IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 9, Issue 2 Ver. III (Mar-Apr. 2014), PP 86-90
www.iosrjournals.org

Media optimization in immobilized culture to enhance the content of Kaempferol in *Tylophora indica* (Asclepeadaceae) and Curcumin in *Curcuma longa* (Zingiberaceae).

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Abstract: *Immobilization of plant cell plays a very important role in exploiting plant cell culture for the production of high value phyto-pharmaceuticals. It is the advanced novel biotechnological approach, through which it is possible to achieve a physiological state conducive for metabolite production .That can overcome many limiting factors of suspension culture with distinct advantage of easier separation from product and also being agreeable for biotransformation of low value compounds to higher value product. In the described study, the immobilized culture of Tylophora indica (Asclepeadaceae) and Curcuma longa (Zingiberaceae) derived from leaves and rhizome respectively were used to enhance the production of their active compounds, Kaempferol and Curcumin. The maximum content of Kaempferol and Curcumin were obtained in cultures fed with 5mg/100ml of Cinnamic acid i.e.3.31%/300beads and 3.36 %/300beads respectively at the age of two weeks in Zenk media .This study also has given a clear image of biosynthetic pathway of these compounds.*
Keywords: *Immobilize culture, Tylophora indica ,Curcuma longa , Kaempferol, Curcumin.*

I. Introduction

The production of secondary metabolites using plant cells has been the subject of extended research. It was expected that the biosynthetic capacity of plants could be exploited *in vitro* using plant cells and cell tissue systems. An important requirement for the improvement of secondary metabolite synthesis in plants is the understanding of the metabolic pathways and the enzymology of the biosynthesis of particular products. The 8000 or so phenolic compounds are formed by way of either the Shikimic acid pathway or the Mevalonate /acetate pathway[1].The knowledge of plant metabolic pathways is still very limited. "In-depth,studies" of pathways in whole plants is often difficult because the biosynthetic activities may only be expressed in particular cell types within a specific plant organ or at a certain time of season. Cell cultures have a higher rate of metabolism than intact differentiated plants. This is the most important advantage of plant cell cultures as model systems for the study of biosynthetic pathways, as secondary metabolite formation can take place within a short cultivation time (about 2-4 weeks).With emerging trends of exploiting plant cell culture for the production of high value phytopharmaceuticals, immobilization of plant cell has a very important role. It can overcome many limiting factors of suspension culture with distinct advantage of easier separation from product and also being amenable for biotransformation of low value compounds to higher value product.[2]

Plant cell immobilization is a valuable addition to the general techniques used in the plant tissue culture, which induces or increase secondary metabolite production. Enclosure in a support, exerts certain stress on the plant cell leading to restricted growth, the conditions normally considered favourable and as a prerequisite for enhanced metabolite production. In general, cell immobilization provides continuous process operation, re use of biocatalysts, separation of growth and production phases, and a simplified separation of bio catalysts from the culture medium[3], which allows product orientated optimization of the medium and reduction of cultivation periods. Since the immobilization of plant cells was introduced for the production of secondary metabolites, gel entrapment has been the most widely used immobilization method because it is cheap, simple, and reproducible using mild conditions during the immobilization.

Kaempferol (3,5, 7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a flavonoid found in many edible plants and commonly used in traditional medicine. Some epidemiological studies have found a positive association between the consumption of foods containing Kaempferol and a reduced risk of developing several disorders such as cancer and cardiovascular diseases. Numerous preclinical studies have shown that Kaempferol and its some glycosides have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, anti-osteoporotic, estrogenic antiestrogenic, anxiolytic, analgesic and antiallergic activities.

Zingiberaceous plants have been widely used in traditional medicine, as well as a food flavouring and spice agents. Many studies have focused on the bioactive organic compounds from *Curcuma longa*. The reports supported the traditional medicinal use of Curcumin[4]of *C.longa.*Curcumin has been used extensively in Ayurvedic medicine for centuries, as it is nontoxic and has a variety of therapeutic properties including anti-oxidant, analgesic, anti-inflammatory and antiseptic activity. Numerous animal and *in vitro* studies have demonstrated the ability of turmeric and its active component, Curcumin, to suppress the growth of a variety of tumor cells. The postulated mechanisms for its anticancer effects are multiple[5]. Curcumin has been found to possess anti-cancer activities via its effect on a variety of biological pathways involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumorigenesis and metastasis. The actual physiological functions of Curcumin are likely to be in the defense against phytophagous predators (mostly insects) and phytopathogenic microorganisms.[6]

Kaempferol has been widely reviewed *in vivo* and *in vitro* tissue culture of number of medicinal plants[7], which also has been reported in *Tylophora indica* tissue culture . Its content has been enhanced in this system by employing many strategies.[8] In the same way Curcumin has been overproduced in tissue culture of *Curcuma longa*[9].Traditional techniques of callus and suspension culture do not fully explore the range possibilities of cultured cells hence we have used immobilize culture to enhance the production of both compounds . Kaempferol and Curcumin in *Tylophora indica* and *Curcuma longa* tissue culture respectively.

II. Materials And Methods

2.1Collection of plant

The plant of *Tylophora indica* and rhizome of *Curcuma longa* were collected from Kelkar Institute Farm House,Mulund (E),Mumbai.(Latitude: 18°96′50″N Longitude: 72°82′53″E) and established in Haffkine Institute campus.

2.2 Preparation of Alginate beads

The fresh leaves and rhizome (one month old leaves in *Tylophora indica* and two months rhizome in *Curcuma longa*) in the month of January were collected from Haffkine Institute campus , washed with teepol and rinsed in running tap water. Both explants (2gm) were sterilized with 0.1%mercuric chloride for 2 min. followed by three times wash with sterile water, aseptically and homogenized. Homogenate materials were then passed though net (425 µm), to remove the large cell aggregate and fibrous material. Fresh cells were suspended in 2% sodium alginate and the suspension was added drop wise to 50mM Calcium chloride through sterilized needle and syringe. The alginate beads of diameter 2-3 mm were formed and were left in $CaCl_2$silution for 30 min for the stabilization of beads and then washed with sterilized water separately.

2.3 Immobilize suspension culture

Calcium alginate entrapped cells (300 beads) were inoculated in autoclaved Zenk[10]suspension media aseptically, supplemented with 5%sucrose with various concentrations of 40mg/100ml , 60mg/100ml of Tyrosine and 5mg/100ml ,7.5mg/100ml of Cinnamic acid in Erlenmeyer flasks(250 ml.) capacity separately. The flasks were placed in Remi shaker cum incubator at 90 rpm and maintaining temperature of 25± 20 C for one and two weeks (Figure 1.).

2.4 Extraction and Estimation for Kaempferol and Curcumin content

The *Tylophora indica* and *Curcuma longa* beads were harvested at the time interval of one week and two weeks separately .The beads were macerated in methanol for Kaempferol and in ethanol for Curcumin. All filtered extracts were dried, weighed and subjected for further qualitative and quantitative estimation for Kaempferol and Curcumin content .High Performance Thin Layer Chromatography(HPTLC) was used for this analysis with standard reference compounds of Kaempferol and Curcumin (purchased from Sigma Alderich) in Anchrome ,Mulund(E), Mumbai.

2.5 High Performance Thin Layer Chromatography (HPTLC) for Kaempferol

The HPTLC analysis of methanolic extracts(for Kaempferol) were carried out by using High Performance Thin Layer Chromatography at 254 nm Inert gas was used as spray gas in CAMAG LINOMAT `121037 HPTLC equipment, Toluene: Ethyl acetate: Methanol :Formic acid (30:15:1:2) was used as mobile phase (Rf-0.91) and 15%ethanolic ferric chloride was used as derivatizing agent.

2.6 High Performance Thin Layer Chromatography (HPTLC) analysis for Curcumin

HPTLC analysis of all ethanolic extracts(Curcumin) were carried with Standard compound of Curcumin (Rf value 0.51) at 425nm.Inert gas was used as spray gas in CAMAG LINOMAT 121037 HPTLC

equipment, Chloroform:Methanol(9:1) was used as mobile phase,while15%ethanolic ferric chloride was used as derivatizing agent.

III. Results

The immobilized culture (Calcium alginate beads) derived from *Tylophora indica* leaves and *Curcuma longa* rhizome were prepared and used for enhancement of Kaempferol and Curcumin respectively. The High Performance Thin Layer Chromatographic analysis of induced immobilized cultures (fed with various concentrations of Tyrosin and Cinnamic acid) were carried out. Obtained results reveals the enhancement of Kaempferol and Curcumin in *Tylophora indica* and *Curcuma longa* respectively. The maximum contents of Kaempferol was observed 3.31% /300 beads in *Tylophora indica* and 3.36 %/ 300 beads of Curcumin in *Curcuma longa* at the age of two weeks in Zenk immobilized cultures supplemented with 5 mg/100 ml of Cinnamic acid.(Table1.)

S.no.	Media content	Kaempferolcontent%in *Tylophora indica*/300beads		Curcumin content% in *Curcuma longa*/300 beads	
		One wk	Two wks	One wk	Two wks
1.	Tyrosin 40mg/100ml	0.77±0.051	0.88±0.067	1.4±0.041	0.338±0.045
2.	Tyrosin 60mg/100ml	0.19±0.051	0.26±0.031	1.66±0.051	2.346±0.062
3.	Cinnamic acid 5 mg/100ml	2.07±0.061	3.31±0.067	2.03±0.089	3.36±0.051
4	Cinnamic acid 7.5 mg/100ml	1.37±0.0560.	1.06±0.052	1.7±0.051	1.49 ±0.079
5.	Control	0.16±0.0043		1.5±0.0005	

Table 1.Effect of different compounds on Kaempferol and Curcumin production in immobilized culture of *Tylophora indica* and *Curcuma longa* ± Mean S.D.(Annova test) P <0.05

Figure1.Entrapped AlginateBeads in immobilized culture.

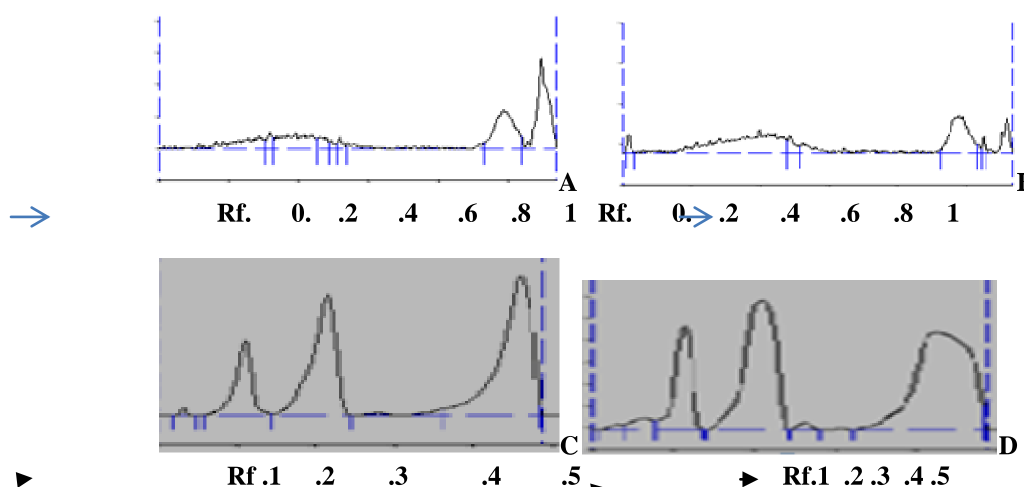

Figure 2.Depicts High Performance Thin Layer Chromatography(HPTLC) A- Analysis of Kaempferol in immobilize culture of *Tylophora indica* fed with 5mg/100ml of Cinnamic acid with standard,B-

Kaempferol(254nm);C-Analysis of Curcumin in immobilize culture of *Curcuma longa* with standard D-Curcumin standard.(425nm)

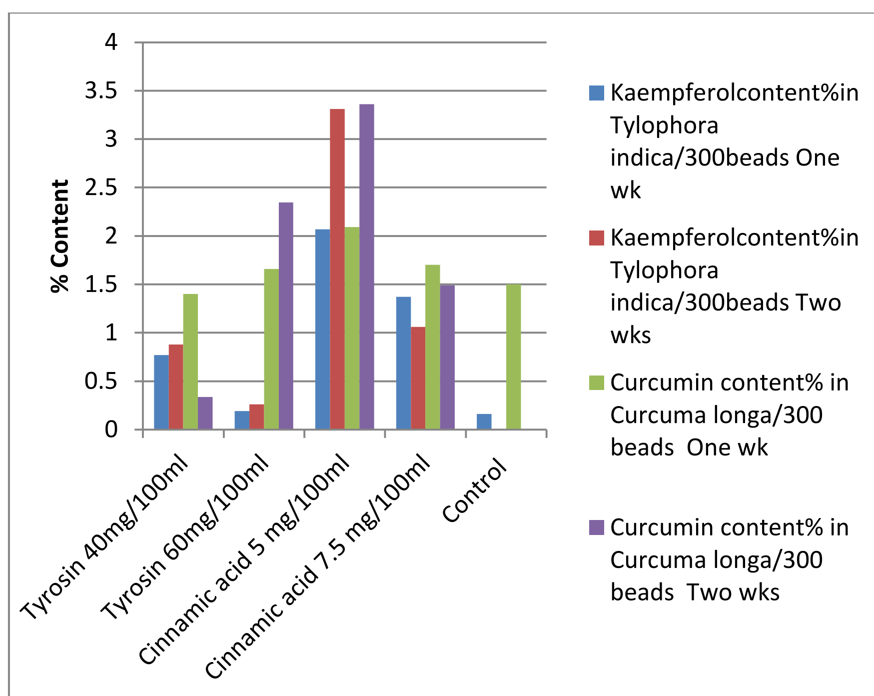

Figure 3.Effect of different compounds on production of Kaempferol in *Tylophora indica* and Curcumin in *Curcuma longa* in immobilized culture

IV. Discussion

Plant cells are biosynthetically totipotent, which means that each cell in culture retains complete genetic information and hence is able to produce the range of chemicals found in the parent plant. The carbon source exerts an effect on production of secondary metabolites in cultured cells. Osmotic stress created by sucrose alone and with other osmotic agents were found to regulate anthocyanin production in *Vitis vinifera* cell suspension cultures [11].The effect of phosphate concentration in the medium plays a major role in the production of secondary metabolites *in vitro*. Higher levels of phosphate were found to enhance the cell growth, whereas it had negative influence on secondary product accumulation (Zenk, 1975).In the described study,5%sucrose in Zenk basal production media(with reduced content of phosphates) was found to obtained better enhancement in Kaempferol and Curcumin production in *Tylophora indica* and *Curcuma longa* respectively .Improvement in the secondary metabolite production in cell cultures is often associated with the organization and differentiation of plant cells[12] and immobilization confines a catalytically active enzyme or cells on a fixed support[13],so keeping these facts, we have used immobilized cultures(entrapped alginate beads) derived from differentiated parts(one month old leaves in *Tylophora indica* and two months old rhizome in *Curcuma longa*)rather than callus for conducting present experiment. The Study of media optimization and enhancement in the production of Kaempferol and Curcumin have proven that this technique is successful to achieve the goal .The results also support the previous views that have been given by Payne et al., 1991. According to them, the product of interest should not be strictly growth associated and immobilized cells should maintain prolonged viability and biosynthetic capacity with high rates of sustainable secondary metabolite production.

Biotransformation can be defined as a process through which the functional groups of organic compounds are modified either stereo or regiospecifically by living cultures and entrapped enzymes.[14]Advantages of the biotransformation include the production of novel compounds, enhancement in the productivity of desired compound and overcoming the problems associated with chemical synthesis. Significantly, the studies on biotransformation lead to basic information to elucidate biosynthetic pathway. In the conducted study, immobilization of cells were carried out by using plant material collected from field directly rather from grown callus, this exercise saved the time as also expenditure. The Kaempferol and Curcumin contents were retained in alginate matrix at the concentration of 3.31% and 3.36 % respectively at the culture age of two weeks in Zenk

media supplemented with 5 mg/100 ml of Cinnamic acid(Table1,Figure3.). The results were obtained by calculating the peak area of HPTLC chromatograms of induced samples with standard compounds of Kaempferol and Curcumin (Fig.2) .Several experiments showed that alginate matrix also induced stress condition in the immobilized cell. Therefore, besides creating more contact among cells, it could also cause the obstruction of cell growth. Since cell growth was reduced in immobilized cultures, substrate consumption and flux of energy was directed to the desired secondary metabolite pathway and so improved product yields of the process. The same result was found in *Datura innoxia* culture which was immobilized.[15] Immobilization using pectin capsulation in *Cruciata glabra* also increased anthraquinone production [16]. These all references support our results.

In the described study, the maximum content of both compounds were obtained in two weeks old culture fed with 5mg/100ml of Cinnamic acid as compared to other samples .The study suggested that Cinnamic acid is the best precursor compound than Tyrosin in immobilized culture. This study also clear a picture of biosynthetic pathway of flavonoids in tissue culture of *Tylophora indica* and *Curcuma longa*.(Fig 4.).The optimization of media and overproduction of flavonoids (Kaempferol and Curcumin) in both plant sps using immobilize culture is a new study.

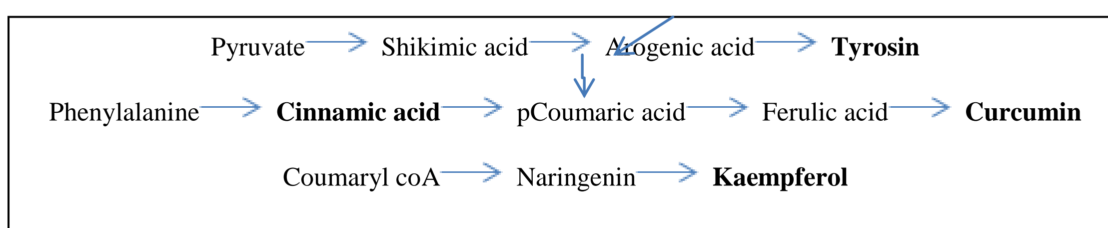

Figure4. Proposed Biosynthetic pathway of Curcumin and Kaempferol

V. Conclusion

The Developed technology favours the optimum production and accumulation of Kaempferol and Curcumin in *Tylophora indica* and *Curcuma longa* respectively in minimum time with reduced expenditure through immobilized culture .Conclusively it can be suggested that Cinnamic acid is better precursor for flavonoids(Kaempferol and Curcumin) production in immobilized suspension culture.

Acknowledgement

Authors are grateful to Women Scientist (Department of Science and Technology) Scheme Funds for providing grant for doing present work

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