Global Journal of Biotechnology & Biochemistry 3 (2): 60-68, 2008
ISSN 2078-466X

Microbial Biosynthesis of γ-Decalactone and its Applications-A Review

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Abstract: This review article deals with the chemistry and biosynthesis of γ-decalactone by various microorganisms. The various potential applications of γ-decalactone like fragrance and bitterness-relieving agent in pharmaceutical products, flavoring agent in manufacturing foods mainly bakery products and instant meals, as thickener for fruit juices and beverages, perfumery ingredients in detergents and cosmetics are reviewed. The metabolic events leading to the differences observed between the species are also reviewed.

Key words: γ-decalactone · Biosynthesis · Industrial application · Fragrances · Aroma

INTRODUCTION

The perception of 'natural' as better than 'artificial' has led to an increased demand for natural flavors and fragrances. With these concerns, there have been strong interests in the biotechnological production of natural flavor compounds during the past decade. Most fragrances are produced by chemical means are expensive, natural fragrances in small amounts are chiefly obtained from plant materials. Chemical synthesis of flavored compounds generally requires numerous steps and often lacks stereoselectivity [1].

Microbial fermentation is regarded as a potential means for producing natural flavor substances and has attracted a great deal of research interest [2,3]. Lactones are molecules of interest for the food industry due to their high aromatic fruity aroma. γ-decalactone which presents a pleasant peachy odor having the molecular formula $C_{10}H_{18}O_2$ and permitted as a food additive by the FDA of USA [4]. γ-decalactone widely used in the flavor industry produced from the genus *Sporidiobolus* was first reported in the year 1973 [5].

Lactones are found to be present in the aromas of more than 120 food stuffs. The increase in the demand of natural aroma in industries results in the development of biotechnological processes for the production of lactones [6]. The biotechnologically produced lactones mainly belongs to the family γ-decalactone, but also to a smaller extent the γ-dodecalactone and γ-octalactone. Generally, patents and papers in this area describe the bioconversion of hydroxy fatty acids into γ-decalactone. The type of lactone produced will be determined based on the presence of hydroxyl group at a particular position [7].

The odorous compounds have been produced from the species of *Sporobolomyces* which belongs to the family *Sporidiobolus* since 1930. This was confirmed in the year 1972 and 1973 by the identification of 4-decanolide and Cis-6-dodecanolide from the peach-like odoured compound produced by the cultures of the yeast *Sporobolomyces odorus*, which was further investigated in fermentor cultures [5,8]. The main problem in the production of fungal γ-decalactone by submerged fermentation is the morphology of the organism such as pellet and free filamentous form. The filamentous form is common in industrial fermentation in which the mycelial enlargement causes high broth viscosities and complex broth rheology, which in turn leads to poor mixing and mass transfer deficiencies. Characterization of morphology is therefore important in fermentations involving filamentous fungi either for physiological studies or for improving the design and operation of bioprocesses [9, 10]. The present review will be devoted to a survey of main achievements in the biosynthesis of γ-decalactone by various microorganisms. Furthermore, the application of γ-decalactone in various fields will also be extensively reviewed, on which much attention has been recently focused.

Metabolic pathway of γ-decalactone synthesis: The commonly accepted pathway ricinoleyl-CoA to γ-decalactone is presented in Fig. 1. The four consecutive β-oxidation steps yields 4-hydroxy - decanoly 1-CoA

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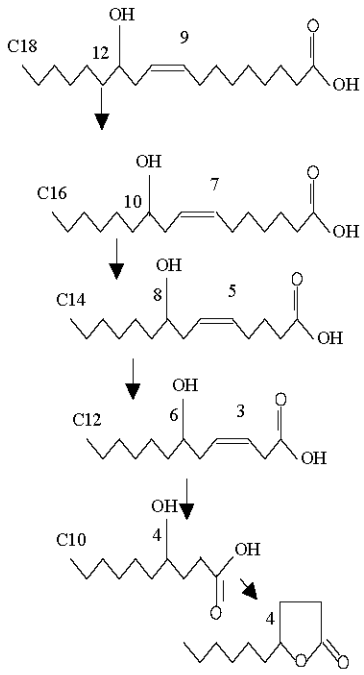

Fig. 1: Fatty acids observed as intermediates in the biotransformation of ricinoleic acid into γ-decalactone [12]

from ricinoleyl-CoA which is then cyclised to γ-decalactone. In some species of *Sporodiobolus* β-oxidation is preferentially localized in the micro body, whereas, in other species it might be localized in the mitochondria [11]. The different steps are now quite well known and recent results highlight the impact of environmental conditions on the biotransformation and on the regulation of β-oxidation fluxes. In this biotransformation, the interfacial area between the organic and aqueous phases is important not only to favour the access of yeast to substrate but also to extract the produced lactone. It is indeed important to subtract it from the degradation by yeast cell membrane contact, which can decrease the yield by perturbing the cell integrity [12, 13]. The three other proposed possible pathways for γ-decalactone synthesis uses methyl ricinoloate a C_{18} unsaturated fatty acid as a source and one of these possibilities was confirmed with the cultures of *Y. lipolytica* and *pichia guilliermondii.*

There are many other hypotheses suggested by different authors related to the metabolic pathway for the synthesis of γ-decalactone. One of the best-studied biotransformations is that of ricinoleic acid from castor oil into γ-decalactone catalysed by yeasts [14, 15]. This process is supposed to involve peroxisomal β-oxidation degradation of the acid substrate [16,17] leading to the formation of 4-hydroxydecanoic acid which then cyclises into γ-decalactone. Despite the economic importance of such a transformation, the detailed pathway leading to γ-decalactone from ricinoleic acid is not yet known. Of special interest in the way yeasts manage the presence of a Z double bond between the alcohol and the carboxylate functions of ricinoleic acid during its degradation. Of two recently proposed pathways [15, 18], one supposed that after three rounds of β-oxidation ricinoleyl-CoA is transformed into 6-hydroxy-3Z-dodecenoyl-CoA. Then the double bond is isomerised in position 2, allowing one more round of β-oxidation and generating 4-hydroxydecanoic acid, the direct precursor of γ-decalactone [15]. This pathway is explained in Fig. 2, which is more complicated as it involves only two rounds of β-oxidation from ricinoley1-CoA, followed by the creation of a new double bond that conjugates dienic E,Z system thus formed shifts from 3,5 to 2,4 positions in all trans stereochemistry. The dienic system is then reduced to a single E double bond located at position 3 which isomerizes to position 2 allowing two new rounds of β-oxidation, leading finally to 4-hydroxydecanoic acid [18].

The metabolic pathway related to the production of γ-decalactone using chemoenzymatic synthesis by the reduction of ethyl 2-hydroxyl-3-Oxoalkanoates by baker's yeast to obtain predominant antidihydroxyalkanoates with high enantiomeric excesses (>95%) have been reported. The diastereosselectivity of the reaction and the enantiomeric excess of the syn product were reported to vary with the substrate and also the ees of the anti and syn products.Only very few authors have consistently observed that the stereochemical outcome of the reduction of prochiral β-ketoesters and α-substituted-β-ketoesters by baker's yeast is strongly dependent on pH of the medium [19]. Thus, for consistent results the α-hydroxy-β-ketoester-3 was reduced by bakers yeast immobilized in calcium alginate at pH4.0 and the resulting dihydroxyester was converted to optically active anti-(4S, 5R)-5-hydroxyl-γ-decalactone [20] as shown in Fig. 3.

γ-decalactone synthesis using immobilized cells: Biosynthesis of γ-decalactone using S*poridiobolus salmonicolor* CRC 21975, immobilized in calcium alginate beads with variable pre culture time, initial pH and bead concentration which influences the production of γ-decalactone were reported. The stability of immobilized *S. salmonicolor* cells in the production of γ-decalacotne was monitored during several consecutive fermentation runs. During fermentation, *S. salmonicolor* cells showed

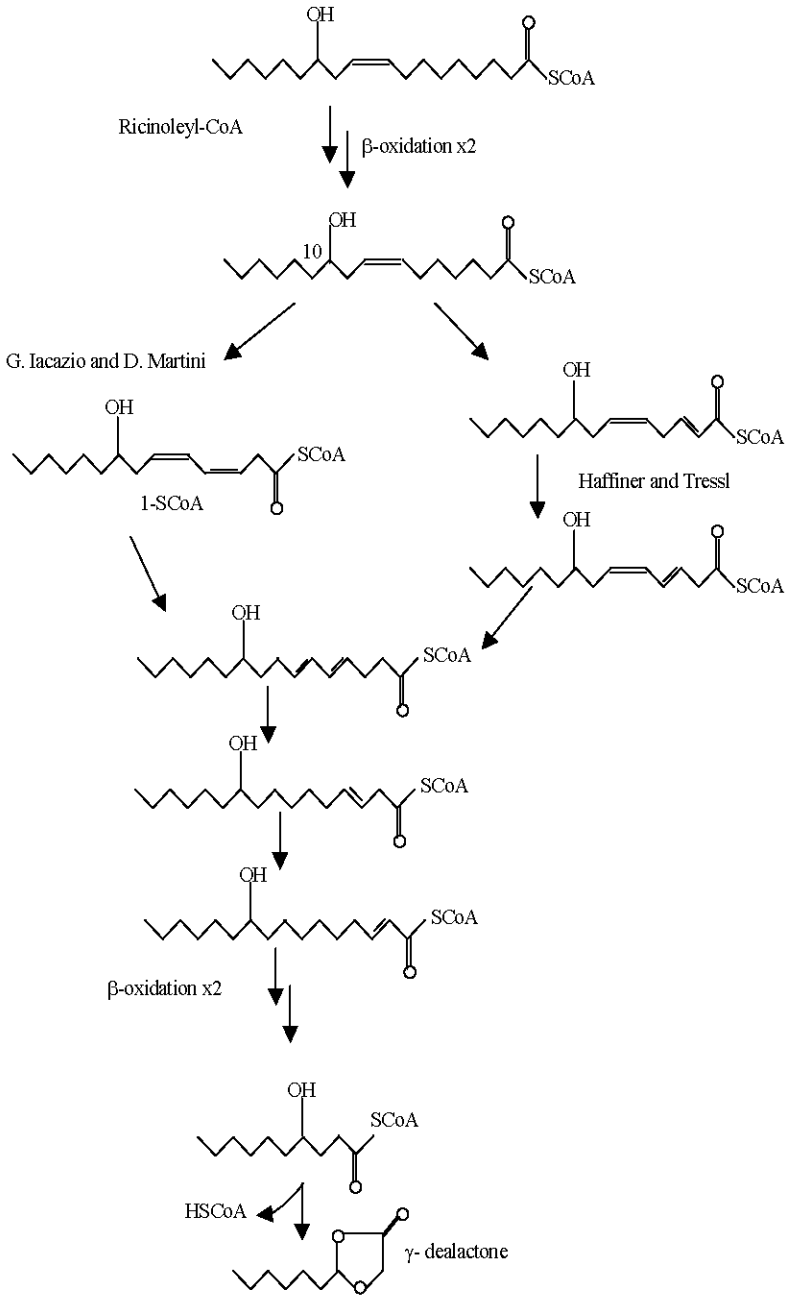

Fig. 2: γ-decalactone biosynthetic pathway from ricinoleic acid catalysed by yeast *P. guilliermondi* [32]

improved tolerance to perturbations in external pH leading to an increase in the production of γ-decalactone. Moreover, the relative insensitivity of immobilized *S.salmonicolor* to pH allows the possibility of using less precise pH control systems during the operation of bioreactors with an immobilized cell system which produced more γ-decalactone than the free cells. There is a better yield in the production of γ-decalactone in 13th cycle about 58.4% as that of the first cycle [21]. Researchers also described the production of γ-decalactone by immobilized *S.salmonicolor* in different polymeric materials. Among the various immobilization methods tested, it was reported that the bead quantity and bead concentration affects the yield of γ-decalactone. This may be attributed by the lower cellular metabolic activity caused by the nutrient deficiency with higher

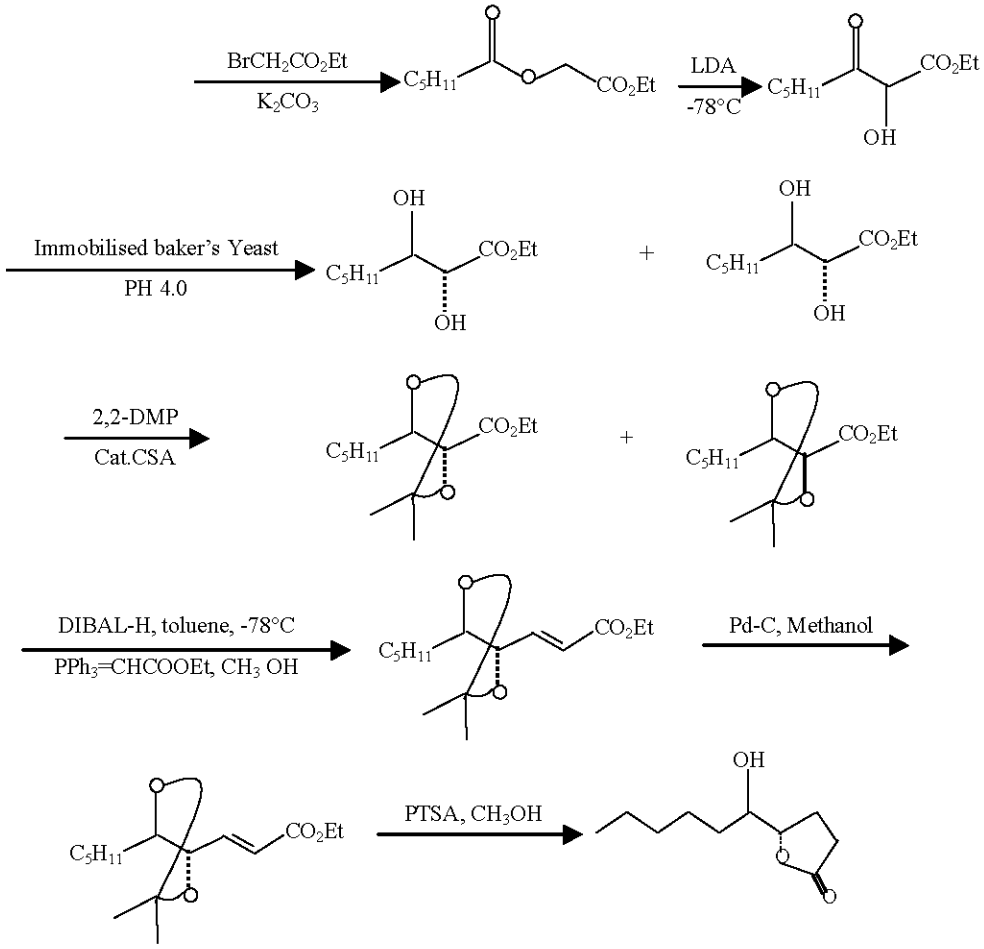

Fig. 3: Chemoenzymatic synthesis of (4S, 5R)-5-hydroxy-γ-decalactone [20]

bead concentration. Immobilization by using alginate is the most adequate procedure for the production of γ-decalactone by *S.Salmonicolor* and it also reveals that the immobilized cells produce a maximum yield of Ca.131.8mg/L after 5 days of fermentation [22].

Production of γ-decalactone

Microorganisms producing γ-decalactone: As each industrial application needs γ-decalactones with specific properties, there is an interest in research for γ-decalactones that could be used in new applications in the industries. Each industrial application requires γ-decalactone with unique properties like specificity, stability, temperature and pH dependence and ability to catalyse synthetic ester reactions in organic solvents. Therefore, isolation of new lactone secreting microorganisms and studies on their flavor production, purification and characterization could provide new lactones with better quality and wider range of applications. γ-decalactone have been synthesized by various microorganisms which include bacteria, fungi and yeasts such as *Trichoderma harzianus* [10], *Yarrowia lipolytica* [12], *Ceratocystis moniliformis* [23], *Pityrosporum species* [24], *Fusarium poae* [25], *Tyromyces sambuceus* [26, 27], *Phebia radiate* [28], *Sporidiobolus salmonicolor* [29], *Sporobolomyces odorus* [30], *Piptoporus soloniensis* [31] and *Pichia guilliermondii* [32] etc., These γ-decalactone producing microorganisms have been isolated from a wide variety of food stuffs and is observed to be associated with aromas described as fruity, coconut-like, buttery, sweet or nutlike etc.,

Production by using s.salmonicolor cbs 2636: Although the γ-decalactone is produced from *S.salmonicolor* CBS 2636 in early 1930's, it was not to investigate the systematic studies but the factors affecting the production of γ-decalactone and to determine conditions

for high yields till 1972-1973 [8]. Ricinoleic acid methyl ester and the size of inoculum were shown to affect the production of γ-decalactone by *S.Salmonicolor* CBS 2636 in static and shake flask cultures. The highest yields were produced when the organism was grown in shake flask using the following medium that contains; Glucose 15g/l; Peptone 0.5g/l; Yeast extract 1g/l; Malt extract 1g/l; KH_2PO_4 2g/l; $CaCl_2(2H_2O)$ 0.13g/l; $FeSO_4(7H_2O)$ 0.01g/l; $MgSO_4(7H_2O)$ 3g/l and 0.1% (v/v) of antifoaming agent. The medium was sterilized at 121°C for 20min and was inoculated with 2% of yeast suspension containing 6×10^8 cells/ ml that have been cultivated previously in the same medium. The inoculated flasks were then agitated or stirred at 250 rpm. In the fermentor experiments, the cultures were aerated (1volume of air per volume of medium per minute) with a volumetric oxygen transfer coefficient of 90 h^{-1}, the pH was maintained at 6.0 by adding 2.5 N NaOH or 2.5 N H_2SO_4. When the cells reach stationary phase, the desired volume of methyl ricinolate was added to the medium to initiate the bio conversion process. Further studies were carried out in order to measure the impact of lactone concentration on fatty acid uptake and different enzymatic systems involved in the activation and catabolism of the fatty acids.

A renewed investigation on the biosynthesis of γ-decalactone using *sporidiobolus salmonicolor* CCRC 2636 derived from the genus *sporobolomyces odorus* in the presence of various individual fatty acids present in castor oil hydrolysate were reported. The fatty acids have stringent effect in the yield and maximum of 135.4µg/l γ-decalactone was obtained after 216 hr [33].

Extensive research have been done on the γ-decalactone production using *Sporidiobolus* strain CBS 2636 with respect to the conditions for γ-decalactone interms of different aspects such as fatty acid accumulation, substrate consumption, the nature and toxicity of the molecule produced [6]. The maximum concentration of 5.5g/l of γ-decalactone was obtained on the 7th day of successive batch cultures of this species and by using $1m^3$ reactors, 9.3 kg of γ-decalactone could be produced per month [34].

Production by using yarrowia lipolytica atcc 20460: *Yarrowia lipolytica* ATCC 20460 isolated from oily media and used in many processes such as lipase production, decontamination of diesel-contaminated soils and olive-mill waste waters, production of proteins, alkanes and of aroma compounds. Much of the work on the production of γ-decalactone by *Yarrowia lipolytica* ATCC 20460 reported that approximately 4-5g /l of fermentation broth [35, 36] was produced along with by-products such as γ-octalactone and γ-dodecalactone in the medium containing methyl recinoleate. For the better understanding of an mechanistic pathway that led to γ-decalactone formation by the strain ATCC 20460, the role played by oxygen-mass transfer [37] and the major medium components was further investigated in detail [38-40]. In case of no additional ricinoleic acid in the medium, γ-decalactone was hardly produced. These results suggested that the ricinoleic acid added is merely an activator for enzymes in the pathway of γ-decalactone synthesis in *Yarrowia lipolytica* ATCC 20460. To enhance the efficiency of γ-decalactone production, a small amount of ricinoleic acid is needed. The schematic pathway for γ-decalactone synthesis from fatty acid or ricinoleic acid is explained in Fig.1. The enzymes from *yarrowia lipolytica* ATCC 20460 that mediates stepwise lactonization of ricinoleic acid or of other hydroxylated fatty acids [41] is shown in Fig.2.

Production by using trichoderma harzianum imi 206040: Trichoderma harzianum IMI 206040, a fungi is being capable of producing γ-decalactone when grown in a culture medium containing disodium hydrogen phosphate, ammonium salt and castor oil as sole carbon and nitrogen source respectively The maximum production of 260 mg/l was obtained after the 7th day of culture of *T.harzianum* in a medium composed of $(NH4)_2SO_4$ 9.5g/l; KH_2PO_4 7.8 g/l; Na_2HPO4 2.2 g/l; $MgSO_4.7H_2O$ 1.7 g/l; $CaCl_2.6H_2O$ 0.11 g/l; $FeCl_3.6H_2O$ 0.009 g/l; $ZnSO_4.7H_2O$ 0.00011 g/l and castor oil 166.7 g/l. The culture was kept at 29°C, stirred at a speed of $3.3S^{-1}$ and was sparged with air at a flow rate of 10 vvm. pH was maintained at 5.6 by the addition of 2N NaOH. The production of γ-decalactone by *T.harzianum* is mainly non-growth associated with challenging and interesting hydrodynamically related aspects because; the fermentation involves mixing of two immiscible phases (oil and water). The broth is highly viscous and pseudoplastic which leads to the formation of stagnant zones with in the fermentor [10]. The biosynthesis of the molecule was associated to a high metabolic activity during the non-growing phase and the rheology, biomass morphology, mixing, shear sensitivity, kinetics are involved and interrelated.

Production by using pichia guilliermondii: All of the yeast discussed so far has produced γ-decalactone under aerobic conditions in which oxygen has generally been

used as an electron acceptor for the effective production of energy for cell growth and biosynthesis of products. It is well established that oxygen availability has significant effect on cell growth, carbon source utilization, γ-decalactone yield and specific productivity in many γ-decalactone producing yeasts. In order to maintain sufficient oxygen supply for efficient γ-decalactone production, the separated or combined use of Oxygen-enriched air, modified impeller design or a complicated and highly controlled aeration system is needed. Therefore, it might be more economical to replace gaseous oxygen with some other ionic electron acceptor. *P.guillermondii* was initially isolated from a marine environment polluted by the hydrocarbons. In order to understand better, the biosynthesis of γ-decalactone in yeasts, the addition of ricinoleic acid during stationary phase could be of interest [32].

Large-scale production of γ-decalactone: For the commercial application of γ-decalactone in large amounts, it is necessary to enhance its production. Several researchers have attempted to search for suitable conditions on γ-decalactone production in industrial scale. France researchers have demonstrated a simple strategy for the production of γ decalactone by *Sporidiobolus salmonicolor* CBS 2636 in a 7 litre bioreactor containing 5 litre of the medium. The dissolved oxygen was initially controlled at a level of 1.5 vvm by supplying air and/ or pure oxygen and by controlling the agitation speed up to 300 rpm. When ricinoleic acid 0.06% was added to the medium before cultivation time or after fermentation to initiate the bioconversion. Under these conditions, maximum yield of 208 mg/l γ-decalactone after 7days of cultivation was reported [42].

Recently, the large-scale production of γ-decalactone by *T. harzianum* IMI 206040 using a 14 litre fermentor was reported. The fungi were cultivated in PDA medium containing the following composition in grams/litre: $(NH4)_2SO_4$, 9.5; KH_2PO_4, 7.8; Na_2HPO_4, 2.2; $MgSO_4.7H2O$, 1.7; $CaCl_2.6H_2O$, 0.11; $FeCl_3.6H_2O$, 0.009; Z $SO_4$7H Q, 0.00011 and castor oil, 166.7g. Tween40 was used as antifoaming agent to maintain a stable cultivation without foaming. The medium pH was adjusted to 5.6 by the addition of 2N NaOH. The temperature and agitation was maintained at 29°C and 200 rpm respectively. The aeration was maintained at 1.5 vvm until 72 hr and then at 2 vvm afterwards. Under these optimum conditions, the maximum γ-decalactone yield was found to be 260 mg/l after 7th day of cultivation [10].

Recovery and purification of γ-decalactone: In most of the species of *Sporidiobolus* (or) *Sporobolomyces* the γ-decalactone was secreted and released outside the cells. Due to this reason the purification of γ-decalactone is made simple. It generally involves three steps; the removal of cells by centrifugation or filtration. The supernatant (both aqueous and oil phases) was mixed and acidified to pH 2 by using HCl (37%). The internal standard (γ-undecalactone) was added and the mixture was extracted with equal volume of diethyl ether in a separating flask by gentle shaking for 5minitues. The ether phase was then quantified in a gas chromatography with a capillary column (30.0m x 320μm x 0.25μm) using N_2 as a carrier gas at a linear flow rate of 4.3 ml/min. The split injector (Split ratio: 7.1:1) temperature was set to 250°C and the FID detector's to 300°C. The oven temperature was programmed from 60 to 145°C at 5min^{-1} and then at 2°C min^{-1} to 180°C.

Applications of γ-decalactone: A safe and natural composition containing γ-decalactone is mainly used as a flavoring agent in peach, mango, strawberry and chocolate-flavored foods. It is considered as GRAS to be used as a food additive by the US Food and Drug Administration [5]. Generally, the fragrance material will be present at the level of 0.01% to about 14% of the total dry weight. γ-decalactone is used as a perfumery ingredient in detergents and soaps, hair preparations such as lacquers and shampoos, cosmetic preparations such as creams, deodorants, perfumes, colognes, hand and sunscreen lotions, powders such as talcs, dusting powders, face powders, house care products, body care products (such as toothpaste, tooth gel, dental creams, dental care gum and mouth wash), textiles and in odor absorbing substances, tobacco wares etc. [43].

There is an increase in the demand for fragrance application with pharmaceutical products such as dissolvable tablets, chewing tablets, throat or cough lozenges, pharmaceutical powders or granulates [44]. For the chewable tablets, pleasant, long-lasting, consistently strong rich citrus flavors are added which remain even after the period of 12 minutes of ingestion.

In today's market, it is frequently desirable to identify flavor components to be added with food items as being "natural flavors". It is generally recognized in the industry that a flavor compound have been prepared by microbial processes can be designated as a natural product and therefore have an important place in the commercialization of products containing them. As a result, the industry has

devoted considerable time and effort to develop methods for the production of flavoring components and in particular for the production of lactones which can be called as "natural flavors". It is used as a part of the main constituents of food substances, or used as ingredients. Proteins have the capacity to bind flavor molecules. It has been shown that adding γ-decalactone to one or more substances having a bitter odor, it greatly relieved bitterness. The addition of γ-decalactone during the manufacture of foods mainly bakery products such as cakes, waffles or wafers, snacks, instant meals as well as other instant products such as soups, sauces, powdered and granulated drinks, tea bags, spice mixtures results in the prevention of aging and the improvement of textures and further it also contributes the shape retention of food. The use of γ-decalactone as thickener with fruit juices, beverages such as wine and brandy and soft drinks improve odor, taste and drinkability. Animal feed containing γ-decalactone as an additive has also been reported [45].

CONCLUSION

γ-decalactone has been known for more than 70 years and numerous researches have been carried out on this aroma compound. The mechanism and even the principle substrates involved as precursor in flavour formation are still not fully understood and often serious conflicts exist with the literature. Even so for, some bio synthetic routes have been proposed for several strains. The fact that there are different mechanistic systems for γ-decalactone production in different microbes, indicating that γ-decalactone production is more diverse with different microbes. The physiological information about the production of γ-decalactone gives us an information about its independent synthesis from ricinoleic methyl ester and also the type (γ or δ) of decalactone to be produced in a process based on the structurally unusual position of the hydroxyl group. Attractive properties of γ-decalactone is that it is a organic solvent soluble, colorless liquid, fruity, peach odor and edible compound. In addition to the above said features, the application of γ-decalactone is versatile, safe and environmentally friendly. So there is a day-to-day increase in the interest of its applications in the field of pharmaceutical, food, cosmetics, body care products, house care products, laundry, textiles, air fresheners and many others. Furthermore, the production of lactone will be established on the industrial scale because it is an extracellular product and can be produced easily with higher yields by culturing yeast in a fermentor. Moreover, it can be produced from sustainable resources. Therefore, the development of this ecomaterial is both economically and environmentally valuable.

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