esterification and transesterification reactions have been performed using lipases to produce, tailored triglycerides and numerous flavour esters. Many acetate and butyrate esters are components of natural flavours. However, reports on the production of acetate esters are scarce in various organic solvents. Due the toxicity of acetate on lipase activity in enzimic acetylation, the use of acetic acids an acyl donor in transesterification and direct esterification reactions was previously attempted with little or no success (Güvenç et al. 2002).

The Food Biochemistry Laboratory at UNICAMP, Brazil has isolated several microorganisms which producing lipases. One of the potent producer of lipases isolated is the strain *Rhizopus* sp. In our laboratory we have tested the ability of the lipase from *Rhizopus* sp to catalyse the

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formation of citronellyl esters with acetic and butyric acid by direct esterification and transesterification reactions.

Materials and Methods

Chemicals

(R,S) citronellyl were purchased from Fluka Chemika; acetic acid, butyric acid were obtained from Merck (Darmstadt, Germany), ethyl acetate, butyl acetate, n-hexane, decane were purchased from Aldrich Chemicals. Molecular sieves 4 Å obtained from Acros Organics (New Jersey, USA).

Lipase production and activity assay

Lipase from *Rhizopus* sp was produced in a solid medium (60% wheat bran and 40% water) at 30ºC. After 72 hrs., water was added to the solid medium, followed by homogenization,1 hrs. of incubation followed by simple filtration. Supernatant were treated with ammonium sulphate (80% saturation). The precipitates were dialyzed in sodium phosphate buffer, pH 7.0, and lyophilized for use as crude lipase preparation in powder form. The lipase activities preparations were quantified by the hydrolysis of triolein (Macedo et al. 1997). One unit (U) of lipase activity was defined as one mmole of oleic acid released per minute at 40ºC. A Lowry method was employed to determinate the protein content in lipase crude power.

Esterification reaction

(R,S) citronellyl alcohol was mixed with acetic acid and butyric acid in equimolar (1:1) ratio, molecular sieves (4% w/w reactants) and lipase (10 Units). Ester synthesis was carried out in screw capped tubes incubated at 45ºC under constant agitation at 200 rpm. System #1 was solvent-free system and System #2, 2 mL of n-hexane was added. A control tube without lipase was prepared and incubated under the same conditions. Samples of 20 µL was withdrawn after 2, 5, 7, 10, 24, 48 and 72 hrs. of reaction time, and analysed by gas chromatography.

Transesterification reaction

Citronellyl acetate synthesis was carried out in screw capped tubes containing equimolar ratio of citronellyl alcohol and ester (ethyl acetate, butyl acetate), molecular sieves (4% w/w reactants) and 10 lipase units. The experiments was conduced with and without n-hexane. Samples of 20 µL was withdrawn after 2, 5, 7, 10, 24, 48 and 72 hrs. of reaction time.

Analysis by gas chromatography

At the end of incubation period, the reaction mixtures were cooled. Two hundred of internal standard (hexanol) was added to each sample. A one µL aliquot was injected in a split (1:100) mode into a CHROMPACK® CP9001 (Middle Burg, Holland) gas chromatograph equipped with a flame-ionisation detector. A CP WAX 52 CB (Chrompack®, Middle Burg, Holland) fused silica capillary column (30 m x 0.32 mm i.d.; film thickness 0.2 mm) was used. Injector and detector temperatures were set at 220ºC and 250ºC, respectively. Oven temperature ranged from 50ºC to 220ºC. The carrier gas was helium at 1ml/min. The extent of synthesis (yield) was determine calculated basis on the consume of alcohol injected and quantified with standard curves of alcohol. The results were calculated as the equation 1:

Conversion rate (%) = Co – C / Co x 100 (1)

Where:

Co: initial concentration of (R,S) citronellyl

C: concentration of alcohol at a given time

Results and Discussion

Synthesis of citronellyl esters by direct esterification

Several lipases showed the ability in catalyse acetate and butyrate citronellyl esters by direct esterification. However, the synthesis of citronellyl acetate usually display very low yields (Claon and Akoh, 1994). *Rhizopus* sp lipase presented maximum yield of 10% after 24 hrs. of reaction time decreasing the yield after that time. Previous reports indicated an inhibitory effect of acetic acid on lipase-catalysed reactions (De Castro et al. 1997). The effect of acetic acid concentration on esterification reaction using lipase SP435 was investigated by Claon et al. 1994. In this study, increasing concentrations of acetic acid (0.4 to 0.7 M) inhibited SP435 lipase activity resulting in low conversion yields for acetate esters. According to Claon and Akoh, 1994 the presence of acetic acid can damage the hydration layer-protein interaction of the enzyme structure causing lipase deactivating during the esterification process. Probably *Rhizopus* sp lipase is vulnerable to acetic acid in reaction medium as other lipases reported.

Synthesis of citronellyl butyrate exhibited better yields. The maximum ester conversion achieved was 95% after only 24 hrs. of reaction time. In Figure 1 the experimental results are shown for solvent-free system and n-hexane reaction system. Ester conversion obtained without n-hexane was not much lower than the yields observed using n-hexane as organic solvent during esterification reaction. The results suggest that n-hexane is not necessary for citronellyl butyrate direct synthesis using *Rhizopus* sp lipase. This fact is of great interest for food industry since the step of solvents recovery (that are toxic) could be eliminate reducing costs and pollution concerns.

Synthesis of citronellyl acetate by transesterification

As direct esterification does not produced satisfactory yields for citronellyl acetate ester, transesterification was

tested. The reaction mixture was composed by ester as acyl donor (ethyl acetate or butyl acetate) and alcohol citronellyl. Figure 2 shows the results obtained for both preparations. Since the presence of n-hexane was not significant in the yields obtained, transesterification reaction was carried out without n-hexane. *Rhizopus* sp lipase catalysed acetate ester with maximum yield of 60% after 48 hrs. using ethyl acetate as substrate. *Mucor miehei* lipase also showed affinity for lower molecular mass acyl donor for geranyl acetate synthesis according to Chulalaksananukul et al. 1992. The chain length of acyl donor could be affecting the yield of ester synthesis either in direct esterification or transesterification reaction using *Rhizopus* sp lipase. Thus, findings indicate that overall a higher affinity was shown for butyrate than for acetate and that the chain length of the acyl donor could have an effect on the yield of cytronellol esters. *Rhizopus* sp lipase showed promising results and immobilizing the enzyme could also improve yields as well as reduce costs. Therefore, reaction conditions and parameters need to be studied more extensively to develop optimum product yields.

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APPENDIX

Figures

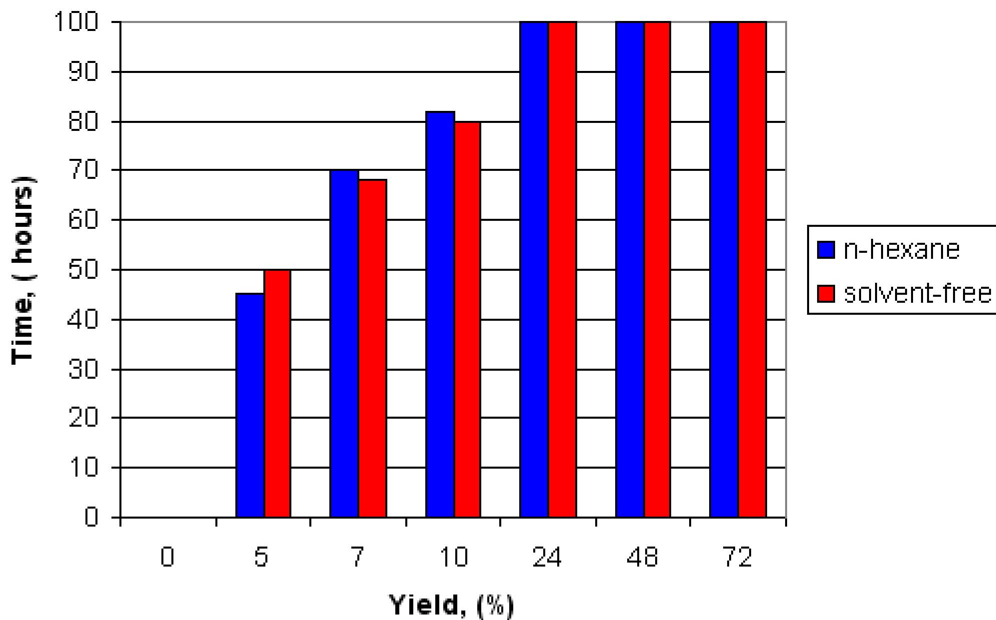

Figure 1. Time course of citronellyl butyrate synthesis using *Rhizopus* sp lipase.

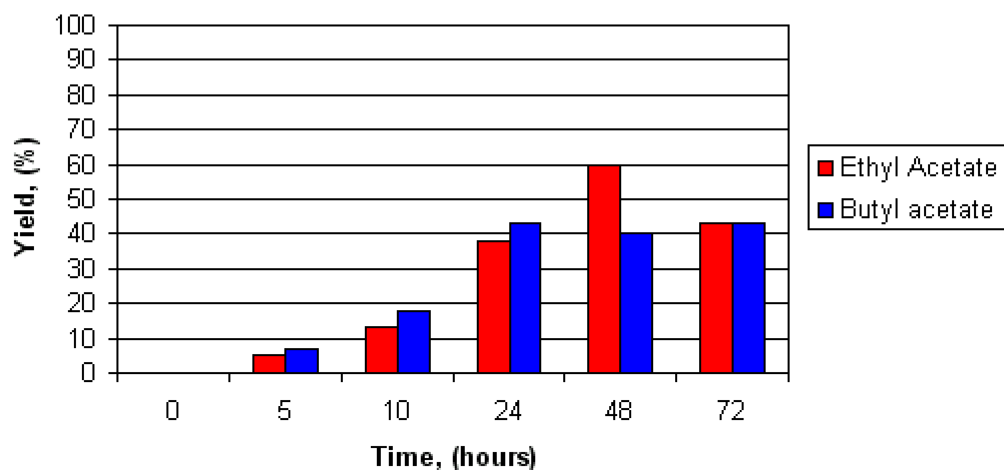

Figure 2. Time course of citronellyl acetate synthesis by *Rhizopus* sp lipase.