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Enzymatic synthesis and parameters affecting on citronellyl acetate ester by trans-esterification reaction

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Citronellyl acetate is a crucial component of flavor and fragrance in the food, cosmetic, and pharmaceutical sectors. In non-aqueous media, it can be successfully produced via lipase catalysis. This study focuses on the lipase-catalyzed trans-esterification of citronellol alcohol with geranyl acetate ester in a non-aqueous medium to produce citronellyl acetate. For the synthesis of citronellyl acetate, crude acetone powders isolated from several plant seedlings of black cumin, fenugreek, coriander, flax, and rape seed were examined for lipase activity. Black cumin seedling lipase had the highest level of citronella acetate production with a yield of 76.32% in 72 h of reaction time. To assess the impact of different reaction parameters on citronellyl acetate production in organic solvents, hexane was chosen as the best solvent, and black cumin seedling lipase was selected as the best biocatalyst. The highest conversion yield of ester (76.32%) was found when 0.25 M of geraniol acetate and 0.25 M of citronellol reacted at 41 °C after 72 h in the presence of 0.25 g of seedling lipase enzyme in n-hexane. It has been determined that crude black cumin seedling lipase is inexpensive yet effective and has the potential to be used industrially for the synthesis of terpene esters.

Keywords: Trans-esterification, seedlings lipase, flavor ester, citronellyl acetate, organic media.

INTRODUCTION

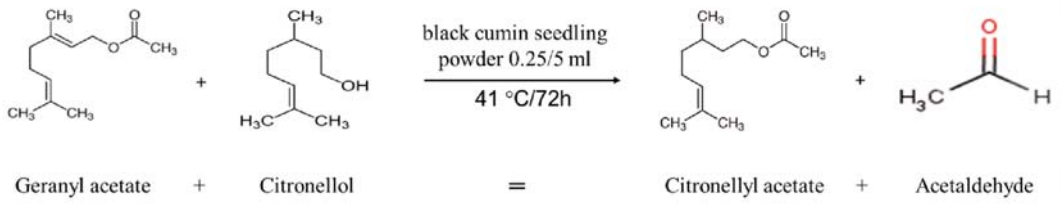
Citronella, rose, and magnolia volatile essential oils contain citronellyl acetate, a terpene ester with a distinctive scent that is widely manipulated in a variety of perfumes with scents of rose, lily, lavender, carnation, apple, and lemon[1]. Additionally, it is employed in producing food, medicine, and cosmetics[2]. Because of this, there is a high demand for citronellyl acetate in the market, but the natural form obtained by directly extracting rose or other plants does not fulfill the specifications. It can be synthesized by microbial, seedling, and fungi lipases. Therefore, it is essential to discover a synthesis technique that is more effective, eco-friendly, and affordable[3], to prepare esters of demands. The use of enzymes in manufacturing produces goods with high purity on par with those obtained by natural extraction and has a high market value[4].

Although lipases from higher plants may exhibit diverse characteristics that could be used for technical or industrial purposes, they have not yet been thoroughly studied[5]. In plant lipase activity has been identified in various tissues but a relatively high concentration was found in oil seeds[6]. The biotransformation of oils and fats primarily uses lipases from plant seed[7]. Lipase activity is said to be absent in dormant (un-germinated) seeds and to increase quickly as germination progresses[8]. However, dormant seeds like peanuts have occasionally been observed to have lipolytic action[9], castor bean[10], physic nut[11], African oil bean[12], and black cumin seedlings[13].

Black cumin seedlings are used as condiment or spice in cooking[14]. It has been used to catalyze esterification and trans-esterification at ambient temperatures[15]. The ground black cumin seedling as well as the pressed seeds or crude acetonic powder proved to be an effective biocatalyst for the synthesis of an ester[16].

Similar to hydrolysis and esterification, transesterification is the displacement of alcohol or acid from an ester by another process[17]. Trans-esterification does not produce water and is not inhibited by acid or alcohol because of this reason it is believed to be more effective than esterification for the synthesis of flavors[18]. However, the conversion yield is accelerated by the presence of a catalyst (a strong acid or base)[19]. Fatty acid alkyl esters and glycerol are produced during the esterification and trans-esterification of triglycerides. At the bottom of the reaction vessel, the glycerol layer accumulates. The intermediaries in this process are di-glycerides and mono-glycerides. The high viscosity of triglycerides has been significantly reduced by using this technique. The general equation's representation of its reaction is present in Scheme 1.

Scheme 1. Lipase-catalyzed trans-esterification of citronellol with geranyl acetate to produce citronellyl acetate in hexane



The purpose of this work was to study the synthesis of citronellyl acetate flavor ester using 5 plant seedling lipases in n-hexane. Crude acetone powder of various days germinated seedling were obtained from black cumin (*Nigella sativa*), rape seed (*Brassica napus*), fenugreek (*Trigonella foenum- graceam*), linseed sp (*Linum usitatissium*), and coriander (*Coriandrum sativum*). Crude acetone powder from seedlings are inexpensive form of biocatalyst for organic phase biocatalyst (OPB). Crude black cumin seedling lipase was further investigated to study the effects of parameters such as enzyme concentration, solvent choices, effect of substrate concentrations, temperature, and incubation time for trans-esterification reactions. The innovative feature of this study was that such terpenic esters were never synthesized in the literature using spice plant seedlings, especially black cumin, fenugreek, and coriander.

MATERIALS AND METHODS

Chemicals

Acetic acid, citronellol, hexane, toluene, acetonitrile, pentane, heptane, sodium hypochlorite solution, and geranyl acetate were among the chemicals of reagent grade that were purchased from Sigma-Aldrich Pvt Ltd, Fishers, Across, Supleco, and Honey Well Pvt Ltd.

Screening of Plant Enzymes for Citronellyl Acetate Synthesis

To esterify citronellol and geranyl acetate ester, five crude seedling lipases were studied. 250 mg of the appropriate seedling lipase was added to the mixture of 0.25 M citronell-ol and geranyl acetate and the total volume of the reaction mixture was raised to 5 mL by adding hexane. At predetermined intervals, samples were produced and subjected to GC analysis.

Seed Material and Germination

Plant seeds were purchased from seeds distributor company Swabi, Pakistan. Whole seeds were sterilized by soaking in 0.1 percent sodium hypochlorite solution for 10 minutes. The entire batch of seeds was washed in tap water and steeped for 24 hours at 30 °C in a dark incubator. The first day of germination was defined as these 24 hours. To help kick start germination, in plastic trays filled with moist sand, seeds were spread out on damp filter paper. After the fourth day of germination, seedlings were collected to make acetone powder. Previous research has indicated that the lipase activity was at peak in the first four to five days after germination[20].

Preparation of Acetone Powder from Seedlings

Acetone powders were made using the procedure described by[20]. A batch of recently germinated seedlings rinsed with three rounds of distilled water, and then chop into little pieces with scissors and put in a refrigerator at 4 °C for 20 min. The seedlings were grounded using a blender, then homogenised for one minute in five volumes of cold acetone (–4 °C or below). Afterwards, the entire mixture was transferred into a Buchner funnel and the acetone extracts were extracted using Whatman No. 1 filter paper that was fastened to a suction filter. The powder was then filtered four more times using five volumes of cold acetone, and it was then allowed to air dry for 10 h while being dried beneath a hood.

Trans-esterification Reaction Conditions

To achieve the trans-esterification reaction, the method outlined[21] was employed with a few minor modifications. In 5 mL of hexane in screw-capped glass vials, 0.25 M of ester and 0.25 M of alcohol were added with 250 mg of acetone powder (5% w/v of total reaction volume) as an enzyme source. In a water shaker, the synthesis was carried out by shaking reaction containers that were maintained at a 40 °C temperature.

The total quantity of esters produced was determined after taking 2 mL samples from the reacting mixture at 1, 10, 24, 48, and 72 h. These samples were then centrifuged for 20 min in a refrigerator-based centrifuge at 13000 rpm and 4 °C temperature. Aliquots of 1 mL from the supernatant were collected, stored at –20 °C, and typically utilized within 24 hours. The quantity of esters, acids, and alcohols was measured using gas chromatography after the frozen samples had warmed to room temperature. Each synthesis experiment was carried out three times using different reaction vials.

Product Yield

Liaquat *et al.*[21] have outlined the process for recognizing alcohol and esters. The formulas below were used to compute molar conversion or percent ester yields:

$$Molar\ conversion\ (\%) =$$

$$= \frac{Initial\ conc.of\ alcohol - Final\ conc.of\ alcohol}{Initial\ conc.of\ alcohol} \cdot 100\%$$

Gas Chromatographic Analysis

A merged silica linear column Elite-5MS Perkin Elmer (30 m 0.25 mm ID and film thickness 0.25 m) Nitrogen gas was employed as the carrier (2 mL/min, split ratio 2:20) were employed in a gas chromatography system (PerkinElmer Shelton, USA, Model Clarus 590) with an FID-detector, it was possible to determine the ester that was formed and the remaining acids and alcohols. The oven›s temperature was maintained at 50 °C for 2 minutes before being raised to 210 °C at a continuous rate of 15 °C per minute and maintained for 4 minutes in a row. The oven's temperature was increased even further, by 15 °C per minute, and held there for five minutes. Detectors and injectors were maintained at a constant temperature of 260 °C. The gas chromatography was linked to a computer system that captured periods of retention and peak areas in chromatograms.

Esters Identification and Quantification

Ester and alcohol were distinguished based on their chromatogram retention durations and by comparisons to findings obtained with standards. A calibration graph plotting peak regions and known alcohols (citronellol) was created. By dilution in n-hexane, various alcohol concentrations (0.05 M to 0.3 M) were created. Then, 0.2 μL of each sample was fed into gas chromatography. Three injections were given from each vial.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Esters were identified using GC-MS using from the Agilent 7890A Series. The GC-MS was outfitted with an electron impact ionization system, a 5-MS column (30 m x 250 m x 0.25 m), and a multimode injector.

Using nitrogen as the carrier gas and injecting 1 μL into the column in splitless mode at 1.5 mL/min. Temperatures for the quadruple and ion sources were 150 ^{o}C and 230 ^{o}C, respectively. Elution was carried out as previously described. To determine the ester, the fragmentation pattern has been compared to the NIST 2011 MS Library. The ester was successfully identified by full-scan mode scanning between 35 and 6008ha m/z with a gain factor of 5.

Factors Effecting Reaction Conditions for Citronellyl Acetate Ester Synthesis

Reaction Temperature's Impact

Investigations were made on how temperature affected the production of citronellyl acetate by black cumin seedling lipase. 250 mg of lipase, 0.25 M geranyl acetate, and 0.25 M citronellol in n-hexane were all included in the reaction mixture. For 72 hours, the reaction mixture was heated to a range of 20 to 50 ^{o}C at intervals of 5 ^{o}C. Gas chromatography was used to assess the amount of citronellyl acetate ester that was produced and the alcohol that was still present in each case.

Effect of Solvents

Using 5 organic solvents of varying hydrophobicity (acetonitrile, toluene, heptane, n-hexane, and pentane), the interaction of geraniol acetate with citronellol was studied. Except for the optimal temperature found in a prior experiment, all other reaction conditions were as detailed below.

Effect of Reaction Time

Under the ideal circumstances mentioned above, the impact of reaction time on the process of geranyl butyrate ester production has been examined while keeping the numbers of other parameters constant. Alcohol and ester were responded to at an ideal temperature for periods of 1, 10, 24, 48, and 72 hours in a water shaker at an equimolar ratio of 0.25 M of each.

The Impact of Enzyme Concentration

For the production of citronellyl acetate ester, the effect of raising the enzyme concentration was assessed while maintaining the other parameters constant. The best concentrations of ester and alcohol were used to react with enzyme concentrations of 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 g. Gas chromatography was used to determine the ester that was produced in each situation.

Effect of Substrate Concentration

It was determined what would happen if one substrate's concentration was raised while the other remained constant. The reaction between varying quantities of geranyl acetate (0.05 M, 0.1, 0.15, 0.2, 0.25, and 0.3 M) and a constant concentration of citronellol alcohol (0.25 M) took place. In the opposing investigation, citronellol alcohol concentration was adjusted (0.05 M, 0.1, 0.15, 0.2, 0.25, and 0.3 M) while geranyl acetate concentration was held constant at 0.25 M. The best organic solvent was chosen, and the temperature was tuned.

Statistical Analysis

All of the tests had a factorial design with three replicates. Data from gas chromatography were statistically analyzed using Statistics 8.1 software. Tukey's test was used to compare the treatment averages ($p \leq 0.05$).

RESULTS AND DISCUSSION

Screening of Plant Enzymes for Citronellyl Acetate Synthesis as a Function of Time

The best plant enzyme for trans-esterification involving geranyl acetate ester and citronellol alcohol was chosen after screening plant seedlings lipase. Black cumin, rapeseed, fenugreek, flax, and coriander plant seedling acetone powders were used in the procedure along with hexane as a solvent.

The ability of five seedling acetone powders to biocatalyze the formation of citronellyl acetate as a function of time (Fig. 1). Synthesis typically increases over time. With a yield of 76.32% after 72 hours, black cumin seedlings had the highest transesterification activity among the five plant lipases that had been screened. In a nutshell, following a 72-hour reaction period, acetone powders from rapeseed and fenugreek seedlings produced yields of 69.43% and 56.73%, respectively. When compared to other powders, coriander seedling acetone powder performed badly and produced a final yield of 23.56% in only 72 hours. Ester generation in this research was less effective than direct esterification studies[22]. After 48 hours, two green note esters, geranyl butyrate, and citronellyl butyrate, were generated through direct esterification using black cumin seedling powder, with yields of around 96% and 94%, respectively. The reasons for varying synthesis yields are several. First off, because crude lipase was utilized, the yields were impacted by the varying lipase activities in the various powders[23]. Second, because different substrates were utilized in direct esterification and trans-esterification tests, it is anticipated that lipase specificity may alter the conversion yields[22].

GC and GC-MS Results

Figure 2 displays the gas chromatograms of citronellyl acetate following a 72-hour reaction period. Figure 3 displays the GC-MS analysis of a library and synthetic citronellyl acetate. The fragmentation patterns shared a lot in common. Such GC-MS results allowed for the identification of the generated ester. Although GC-MS is presently the most effective method for identifying and separating volatile compounds in complex combinations, gas chromatography is a useful tool for the precise segregation, verification, and quantification of compounds in complicated mixtures. The fingerprint of the compound discovered is produced by combining GC and MS, resulting in a highly precise identification.

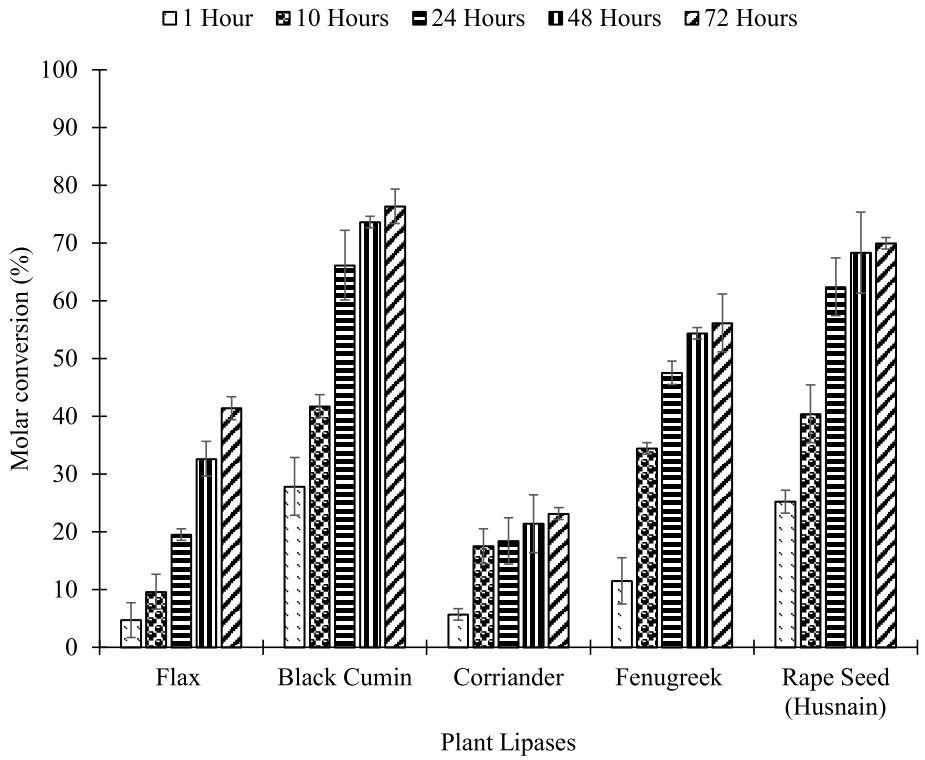


Figure 1. Time series demonstrating the yields of citronellyl acetate from different plant seedlings catalyzed by acetone powder. Specifications for the reaction were: 250 (5% w/v) mg of enzymes, 5 mL of hexane, and 0.25 M of geranyl acetate and citronell-ol. Samples were collected at different periods in a water shaking at 40 °C

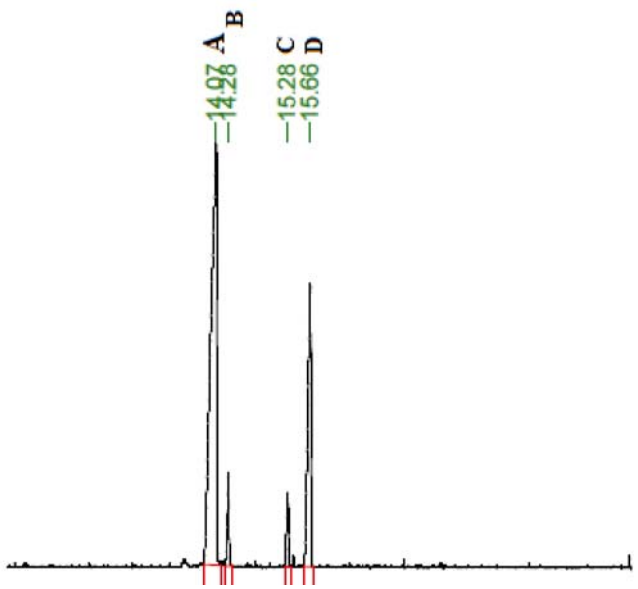


Figure 2. Analysis of samples from geranyl acetate transesterification studies with citronell-ol during a 48-hour reaction at 40 degrees Celsius using a gas chromatogram: Citronellyl acetate (A), Butyric acid (B), Citronell-ol (C), geranyl acetate (D)

Enhancement of Geranyl Butyrate

In light of the findings of our investigation, black cumin seedlings were selected as the greatest source of lipase enzymes. Black cumin seedling lipase's is model enzyme to catalyze citronellyl acetate ester (Scheme-1).

Effect of Incubation Time on Citronellyl Acetate Synthesis

Figure 4 shows the time scale for the synthesis of citronellyl acetate. Extended reaction periods resulted in an increase in the ester yield percentage. At 41 °C, the synthesis of citronellyl acetate was completed in 72 hours. A product yield of 76.32% was reported for the baseline research with no additional increase. This conversion came as quite a shock that there was no attempt taken to prevent water from accumulating in the reaction mixture. This response was substantially faster than the butyl caprylate ester reaction, which was catalyzed by psychrotrophic Pseudomonas fluorescents P38 lipase, achieved balance at 20 °C after 96 hours, and resulted in an overall molar conversion of 75% [24]. Two crucial objectives in this study are product yield and reaction time. A short incubation period lowers the cost of the entire reaction cost, the quantity of substrates needed, and the amount of energy required. The amount of biocatalyst employed, the concentration of co-substrates, the temperature, the organic solvents, The amount of speed at which sonication, churning, and shaking affect mass transfer are all factors that affect how long an incubation will take place[25].

Reaction Temperature's Effect

The temperature dependence of citronellyl acetate ester via trans-esterification is shown in Figure 5. Temperature ranges of 20–50 °C produced a maximum yield of 74.5%. Citronellyl acetate ester can be formed maximum at 41 °C, according to my observations. This functionality should result in significant energy cost savings for biotransformation. In contrast to this result, Using papaya (Carica papaya) lipase's the ideal ambient temperature for ester production was 63 °C, while for *Pseudomonas P38* lipase's the optimal temperature for the synthesis of butyl caprylate was 20 °C[26, 27]. The lipase becomes inactive at temperatures above 45 °C, which leads to a reduction in the synthesis of esters[28]. The enzymes that have low specific activity at low temperatures are stable in hydrophobic solvents, but it may be possible to

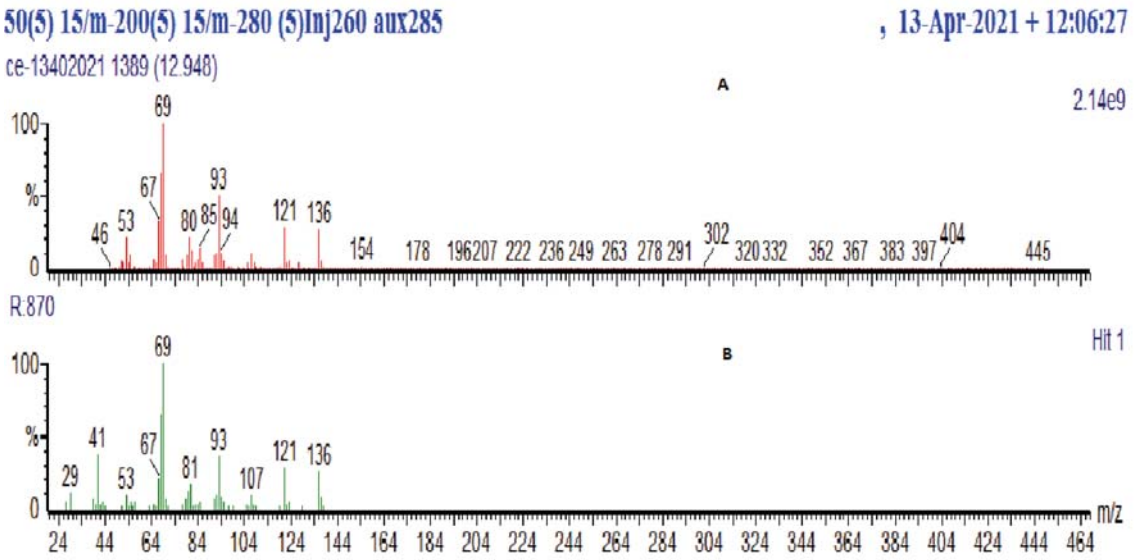


Figure 3. Citronellyl acetate was recognized in the mass spectra of peak (A), and its identification was confirmed in the transesterification sample (B)

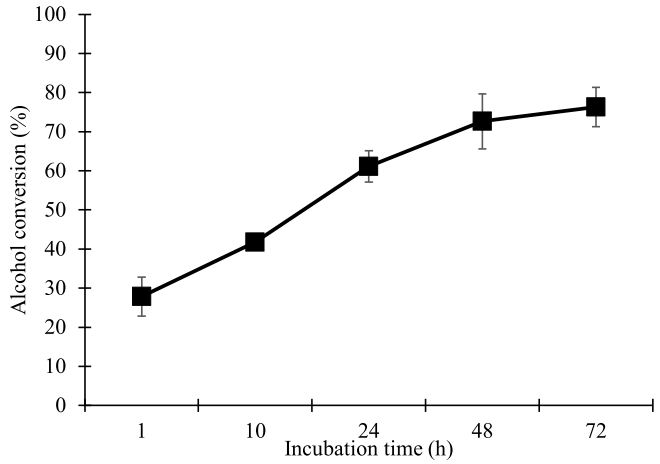


Figure 4. The production of citronellyl acetate over time. The reaction mixture included 5 mL of hexane, 0.25 M of geranyl acetate, and citronell-ol at 40 °C, 250 mg of powdered black cumin seedling acetone was utilized as the catalyst

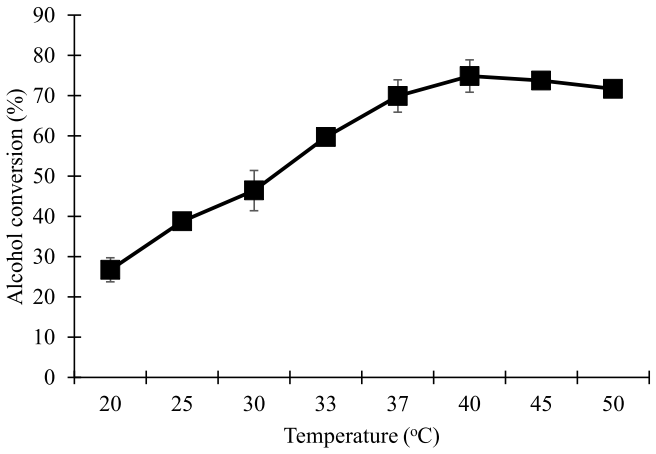


Figure 5. Impact of varying temperatures on the transesterification process citronellyl acetate ester formation. The reaction mixture consists of 5 mL of hexane, 0.25 g of acetone powder from day 4 of sprouted black cumin seedlings, and 0.25 M of each of geranyl acetate and citronell-ol at 37 °C

link an enzyme with a high specific activity in a hydrophilic solvent to one that is exceedingly adaptable and moderately thermo labile[29].

Organic Media's Impact on Ester Synthesis

Clearly, non-polar and light polar solvents had the highest citronellyl acetate % yield. After 72 hours at 40 °C a yield of 76.21% was observed in n-hexane and 74.39% was observed in pentane[30]. All report that hydrophilic solvents are linked to low ester yield. Acetonitrile, Tetra-hydrofuran, and Dioxane are hydrophilic solvents that have a coating that absorbs water from the biocatalyst, rendering it inactive[31, 32]. The water layer that surrounds the enzyme particles is preserved in non-polar solvents including pentane, hexane, and heptane, which helps to sustain enzyme activity and leads to high product yields[32, 33].

Effect of Enzyme Concentration

A number of studies were conducted in the range of 0.05 g – 0.3 g enzyme loading under similar circumstances to determine the right amount of enzyme (Figs. 5–8).

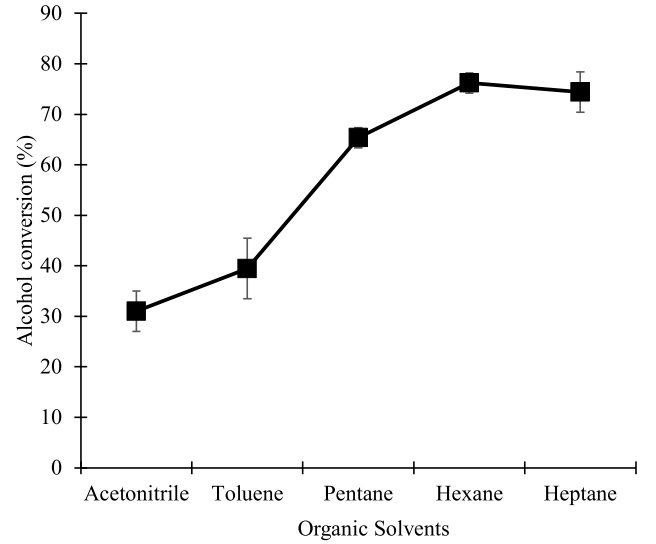


Figure 6. Impact of different kinds of organic solvents on the production of citronellyl acetate ester. The reaction mixture contains 0.25 g of powdered fourth-day sprouted black cumin seedling acetone together with 0.25 M of each geranyl acetate and citronell-ol alcohol at 40 °C

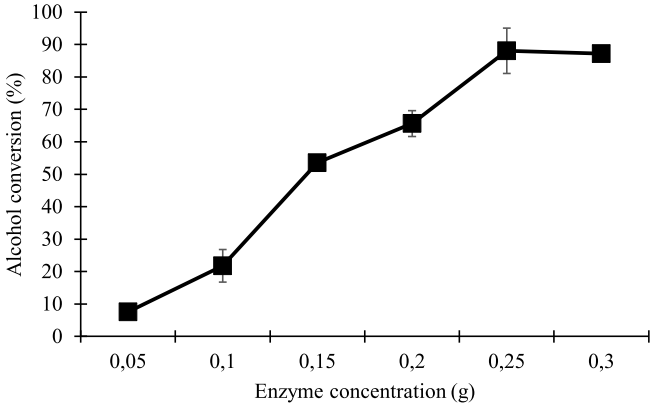


Figure 7. Impact of enzyme concentration on the percentage production of citronellyl acetate ester. The reaction mixture is composed of 5 mL of hexane having varying amounts of black cumin seedling acetone powder for 72 hours, along with 0.25 M of citronell-ol alcohol and geranyl acetate at 40 °C

Figure 7 displays the conversion profiles obtained with varied enzyme loadings. Up to 0.25 g of enzyme loading, there was an increase in conversion and reaction rate, after which there was only a slight rise. Increased enzyme loading will initially speed up the reaction and improve conversion. While high enzyme loading may cause the enzyme particles to react with one another and have a propensity to attach one another and form the enzyme aggregate. The enzyme particles on the aggregate's outside surfaces are readily exposed to the substrate, but the mass transfer rate may dramatically reduce the amount of substrate available to the enzyme particles inside the aggregate[34].

Substrates Loading Impact

The objective is to raise the citronellyl acetate ester's % yield. The ester yield in this investigation was controlled by the substrate concentration (Figures 8 and 9). While the other co-substrate was held constant at 0.25 M, the product yield increased as the concentration of geranyl acetate or citronellol alcohol increased (from 0.05 to

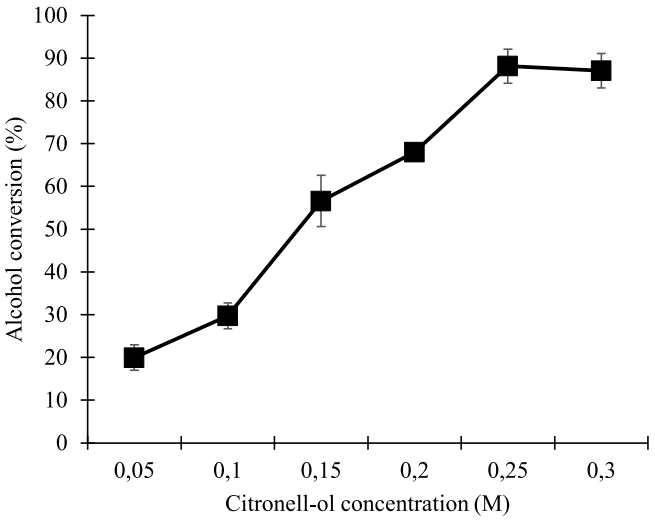


Figure 8. The citronell-ol concentration effected the citronellyl acetate ester Production. Several Citronell-ol concentrations were explored while maintaining a uniform, geranyl acetate concentration equal to 0.25M. The reaction mixture contains 0.25 g of black cumin seedling acetone powder in 5 mL hexane. Every reaction was run for 72 hours at 40 °C

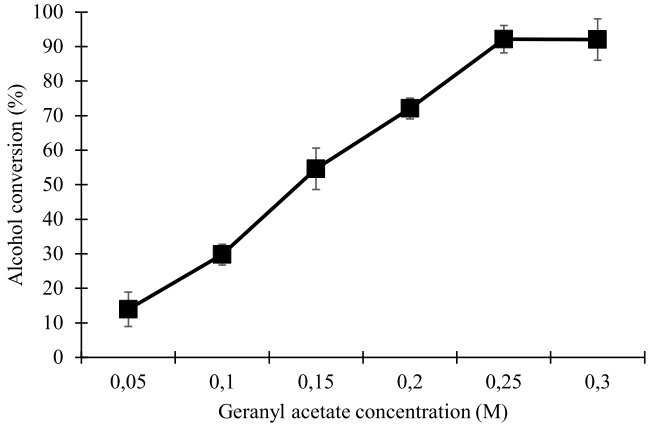


Figure 9. The geranyl acetate concentration affected the citronellyl acetate ester production. Several ester concentrations were explored while maintaining a uniform alcohol concentration equal to 0.25 M. Reaction mixture containing 0.25 g of black cumin seedling acetone powder in 5 mL hexane. Every reaction was run for 72 hours at 40 °C

0.3 M). This continued until the geranyl acetate or citronellol concentrations reached 0.25 M. A linear or diminished synthesis resulted by further raising the ester or alcohol concentration. The loss of efficiency happened when the concentration of substrates reached above a certain point, which may have been caused by a change in the medium's polarity[23]. It has been shown that the dehydration effect on black cumin seedlings' lipase stability diminishes the effectiveness of synthetics in higher alcohol concentrations[35]. Alcohols have been shown to prevent the lipase activity from *Bacillus licheniformis*[36], *M. miehei*[37], *Candida antarctica*[38], *Candida cylinracea*[39], and goat pregastric lipase[40]. Shorter-chain alcohols especially methanol, ethanol, and butanol have been discovered to have a deactivating effect when compared to long-carbon chain alcohols like geraniol and citronellol. This corresponds to how acid co-substrates deactivating effects correlate with their respective pKa values, which increase (acidity decreases) with chain lengths[23]. When employing different lipases as an organic phase biocatalyst, high concentrations of geranyl acetate were demonstrated to be susceptible[41], but not when using acetic acid[20, 42–43].

CONCLUSION

An exceptionally aromatic substance is citronellyl acetate. It is a short-chain ester with a fruity smell that is widely utilized in the food sector. The ability of lipases to catalyze the trans-esterification of geranyl acetate with citronellol alcohol to create citronellyl acetate was examined in crude acetone powders of five different plant seedlings. With black cumin seedling lipase, a maximum output of ester (76.3%) ester was attained after 72 h of reaction time. For the best yields, the ideal trans-esterification conditions were: 0.25 M substrate concentration for 72 h; n-hexane as the organic solvent; and 250 mg/5 mL (5% w/v) crude black cumin seedling lipase at 40 °C. The experimental data and the simulated data had very good agreement.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIALS

These seed spices are mainly used as condiments and have other ingredients for the development of functional foods and nutraceuticals.

First-time acetone powders from 3 spices and two oil seeds before and after germination have been evaluated for the synthesis of unique short-chain terpenyl esters.

Synthesis of esters increased with germination crude black cumin seedling gave a yield of 97% which was comparable to purified microbial enzymes.

Concentration terpenyl esters was determined by Gas chromatography and confirmed by GC-Mass Spectroscopy.

ABBREVIATIONS

μL:	Microliter
μmol:	Micromole
TSS:	Total Soluble Solid
ADH:	Alcohol dehydration
CRD:	Complete Randomized Design
mmol:	Millimole
Min:	Minutes
μg:	Microgram
Temp:	Temperature
UV:	Ultraviolet
MUB:	Methyl Umbelliferyl Butyrate
NaOH:	Sodium Hydroxide
HCl:	Hydrochloric Acid
NA_2HPO_4:	Disodium phosphate
H:	Hours
Mg:	Milligram
MUP:	Methyl Umbelliferyl Phosphate
MU:	Methyl Umbelliferone

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