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REVIEW ARTICLE

IN VITRO PRODUCTION OF CAPSAICIN THROUGH PLANT TISSUE CULTURE

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ABSTRACT

Capsaicin, a secondary metabolite produced in capsicum, is in high demand in pharmaceutical industry because of its various medicinal properties. Currently, the supply of capsaicin depends upon its extraction from capsicum fruits. This limits the production of capsaicin as it depends upon agricultural produce. The current review has compiled information from various literature published on chemistry and importance of capsaicin along with its method of production. It also reviews the process of *in vitro* production of capsaicin through plant tissue culture, strategies of increasing capsaicin accumulation and its advantages over extraction from fruits and artificial synthesis.

Keywords: Capsaicin, Plant tissue culture, Suspension culture, Elicitation and cell immobilization

INTRODUCTION

Capsicum is a dietary species that is very common in Indian household. Of all the spices, capsicum fruit is the most widely used throughout the world. Chilli is a spice that is one of the basic ingredients of the different recipes all over the world. It is used to provide flavour, aroma and colour to the food. It is either used as whole or in grounded form, alone or in combination with other spices. Whatever the process may be, it imparts a long lasting taste. Capsicum is a vascular (tracheobionta) flowering (magnoliophyta) plant belonging to the family Solanaceae. It is highly pungent and has a hot burning sensation associated with it. Capsicum varieties are classified on the basis of their hotness which is measured on a scale called Scoville Heat Unit (SHU). Higher the SHU value of a pepper, more will be its hotness. A panel of experts taste the chilli samples, which is diluted repeatedly until it no longer tastes pungent. This dilution, at which capsicum loses its pungency, is referred to as SHU [1]. This method was evolved in 1912 and despite many scientific methods for measurement of pungency, it is still in use. The hotness of different types of capsicum are tabulated below (table 1).

Table 1: Different chilli peppers of the world on scoville scale*

Chilli pepper	Scoville heat unit
Carolina Reaper	1,600,000–2,200,000
Trinidad Morgua Scorpion	1,207,764 (Bosland, 2012)
Bhut jolokia	1,019,687 (Bosland, 2012)
Habanero	200,000–300,000
Bird's Eye	100,000–125,000
Carolina Cayenne	100,000–105,000
Habanero Orange	150,000–325,000
Thai	70,000-80,000
Cayenne	35,000–40,000
Chile de Arbole	15,000–30,000
Jalapeno	30,000–50,000
Super Chille	30,000–40,000
Hottie	30,000
Jalapeno	7,000–25,000
Yellow wax	15,000–17,000
Bell Pepper	<200

Courtesy: National Hot Pepper Association

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Origin of Capsicum has been presumed to be in the area between the mountains of South Brazil, Bolivia, Paraguay and Northern Argentina. All the major domesticated species of Capsicum is cultivated in this region [2]. It was Christopher Colombus who introduced chilli to Spain [3]. However, according to Pickersgill (1984), when the Mongolians migrated to Europe in the last Ice–Age, they found some highly pungent plants which are now thought to be Chilli [4]. However, the exact time of the introduction of chilli in India is still not certain.

Capsicum also possesses a high nutritive value. It contains vitamin A, C, E and B–complex along with some essential minerals like molybdenum, potassium and manganese. In addition, they are excellent antioxidants–a property which is attributed to beta-carotenoids and vitamin A and C. The various colours of capsicum are due to a combination of compounds, namely, capsanthin, capsorubin, zeaxanthin, cryptoxanthin and other carotenoids.

Chemistry of capsaicin

The hotness of the chilli is attributed to a compound named capsaicin ($C_{18}H_{27}O_3N$) that belongs to a group of alkaloids known as capsaicinoids. There are different types of capsaicinoids which differ with respect of aliphatic side chain, branching point and their relative pungency. Most of the capsaicinoids are pungent, however, some varieties like ω-hydrocapsaicin are non–pungent [5]. A group of compounds called capsinoids have been recently discovered. This aliphatic side chain of this group is same as that of the capsaicinoids but differ with respect to aromatic group [6].

Capsaicin is a secondary metabolite synthesized in the chilli fruits as a defence mechanism to save it from the predators. A wide variety of organic compounds are produced in plants, most of which are not directly involved in plant growth. These substances, also known as secondary metabolites, are synthesized in a few plant species [7]. They don't have any function in plant's primary metabolism but may serve ecological function. They may serve as pollinator attractant, provide adaptation to the environmental stresses, provide defence against microorganisms, insects or predators. In comparison to primary metabolites, they are produced and stored in plants in a very small quantity. Also, they are synthesized in specialized cell types and at distinct developmental stages [8; 9]. About 89% of the total capsaicin is located in the placenta whereas pericarp contains only 5-6% capsaicin [10].

Capsaicin [N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-transenamide, ($C_{18}H_{27}NO_3$)] is crystalline, lipophylic, colourless and odourless in nature with a molecular weight of 305.199 g/mol. Capsaicin possess a melting point of 64.5 °C and a boiling point of 210–220 °C at 0.01 mm of Hg. It is highly soluble in fat, oil and alcohol; slightly soluble in carbon disulfide and hot water, and completely insoluble in water at room temperature. There are many homologs of capsaicin of which dihydrocapsaicin and nordihydrocapsaicin are the most prominent ones. Dihydrocapsaicin [N-(4-hydroxy-3-methoxybenzyl)-8-methylnonamide, ($C_{18}H_{29}NO_3$)] is crystalline, white and odourless in nature with molecular weight of 307.215 g/mol and melting point of 65.6–65.8 °C. It exhibits same solubility as capsaicin. Nordihydrocasaicin [N-(4-hydroxy-3-methoxybenzoyl)-7-octanamide, ($C_{17}H_{27}NO_3$)] is a white crystal with molecular weight of 293.199 g/mol and melting point of 65.6 °C [11].

Biosynthesis of capsaicin

Capsaicin is synthesized by the condensation of a short branched fatty acyl chain, synthesized from branched amino acid, with vanillylamine produced through phenylpropenoid pathway.

A large number of genes participate in capsaicin biosynthesis, however, little knowledge is available about their location. *Pun1* gene is associated with the spiciness of capsicum. It encodes for AT3 which is specifically found in the placenta of pungent varieties of chilli. Product of *Pun1* gene is associated with the development of vesicles for the accumulation of capsaicin. *Csy1* is another gene playing a vital role in capsaicin synthesis. It encodes for the Capsaicin synthase enzyme catalyzing the condensation of vaniyllamine and fatty acid [10].

Mechanism of action

Being a member of vanilloid family, capsaicin binds the vanniloid receptor subtype 1 (TRPV1) which is an ion–channel receptor [13; 14], expressed in sensory neurons. When it is bound by capsaicin, there is an increase in the level of intracellular calcium, which in turn, leads to the release of neuropeptides. This sends the signal of excessive heat to the brain. In effect, there is no actual tissue damage but a neuro signal is send as if it has suffered a burn and thus an extreme exposure can cause the body to trigger an inflammatory response causing tissue damage.

Capsaicin: Clinical uses

Analgesic: Capsaicin helps in reducing tissue soreness and pain from rheumatoid arthritis, neuralgia and diabetic neuropathy [15]. Tropical application of capsaicin initially causes an uncomfortable burning sensation but ultimately cuts out the pain by increasing intracellular Ca^{2+}that desensitize nociceptor fibres degenerating pain signalling pathway [16; 17].

Cancer control

Capsaicin has been noted for its anticancer properties. When capsaicin was used against cultured cancer cells, it was found to prevent the relocation of breast cancer cells; it also successfully killed the prostate cancer cell. When dihydrocapsaicin was added, it induced apoptosis in human colon cancer cells [18; 19; 20]. Various mechanisms have been put forward to demonstrate the cancer preventing mechanism of capsaicin. Capsaicin induces apoptosis by the obstruction of mitochondrial respiration [21]. It also suppresses plasma membrane NADH-oxidoreductase (PMOR). Capsaicin also arrests the endothelial cells in G1 phase of cell cycle by downregulating cyclin–D and VEGF–induced angiogenic signalling pathway [22]. STAT3 (Member of signal transducer and activator family), which is linked to the cell cycle regulation, chemoresistance and angiogenesis [23; 24; 25], is associated with tumor formation [26]. Capsaicin prevents the activation of STAT3 and also suppresses its DNA binding [27].

Anti-obesity

It has been reported that the consumption of red pepper increases calorie burn and lipid oxidation [28] and at the

same time reduces food cravings [29]. In one study, the addition of 30 mg to high fat and carbohydrate breakfast resulted in decrease of food consumption. Also after the intake of a fat rich breakfast containing capsaicin, a decrease in appetite was reported [28]. A small human trial (36 people of which 22 were women, average BMI 26.3 kg/m2), showed that the consumption of food containing chili, insulin resistance decreased by about 60%. Also, C-peptide: insulin ratio, a measure of insulin metabolism, also improved on chilli consumption. The result showed that regular consumption of chilli is beneficial in improving the condition of meal-induced hyperinsulinemia [30]. Capsaicinoid treatment on humans with 6 mg/day showed that when consumed orally, it was safe and resulted in the loss of abdominal fats. It also significantly increased fat oxidation [31]. Oral intake of capsaicin also decreases glucose intolerance in obese subjects by restraining the inflammatory responses and augmenting the fatty acid oxidation in adipose tissue and liver, tissues involved in insulin resistance. Several mechanisms have been proposed for the anti-obesity effect of capsaicin. Capsaicin binds the peroxisome proliferator activated receptor (PPAR) alpha and also affects TRPV-1 expression/activation that in turn increases fats metabolism in adipose tissues and stimulates liver activity [32]. It increases the activity of sympathetic nervous system [28], blood flow and secretions of the gastrointestinal tract [17], and increased secretions of norepinehrine by stimulated sensory neurons [29] resulting into stimulating energy and lipid metabolism. When mice were given a high-fat diet containing capsaicin, lower body weight and higher TRPV1 expression was observed as compared to those given capsaicin lacking high-fat diet [33]. This study proves that the TRPV1 activation and increased level of calcium in cytosol inhibits adipogenesis.

Antioxidant activity

Due to the presence of pungent capsaicinoids, peppers are a rich in dietary antioxidants [34]. Capsaicin curbs lipid peroxidation induced by iron-(Fe^{2+}) and quinolinic acid reducing the production of superoxide anion in rat brain produced by injecting 1 mmol cyanide [35; 36]. The phenolic portion of capsaicin is responsible for its antioxidant property [37]. However, in another study, it was found that instead of the phenolic-OH group, C7-benzyl carbon exhibits the antioxidant and free radical eliminating properties [38; 39].

Antimicrobial activity

The antimicrobial activity of capsicum has been known since ancient times. They were used to treat infections in the Mayan civilization [40]. The hot and cold extracts of Capsicum were effective against *Bacillus cereus*, *Bacillus subtilis, Clostridium sporogenes, Clostridium tetani,* and *Streptococcus pyogenes* to varying degrees [39; 41]. The ethanolic extracts of the Capsicum fruit were also found to be potent against Gram (+) and Gram (−) bacteria, and fungi. Capsaicin was found to be the main component that disrupts the microbial membrane [42; 31, 43]. High concentrations of capsaicin impeded the growth of *Escherichia coli* and *Pseudomonas solanacearum*, and the growth of *Bacillus subtilis* was repressed [44-46]. Bactericidal effect of capsaicin also has antibacterial activity against *Helicobacter pylori*. Pure capsaicin completely inhibits the conidial germination of fungus [47].

Cardiovascular effects

Capsaicinoids are beneficial in the ailments of the cardiovascular system in humans like coronary heart disease, myocardial infarction, hypertension and atherosclerosis. [48; 49]. The cardiovascular systems are associated with sensory nerves having sensitivity to capsaicin that activate TRPV and Substance P that resulting in the release of various neurotransmitters like CGPR thus regulating cardiovascular functions [50; 49; 51]. It inhibits platelet deposition and the activity of Factor VIII and Factor IX, a property that makes it effective in preventing the induction of cardiovascular diseases. Plasma membrane of platelets is permeable to capsaicin thereby altering the membrane fluidity [52; 53]. Capsaicin causes the release of Ca2+from intracellular platelet stores which promote the release of ADP and thrombin thereby promoting the activation of platelets. In *in vitro* studies it was found that capsaicin slows down the rate of oxidation of low density lipoprotein (major cause of atherosclerosis) thus increasing body's resistance against it. Oxidation of serum lipoproteins in adult humans has found to decrease after regular consumption of chili [30]. This shows that capsaicinoids are effective in the prevention of various heart cardiovascular diseases.

Gastrointestinal effects

Gastrointestinal system is associated with sensory nerves that have sensitivity to capsaicin. They help in maintaining gastrointestinal mucosa integrity against diseases. Depending upon the dose and/or drug treatment, capsaicinoids effects on gastrointestinal tracts may either be beneficial or harmful. In high concentration, capsaicinoids damage these nerves thereby damaging the gastrointestinal systems [54; 49]. However, in lower concentration, capsaicinoids increase the blood flow in the mucosal lining thereby increasing the secretion of mucus in the gastrointestinal tract and promoting defence activity against microbes [54].

Objectives of this review

In this review, a detail literature survey of chemistry and application of capsaicin has been done. Moreover, the present methods of capsaicin production and their limitation alongwith its *in vitro* production through plant tissue culture and how it is advantageous over the traditional methods has been discussed. This review will aid the researchers and industrialists in better understanding the benefits of *in vitro* method and its application on industrial level.

Artificial methods of capsaicin production

Many efforts have been applied to synthesize its chemical analogues with properties similar to natural capsaicin. Enzymatic synthesis has an advantage over chemical synthesis that there is no presence of non-toxic reagents and is substrate specific. Synthesizing capsaicin and its analogues by lipase catalyzed amidation reaction has been extensively studied [55]. Condensation product of vanillylamine and the derivatives of fatty acid is used as a substrate in the oleose phase, 40-59% capsaicin was produced [56]. Different non-pungent analogues of capsaicin can be synthesized using aromatic ring containing different functional groups and acyl chain of varying lengths [38] The by-products were two highly pungent and some low pungent analogues having promising utilities. Commercial capsaicinoid production is achieved by heating chlorinated fatty acids and amines at temperatures between 140°C and 170 °C in the presence of low pressure [57-59]. Choi and Yoon, conveyed the use of bioisosterism to synthesize a capsaicin analogue, namely, 1-hydroxy-2-pyridone showing activity similar to it [39]. A study was

conducted for the production of vanillyl nonanoate using vanillyl alcohol and nonanoic acid as reactants and tetrahydrofurane as reaction medium in the presence of di-isopropyl azodicarboxylate and triphenyl phosphine in equal proportions with respect to molarity [39]. This reaction resulted in the synthesis of 67% vanillyl nonanoate after being incubated at room temperature for 24 h. In a different study cerium chlorate (III) catalyzed the selective esterification of phenolic alcohols which resulted in the synthesis of 70% vanillyl nonanoate [60]. As a result of the toxic level of the substrates and catalysts involved, enzymatic synthesis is preferred over chemical synthesis. Capsinoids (capsiate, dihydrocapsiate and nordihydrocapsiate) are closely similar to capsaicinoids with respect to its structure, except their central linkage: capsaicinoids have amide moiety whereas capsinoids have ester moiety [62]. Capsinoids are as potent as capsaicinoids although they are not as pungent as the latter and do not cause any sensory irritation. However, action mechanism of the former is less known. They naturally occur in a few species. They are also synthesized chemically which is an intricate process since they have an end chain methyl group and contain a double bond [62].

Chemical extraction of capsaicin

Various chemical methods are available for the extraction of capsaicinoids from capsicum. Different organic polar and non–polar chemicals are used as solvents for extraction of capsaicin [63]. Chemical extraction is based on the principle that the sample matrix must produce at least two fractions with varying properties, under the effect of a third component (i.e., extraction agent or solvent). The separation procedure is chosen based on whether the sample matrix is heterogeneous or homogeneous. For heterogeneous matrix, mechanical processes such as filtration, centrifuging or pressing can be used for separation. In case of homogeneous matrices, different physiochemical properties of every component should be considered. The extraction of natural products is based on the principle of percolation where solvent diffuses into the pores of the sample matrix and extracts the solutes. For the selection of the extraction process, properties of both the solute to be extracted and the solvent have to be considered. Factors such as density, viscosity and surface tension of the solvent have to be considered carefully.

The extraction method that is used largely affects the capsaicin content in the extract. Soxhlet method yields up to 9 times capsaicin when compared to vacuum filtration. Recirculation of the solvent through the material results in the extraction of various other components in addition to capsaicin. Amrutraj *et al.* [64] used different non-polar, Polar Aprotic and Polar Protic solvents for the extraction. HPLC analysis showed the pungency level of 5,948,120 SHU and 11,161,030 SHU in the extracts of acetone and acetonitrile respectively. Pungency level was less than 1,000,000 SHU, in the extracts of non-polar solvent (hexane, benzene, chloroform) and polar protic solvents (methanol and water). Both UV-Vis spectrophotometer and HPLC analysis showed that acetone and acetonitrile yield highest amount of capsaicinoids when compared to the other solvents [64].

Need of biotechnological approaches

With the application of biotechnological approaches, one can exploit cell, tissue, organ or entire organism by growing them *in vitro*. It also enables us to get desired compound from plants through genetic manipulation. The rapid increase of human population in the world, availability of cultivatable land has decreased. Hence, the basic aim of agriculture must be to feed the increasing population. Using available agriculture produce for other purposes like extracting chemicals from them will exert unnecessary pressure on agriculture. Hence, it is judicious to try to develop modern technologies for obtaining chemicals from plants without depending on agricultural produce.

The use of plant cell and organ culture methods has resulted in the high yield of plant products independent of plant itself. Researchers are taking great pain in developing highly efficient bioreactors for the higher product yield using plant tissue culture. Developments in molecular biological research have given a new direction to *in vitro* culture of plant cells which led to high production of plant products. Development of genetically engineered plants has enabled us to produce large amount of products alongwith some novel products. Furthermore, the demand of drugs based on natural products with minimal side effects has promoted the search of various medicinal plants and the use of plant products with health benefits. These factors have ensured the need to use novel biotechnological approaches for producing plant based chemicals naturally. Use of plant tissue culture systems is a better alternative for producing high-value secondary metabolites [65-68, 70]. Plant cells exhibit the property of totipotency, i.e. plant cells in culture possess complete genetic information and cellular machinery to produce any chemical produced in the whole plant.

There are many ways in which use of plant cell cultures are advantageous over conventional agricultural methods for secondary metabolites production:-

- Production is independent of any geographical and climatic factor.
- It ensures round the year production of the desired product in uniform quantity.
- It allows the production of novel compound not produced in parent plant.
- It ensures the production of compound in higher quantity as compared to the parent plant.
- Cells in culture can be genetically modified to produce higher yield.
- It lessens the pressure on cultivatable land and agricultural produce.

***In vitro* production of capsaicin**

In vitro synthesis is a very effective alternative for the production of capsaicin and its analogues. *In vitro* production involves following steps:-

- Callus induction from chilli fruit.
- Establishment of cell suspension cultures from callus.
- Selection of cell lines for high yield of capsaicin.
- Plant cells immobilization for capsaicin production.
- Elicitation of culture system for augmenting the yield.
- Capsaicinoid extraction from cell culture system.

Callus induction from chilli fruit and establishment of suspension cultures

Callus induction is the first step for *in vitro* capsaicin production. When the explants are inoculated in plant tissue culture media enriched with high concentration of auxin and low concentration of cytokinin, callus formation takes place [71]. Since 90% of capsaicin in present in the

placental region of chilli fruit, it is preferred to use it as explant.

Selection of high yielding cell lines

For getting maximum yield, it is important to select those cell lines that give high yield of capsaicin. In order to get high yielding cell lines, it is necessary to screen callus induced from different parent plant. Heterogeneous population of different cell clones are tested to select the ones producing highest amount of desired product.

The differences in the biochemical activity within a given cell population has been studied to select cell lines with maximum productivity [72]. If the product of interest is a pigment, selection can be easily done. For instance, extensive screening of cell clones in cultures of *L. Erythrorhizon,* enabled 13–20 times increment increase in shikonin production [73]. Other biochemical techniques such as HPLC and RIA can also be utilized to select promising cell lines [74; 50].

Mutation techniques have also been used to select cell lines giving high yield. Selective agents can also be used for this selection process [1]. When a cytotoxic inhibitor or environmental stress is employed to a large population of cells, only the resistant cells will grow. Different selective agents used to select high–yielding cell lines include 5-methyltryptophan, glyphosate, biotin etc. [75; 76; 77].

Strategies to increase yield of capsaicinoids

The chemical composition of the plant tissue culture media is a major determinant of secondary metabolite production [78; 79]. By altering certain aspects of the culture environment, one can increase the product yield.

In cultures, plant cells follow heterotrophic mode of nutrition. They include simple sugars as carbon source and other inorganic nutrients for various physiological activities. It has been shown that the level of sucrose affects the accumulation of secondary metabolites in plant cell cultures. The osmotic stress created by sucrose is a probable factor that is responsible for increase of the secondary metabolite. 8% (w/v) sucrose concentration was found to be optimal in the tested concentration range of 4–12% (w/v) for the accumulation of alkaloids [80]. However, the effect of sucrose on secondary metabolite accumulation also depends upon the plant species and also on the class of alkaloid present.

All of the plant tissue culture media contain both nitrate and ammonium nitrogen source. However, the amount of secondary products accumulated is also determined by the ratio of ammonium to nitrate and overall levels of total nitrogen. It has been found that the reduced levels of total nitrogen increase the yield of capsaicin in *Capsicum frutescens*, anthraquinones in *Morinda citrifolia* and anthocyanins in *Vitis* species [44; 81; 82].

Growth regulator plays an important role in the accumulation of secondary metabolites in cell suspension cultures [83; 84]. The kind and concentration of auxin and cytokinin and the auxin/cytokinin ratio has remarkable effect secondary metabolite accumulation in cultured plant cells [85]. In some instances 2,4-D (auxin) inhibits the production of secondary metabolite. If this happens, media lacking 2,4-D or replacing it with naphthalene acetic acid (NAA) or indole acetic acid (IAA) increases the production [44; 86; 87; 88; 89; 95]. Cytokinins have different effects on secondary metabolite production that depends largely on the metabolite and species concerned [45; 87; 58].

Precursor feeding is a popular method to increase secondary metabolite production in cultured cells. Adding phenylalanine (Precursor for vanillylamine) a constituent of capsaicin isocapric to the cell cultures of *Capsicum frutescens* enhanced the overall accumulation of capsaicin in the culture [81].

Application of immobilized cell system

SPIC (Surface Immobilized plant cell) is the most recent technique in the production of secondary metabolites. Immobilized plant cell is of high importance in plant research and development of plant cell culture. It provides certain prospective benefits such as [91; 92]:

a) The immobilized cells are viable over a longer period both in the stationary and the reproducing stage, enabling to uphold cells for a long time period.

b) Provides the prospective of easily attainable downstream processing (in case of secreted products).

c) Differentiation gets promoted (supposedly), which is coupled with boosting secondary metabolism production.

d) There is higher cell density allowing a smaller bioreactor, hence cutting down production costs and the risk of contamination

e) Less shear sensitivity (especially with entrapped cells)

f) Promotion of secondary metabolite accumulation, in some cases

g) Flow-through reactors can be used enabling greater flow rates

h) Decrease in fluid viscosity, which in cell suspension causes mixing and aeration problems.

An immobilization system makes it possible to maintain viable cells for a longer time and gives higher yield of product in stable form. It dramatically reduces production cost in plant cell culture [63]. However, an immobilized system can also be a bit problematic, as:

a) Immobilization is normally limited to cases where production is decoupled from cell growth

b) The initial biomass must be maintained in suspension

c) Secretion of product into the extracellular medium is crucial

d) Secretion in the extracellular media may cause extracellular degradation of the products

e) In the case of gel entrapment, the gel matrix introduces an additional diffusion barrier.

Immobilization methods widely range from gel to membrane entrapment. Various gels like alginate and carrageen are used for plant cell entrapment. Sometimes gels like agar, agarose and acrylamide may also be used. However one needs to optimize the gel based system best suited for capsaicin production.

Gel entrapment

Brodelius *et al.* (1979) reported the plant cell immobilization using calcium alginate. Since then, different gels, either alone or in combination, are being

used for this purpose [91]. Agar, alginate, agarose and carrageenin are the most widely used gels.

Entrapment in nets or foam

In 1983, Lindsey *et al.* reported that plant cells growing in liquid nutrient medium can be entrapped within polyurethane foam in blocks of 1 cm^3. Gel blocks are submerged in the flasks containing cell suspension culture under continuous agitation. Initially, the cells get washed in and out of the blocks. However, as time passes, the cells get trapped within the inner matrix of the blocks. The cells get combined within the foam compartment and form larger aggregates. After the blocks get completely loaded, the cubes are transferred to a low nutrient media that favours metabolic activity but not cell division. Normally, the lower concentration of nitrate or phosphate in media imparts the characteristic of low growth. When immobilized cells are placed in the low growth medium, they do not divide but remain confined to the foam [93].

Polyurethane matrices do not affect the viability of the cell. They can accommodate any degree of cell aggregate. They do not give any inward or outward barrier to the diffusion of the metabolites. Immobilized cells of *Capsicum frutescens* were found to produce more capsaicin as compared to the cells in suspension under similar conditions. [93].

Entrapment in hollow–fibre membranes

For plant cells immobilization, hollow–fibre membranes are also employed. Tubular fibres made of cellulose acetate silicone polycarbonate are used for cell entrapment [94]. These fibres are packed within the reaction vessel. The cells get confined within the spaces between the fibre membranes. These spaces allow the passage of different precursors and nutrients.

Elicitation of culture to increase total yield of capsaicin

Any material which when is introduced in a cell in culture, increases the production of a particular compound, is called elicitor and the process is known as elicitation.

Elicitors can either be abiotic or biotic depending upon their nature or they can be exogenous or endogenous depending upon their origin. Substances derived from non-biological sources like $Cu^{2+}$and Cd^{2+}ions and physical factors like high pH are abiotic elicitors. 'Biotic elicitors' are those substances which are derived from biological origin. They may be polysaccharides like pectin or cellulose derived from plant cell walls and chitin or glucans derived from micro-organism. They also include glycoproteins or G-protein or intracellular proteins which are coupled to receptors and act by activating or inactivating the signal pathways [95]. Both exogenous and endogenous elicitors are biotic in nature. Substances that are synthesized in extracellular environment, like polysaccharides, polyamines and fatty acids etc. are exogenous elicitors, while the vice versa cell are endogenous elicitors (galacturonide or hepta–glucosides, etc.). Different biotic/abiotic elicitors and endogenous/exogenous elicitors are presented in table 2 and table 3 respectively.

Mechanism of elicitation

When plant is treated with elicitors or attacked by incomatible pathogens, a number of defence mechanisms are triggered. These mechanisms also include the accumulation of various plant secondary metabolites that are defensive in nature. This mechanism occurs both in *in vivo* and cells in cultures. However, the exact mechanism of elicitation is not fully understood. According to some hypotheses, the elicitors act by binding the plasma membrane receptor [42; 96; 97; 6; 5; 98). Gelli *et al.* (1997) reported the Ca^{2+}entry into cytoplasm from outside the cell as a mechanism for elicitation. Rapid changes have been observed in protein phosphorylation patterns and protein kinase activation after the addition of elicitors [99; 100; 92]. Mitogen-activated protein kinase (MAPK) stimulation and G-protein activation has also been observed by some researchers [101; 102; 103; 34; 100; 104]. Armero and Tena (2001) reported the cytoplasm acidification caused by H-ATPase inactivation as the probable cause for enhanced production of secondary metabolite [105]. Pugin *et al.* (1997) and Bolwell *et al.* (1997) found that there was reduction in membrane polarization and enhancement of extracellular pH in cultured cells in response to elicitor treatment[106; 107]. ROS such as superoxides and H_2O_2 were found to be involved in the cross-linking of cell-wall-bound proline-rich proteins [108]. H_2O_2 acts as a secondary messenger and it is involved in the transcriptional activation of defence genes [109]. When defensive proteins get accumulated in the cell, signalling pectic oligomers (endogenous elicitors), hydroxyproline-rich glycoproteins, and protease inhibitors are released [23]. It has also been observed that cell death at infection site results in hypersensitive response 110; 103; 102; 34). Some elicitors could also activate certain defence mechanisms [73; 111; 112; 113]. The actual elicitation mechanism is still unknown. There is interconnection and inter correlation between secondary reactions triggered by elicitors and various defence mechanisms that resulting in secondary metabolite production. Different elicitors follow different modes of action and vary with respect to their origin, specificity, concentration, physiochemical environment, etc.

Table 2: Different biotic and abiotic elicitors

Biotic elicitors	Abiotic elicitors
1. Synthesized by microbes and identified by plant cells (enzymes)	1. Physical or chemical in nature.
2. Synthesized by microbial activity on plant cell wall (fragments of pectin).	2. UV light.
3. Formed in the response to the activity of plant enzymes on microbial cell wall (chitosans, glucans).	3. Denatured proteins (RNase).
4. Compounds synthesized by plants in response to external agent.	4. Repetitive freezing–thawing sequence.
	5. Unwanted media constituents.
	6. High DNA affinity chemicals.
	7. Detergents
	8. Fungicides.
	9. Herbicides.

Table 3: Elicitors on the basis of origin

Exogenous elicitors	Endogenous elicitors
1. Extracellular origin	1. Formed in the cell through secondary reaction induced by biotic or abiotic signals.
2. Polysaccharides: Chitosan, Glucans, glucomannose	2. Hepta-β-glucosides.
3. Polycations: Polyamines, Glycoproteins.	3. Alginate oligomers.
4. Enzymes: Cellulase, polygalacturonase, etc.	
5. Fatty acids: Arachidonic acid, Eicosapentanoic acid	

Effect of different elicitors on capsaicin synthesis

Different elicitors have different effect on capsaicin synthesis. When calcium ionophore A32187 was introduced in the suspension culture, 1.43 times increment of capsaicin was observed. Also, treatments with verapamil and chlorpromazine (Calcium modulators) resulted in lesser cell growth and capsaicin synthesis. This suggests the involvement of calcium in signalling of capsaicin synthesis [114]. When both salicylic acid (SA) and methyl jasmonate (MeJA) were administered together in the cultures of *Capsicum frutecense,* overall production of capsaicin was found to be enhanced. However, when both the SA and MeJA were used in combination, overall accumulation of capsaicin was decreased [114]. Ferulic acid resulted in the enhancement of the level of capsaicin in immobilized cells by 2 fold [70]. Curdlan, along with tyrosine resulted in 8.7 times increment in capsaicin yield [65]. An increment of 1.5–2 times in capsaicin production was observed after treatment with laminarin [115]. Isleck *et al.* (2014) found that treatment with cellulase for different period of time elevates the total accumulation of capsaicin by different degrees. However, at higher concentration and incubation time, capsaicin accumulation tends to decrease [116].

Product recovery

Separation of capsaicin from suspension culture is same as that from the plant. Cells have to be filtered out, dried and ground. The dry callus is then extracted in suitable polar solvent. With the help of HPLC and UV–Vis spectrophotometer, the presence of capsaicin and its amount can be detected.

CONCLUSION

Capsaicin is a promising compound with respect to defence and healthcare. Presently the only source of capsaicin is extraction from chilli. This method is dependent on agricultural produce of chilli. Synthesizing capsaicin artificially is economically unpractical as the complicated chemical structure makes it highly costly. Production through plant tissue culture is a highly promising alternative as it is not limited by agricultural produce, highly economic and large quantities of capsaicin can be obtained.

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