ISSN : 0974 - 7435 Volume 8 Issue 11

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 8(11), 2013 [1451-1457]

Biosynthesis and regulation of paclitaxel in *Taxus*

Ronghua Chen, Xiangli Yu, Shanshuo Zhu, Zhiqi Miao*
School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, People's Republic of (CHINA)
E-mail: zqmiao@sjtu.edu.cn

ABSTRACT

Tremendous progress has been made recently on improving Taxol biosynthesis, such as elicitation and precursor feeding. This article summarizes recent progress on the above area and provides a current overview of research on taxane production by biological method. In addition, the probability and foreground of regulation of taxus by genetic approaches are also discussed.

KEYWORDS

Biosynthetic pathway;
Cloning;
Elicitor;
In situ separation;
Precursor;
Paclitaxel.

INTRODUCTION

Paclitaxel (marked as Taxol® by Bristol-Meyers Squibb) is a naturally occurring diterpenoid alkaloid from various species of *Taxus*[28]. After more than 20 years of development, Taxol gained the first marketing approval from U.S. Food and Drug Administration (FDA) for the treatment of refractory ovarian cancer in 1992 and metastatic breast cancer in 1994, and entered the generic drug market in 2000, and has been thought as a powerful anticancer drug during the last 20 years. In addition, Paclitaxel has shown promising results in drug-eluting coronary artery stents[3] because the surface of paclitaxel stents may help prevent cardiovascular disease and narrow again after surgery in artery implanted to close issue. The annual sales of Taxol and Taxotere reached up to three billion USD in 2004[22] and four billion USD in 2007. From then on, it has ranked top one in international natural anti-cancer drugs.

As one of the most potent chemotherapeutic agents against a range of cancers, the demand for paclitaxel greatly exceeds its limited supply from the bark of natural yews. And now paclitaxel is largely produced by Taxus cell culture methods, and the other commercial production for paclitaxel or its closely related drug, docetaxel is semi-synthetic means from advanced precursor, such as baccatinbIII or 10-deacetyl baccatinb III, that are more readily available from the needles of various yew species as a renewable resource.

For the foreseeable future, the efficient and affordable supply of paclitaxel and its precursor for semi-synthesis will continue to rely upon biology methods.

The paclitaxel biosynthetic pathway involves a series of biochemical reactions. The committed step to paclitaxel biogenesis is thought to be the reaction in which the taxane nucleus is constructed via cyclization of the branch-point intermediate geranylgeranyl diphosphate (GGPP), diverting GGPP into the biosynthesis of toxoid. The entire pathway of paclitaxel biosynthesis, therefore, can be conceptually divided into two parts. The upstream pathway, which also exists in many plants and fungi, is composed of the biogenesis of GGPP. The downstream pathway, the unique part in Taxus, constitutes 18 sequential biochemical reactions (Figure 1),

FULL PAPER

being responsible for the modification to the nucleus mainly by hydroxylation and acylation to get paclitaxel, The hydroxylation, occurring at seven positions of C1, C2, C5, C7, C9, C10, C13, activates the skeleton, and makes it possible for the following esterification via Co-A-dependent acyl/aroyl transfer at five positions of C2, C5, C10, and C13. Among the 18 biochemical steps in paclitaxel biosynthetic pathway, genes encoding TS (the committed enzyme), five hydroxylases (2α, 5α, 7 β, 13α and 10β) and five acylases (TAT, TBT, DBAT, BAPT and DBTNBT) have been cloned from *Taxus*[15].

Engineering the paclitaxel biosynthesis has the dual purpose. The first is to increase the yield of paclitaxel in a biological system and the second is to proofread the biochemical steps in this complex pathway.

ENGINEERING THE BIOSYNTHESIS OF PACLITAXEL BY GENETIC AND ENVIRONMENTAL APPROACHES

According to the theory of metabolic engineering, the flux of any pathway is determined by the levels of intermediates and the activities of enzymes responsible for catalyzing these intermediates.

The most feasible measure to overproduce paclitaxel is feeding precursor, in which exogenous intermediate or substance which can be converted into the above

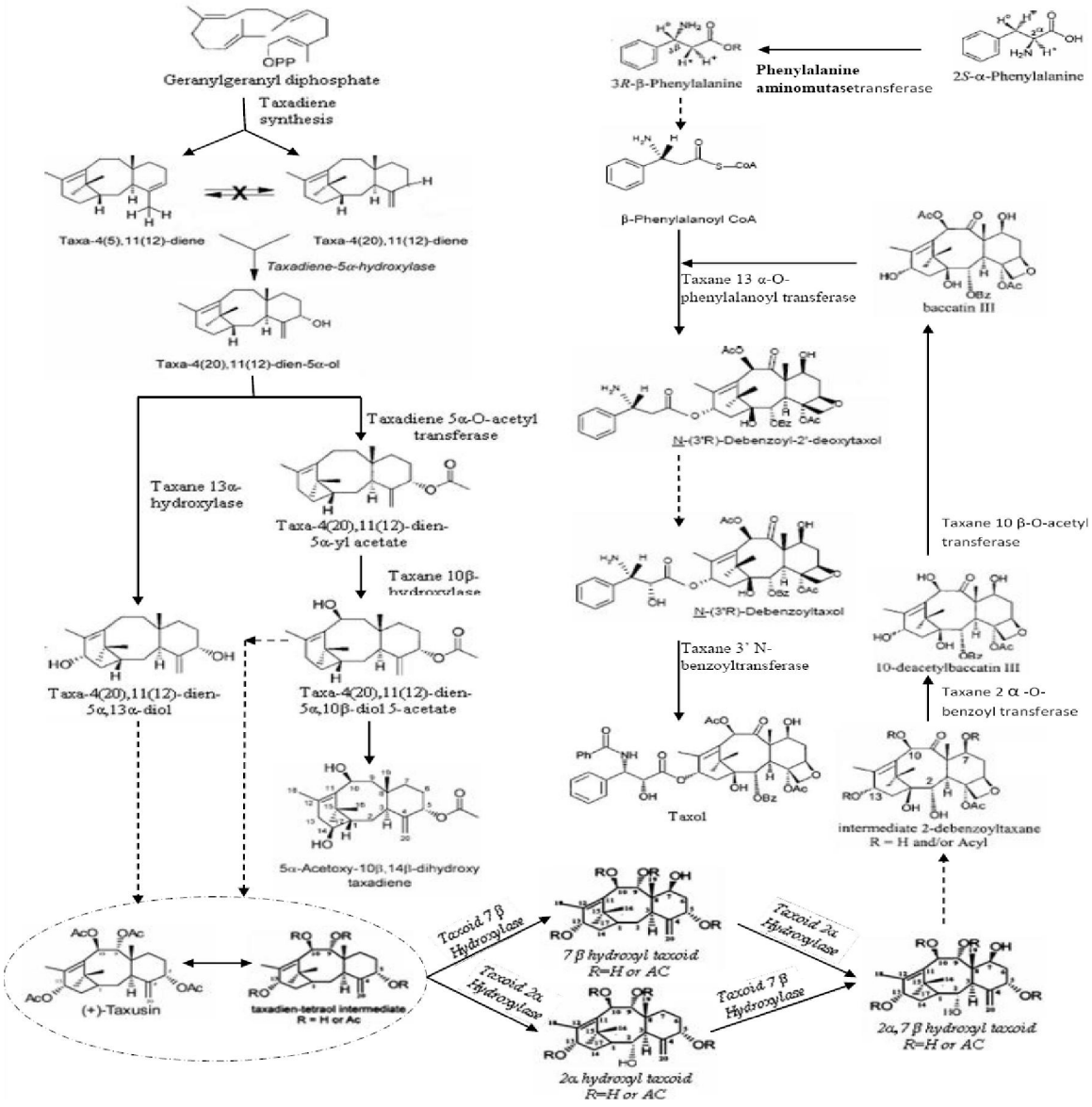

Figure 1 : Outline of taxol biosynthetic pathway

intermediate by cell is added to the culture system, absorbed by the *Taxus* cell, and incorporated into the production of paclitaxel.

The most efficient strategy to improve paclitaxel biosynthesis is regulating, upward or downward, the activity of enzymes responsible for paclitaxel biosynthesis, directly or indirectly, by genetic or environmental approaches.

In altering the production of paclitaxel one has to keep in mind that paclitaxel is only one among more than 400 kinds of taxanes and has a common precursor with any terpenoid. Therefore, inhibiting or even blocking competitive pathways, such as those for the large family of 14b-hydroxy taxoid[12] and other completive terpenoids, such as chlorophylls, quinines, tocopherols, carotenoid, casbene, oryzalexins, *et al.* is favor to the production of paclitaxel.

As other secondary metabolite, metabolic engineering of paclitaxel starts from the overexpression of the putative key enzyme, TS, which catalyzes the slowest branching step and controls the flux to paclitaxel. However, this approach has only limited value, because the effects of modulating single enzymatic steps are often absorbed by the system with strong feedback or the coming of the new rate-limited step. Targeting multiple steps in the same pathway could help to control metabolic flux in a more predictable manner. This might involve activating several consecutive enzymes responsible for paclitaxel biosynthesis, while suppressing those in another competing pathway, or using regulatory genes to establish multipoint control over one or more enzymes in the cell. And the multipoint metabolic engineering is now beginning to supersede single-point engineering as the better way to manipulate metabolic flux. Some of the above proposed regulations have been done through genetic or environmental approaches[30].

Genetic approaches

The establishment of transgenic approaches of *Taxus* is required for metabolic engineering of paclitaxel biosynthetic pathway in order to improve drug production. Methods for gene transfer in plant systems include utilization of Agrobacterium spp., polyethylene glycol, electroporation, and biolistic technologies, et al.

In general, effective transient transformation method is needed in order to identify and characterize rapidly the key regulatory genes before intensive, time-consuming stable transformation efforts can proceed. A variety of promoters (i.e., CaMV 35S and maize ubiquitin) have shown their ability to conduct the transient expression of the reporter genes, such as luciferase, GUS, and DsRed, respectively in *Taxus* by particle bombardment-mediated transformation method[27]. The transit expression experiment can complete within days as opposed to waiting for enough biomass for stable transformation, which can take months.

Based on preliminary works on the putative transformants of *Taxus*[16,27] with ambiguous evidence of stable transformation, and those on the T. cuspidata cell being sonication assisted transformed[26] transiently with the help of Agribacterial, The first stable transformation and long-term maintenance of transgenic *Taxus* cell suspension cultures have been achieved[15,24] after testing the many possible permutations of *Taxus* species and Agrobacterium strains. In the transformation protocol, hygromycin was selected as the selection agent because *Taxus* cell is sensitive to hygromycin, with complete inhibition of cell growth at a typical selection concentration of 2.5 mg l^{-1}, while *Taxus* culture is quite tolerant of kanamycin, with cells still growing, albeit slowly, at 800 mg l^{-1} of kanamycin. Meanwhile, a pCAMBIA1301 bearing intron-containing GUS reporter genes was selected as the binary vector in order to ensure that glucuronidase expression is from transformed plant cells and not from residual Agrobacterium. A more realistic estimate of transformation efficiency, therefore, may be that approximately 1% of experiments result in a stable, maintainable, transgenic cell line. However, the transformation of *Taxus* seems to be difficult, far away from routine, and continues to be the major obstacle to the metabolic engineering in *Taxus*. And now identifying and optimizing factors critical for the successful and reliable transformation of *Taxus*, especially testing the many possible permutations of *Taxus* species and Agrobacterium strains, are under study for developing a more optimal transformation protocol.

From then on, regulating the paclitaxel product ion in *Taxus* by genetic engineering has been listed in the agenda of many laboratories. It seems likely that overexpression of the key enzyme at branch point, TS, or its modification to out-compete other enzymes using

the same substrate GGPP can divert more flux into the biosynthetic pathway of taxane, while competing enzymes and that responsible for the degradation of paclitaxel can also be inhibited directly by the technology of RNA interference, antisense technology and site mutation to avoid the production of undesired substances. If possible, competing branches, such as that for the taxane derivatives with hydroxyl group at C14, can be short-circuited by the import of enzyme shunts that redirect flux in the appropriate direction. Further increases in flux can be achieved by using feedback-insensitive enzymes or those modified to favor unidirectional catalysis in reversible reactions. Instead of modulating individual steps, the future of metabolic engineering will probably involve holistic approaches in which multiple steps are targeted simultaneously. Overexpression of the last three acyltransferase genes simultaneously in transgenic *Taxus* would convert relatively rich intermediate, such as, 10-DAB and baccatin III in *Taxus*, to paclitaxel easily. In addition, regulating the complex biosynthetic pathway of paclitaxel at multiple points by transforming only one exogenous gene is also possible. The transcription factor can be used to coordinately upregulate several enzymes while a polycistronic antisense RNA is being used to suppress several enzymes in competing pathways.

Meanwhile, Some works were suggested to redirect the pathway for the production of novel taxanes with a greater range and potency of anti-cancer activity and decreased side-effects, such as taxoids 10-deacetyl-14α-hydroxybaccatin III, 10-deacetyl-19-htydroxybaccatin III, 13-acetyl-9-dihydrobaccatin III, and etc, from 13-acetyl-9-dihydrobaccatin III, two 9-dihydro-paclitaxel analogues (9-dihydropaclitaxel and 10-deacetyl-9-dihydropaclitaxel) were derived and shown to possess increased water solubility, decreased toxicity, and improved *in vivo* efficacy compared to paclitaxel[12]. The transgenic redirection of the pathway in cell cultures to produce these new drugs directly, to produce additional precursors for semisynthesis, and to produce other rare taxoids for clinical evaluation, can be readily envisioned as an important future direction.

As well as metabolic engineering in *Taxus*, researchers have explored the possibility of transferring the pathway to microbial or fast-growing plant species that are easier to genetically manipulate in order to produce taxoids by metabolic engineering. By expressing both GGPP synthase and a truncated taxadiene synthase gene, taxadiene can be synthesized in the culture of the engineered *Escherichia coli*[11]. Further, eight Taxol biosynthetic genes were transformed and functionally expressed in *Saccharomyces cerevisiae* to obtain more advanced taxanes, such as taxadiene-5α-acetoxy-10β-ol[7]. Because the universal diterpenoid progenitor GGPP is rich in most plant, more attentions have been paid on producing taxanes by transgenic plant, such as Arabidopsis[1], tomato[17] and *Artemisia annua* L (will be published in other place).

It would be keep in mind that metabolic engineering of paclitaxel must be studied in the context of the whole cell rather than at the single pathway level, and that even the simplest modifications can send ripples throughout the entire system. Attention has therefore shifted away from reductionist, single-gene engineering strategies and towards more complex approaches involving the simultaneous overexpression and/or suppression of multiple genes. The use of regulatory factors to control the abundance or activity of several enzymes is also becoming more widespread. In combination with emerging methods to model metabolic pathways, this should facilitate the enhanced production of natural products and the synthesis of novel materials in a predictable and useful manner.

Environmental regulation

Controlled by genetic characteristics of *Taxus* cell, the biosynthesis of paclitaxel can also be regulated by environmental factors other than the genetic one to improve the production of paclitaxel[25]. *Taxus* cell cultures in bioreactor represent a relatively fast-growing biomass which can be maintained under carefully controlled condition in order to increase the production of paclitaxel. Therefore, nearly all studies about regulating paclitaxel biosynthesis by environmental factor were carried out in *Taxus* cell culture system. Three kinds of approaches have been proved to be very effective to increase paclitaxel production: precursor feeding, induced culture, and *in situ* separation[5].

Precursor feeding

Precursor is substance that can be easily directed

into paclitaxel biosynthetic pathway via the complex metabolic network of *Taxus* cell. Therefore, all intermediates from the biosynthetic pathway of paclitaxel and other substances that can be easily converted to them by cell metabolism are precursors to paclitaxel. Addition of precursor is an effective way to increase paclitaxel production by bypassing the endogenous biosynthesis of the precursor, breaking through the limit of its inherent low concentration, and providing more exogenous substance for enzyme resident in paclitaxel biosynthetic pathway.

Though complex and advanced precursors, such as 10DAB and BactinIII, are more efficient to enhance the production of paclitaxel production, simple precursors, such as glyceraldehyde 3-phosphate, 1-deoxy-D-xylulose 5-phosphate, pyruvate, IPP, DMAPP, GPP, and FPP and GGPP, which can be incorporated into the skeleton of paclitaxel, are popularly used because of their lower cost[5]. In addition, acetate, benzyl amino acid, phenylalanine and their salts, linking to the skeleton of paclitaxel as the acyl donator, were reported with the ability to improve paclitaxel biosynthesis in cell culture system[8,9].

Elicitation culture

As a kind of secondary metabolite, paclitaxel does not appear to participate directly in the growth and development of *Taxus*, and it is produced as a protection agent against fungi aggregation or various stresses. It is assumed that the expression of the genes encoding enzymes responsible for paclitaxel production should be inducible to improve the adaptability of *Taxus* to the environmental changes. Therefore, a complex molecular mechanism must have been developed in *Taxus* to regulate the production of paclitaxel at transcription or enzyme level in responsive to these outer stresses.

The factor, which can influence the transcription of genes coding for enzymes resident in the synthetic pathway of paclitaxel, directly or indirectly, is called elicitor. According to the definition, elicitor includes not only the conventional chemical and biological elicitor, but also the physical stimulus such as heat, light, magnetism and others. For example, under the optimized condition, paclitaxel yield increased six folds via heat shock at 35–50°C for 30–60 mins[17]. The mechanism of elicitation begin by signal transduction process: the first step is the identification and binding of elicitor to its receptor; then signal is transduced, magnified and passed to effector like domino card game; finally, the transcription of gene relative to paclitaxel biosynthesis is regulated by the effector, resulting into more corresponding enzyme and more paclitaxel production. However, the detailed mechanism of elicitation in paclitaxel biosynthesis is very complex and has not been clarified up to now. Traditionally, any substance that can increase paclitaxel product but can not be incorporated into paclitaxel has been classified into elicitor.

Methyl jasmonate has been reported as the most effective elicitor and the induced content of paclitaxel increased 19-fold[2]. Both the steady state level of mRNA and enzymatic activity of GGDS and TS *Taxus* were improved when paclitaxel production was induced by methyl jasmonate[29]. By examining the expression profile of paclitaxel biosynthetic pathway genes by RNA gel blot analysis and RT-PCR in the Taxus cuspidata cell, it was found that the early pathway enzyme genes GGPPS, TS, and Taxane 5α hydroxylase are upregulated by MJ elicitation within 6 h andtill 24 h before their abundances decrease. The study reveals the preference for one side of the biosynthetic pathway branch in early taxane synthesis, where transcripts coding for Taxane 5α hydroxylase are abundant after elicitation with MJ but transcripts encoding the two enzymes for the alternative branch (TAT and Taxane 10β hydroxylase) are not highly expressed following elicitation. Transcripts encoding the enzymes TBT and DBAT are also up-regulated upon MJ elicitation. However, the steady-state levels of the two enzyme transcripts (BAPT and DBTNBT) responsible for the C-13 modification are much lower than transcripts of early pathway steps[23]. So far, only one transcription factor, TcAP2, was isolated from Taxus cuspidata by yeast one-hybrid strategy[6]. This factor interacts with jasmonate responsive element and can be induced to express by treatments with methyl jasmonate or salicylic acid.

Salicylic acid, ammonium citrate, silver nitrate, arachidonic acid, ethylene, extracts from fungal cell wall, oligosaccharide were used popularly to enhance the production of paclitaxel[20,21,35], though their mechanisms in paclitaxel biosynthesis have been undiscovered.

Some researchers focused their attentions on the

FULL PAPER

signal transfer process after the addition of elicitor and believed that the oxidative burst induced by elicitors such as salicylic acid, fungal elicitor and oligosaccharide is the key step of the signal process[32,34]. Cell apoptosis was found when the cell of *Taxus* was induced by elicitor[33]. However, the causality between paclitaxel production and cell apoptosis is controversial. Other researchers want to solve the problem at the metabolic level[14,30]. They analyzed the kinetic change of various kinds of taxane in elicitation experiment, inferred and validated the activity change of enzyme responsible for paclitaxel biosynthesis as the result of elicitation. Based on the elicitation kinetics, elicitors have been divided into more than two groups; each group has the same character of elicitation kinetics and is assumed to target to the same part in paclitaxel biosynthetic pathway. The theory of acting point is helpful to study the synergism between elicitor, and one interesting achievement of the theory is that it triumphantly suggests an optimal combination of elicitors: salicylic acid and citrate, one upregulating the biosynthesis of BactinIII, forming the skeleton of paclitaxel, and the other improving the assembly and modification of the C13-side chain. The production of paclitaxel induced by these two kinds of elicitor is 44% higher than the sum of paclitaxel induced by salicylic acid and ammonium citrate respectively[36].

In addition, substances, known as the inhibitor of competitive pathway branched from the pathway of paclitaxel biosynthesis, are also used to improve the production of paclitaxel. It has been reported that the addition of inhibitor of gibberellin biosynthesis, such as CCC, tetramethylammonium bromide and 2, 2-ditmethylhydrazide, inhibited the flux from GGPP to gibberellin and increased paclitaxel production. The similar results were obtained when the inhibitor of carotenoid biosynthesis, including clomazone and MPTA, were added into the system of *Taxus* cell culture[19]. Finally, gibberellin and carotenoid are byproducts of paclitaxel production and exogenous gibberellin and carotenoid may regulate downward the activity of enzyme responsible for their biosynthesis via product feedback inhibition. Therefore, feeding these above substances may inhibit their production and redirect more substance flux into the pathway of paclitaxel biosynthesis.

In situ separation

Separating paclitaxel from cells in a cell culture system as quick as possible would be favorable to decrease the degradation, reduce the toxicity, and ease the product feedback inhibition of paclitaxel. The biosynthesis of paclitaxel was enhanced by 40-70% when the non-ionic exchange resin XAD was added into the culture medium as an adsorbent of paclitaxel[18]. The more heart-stirring increase of paclitaxel (about three or four times higher than that of control) was achieved in two-liquid-phase culture, in which cells were retained in water phase while taxane produced by cells was extracted into the oil phase and isolated from the cells to eliminate the adverse effects of paclitaxel accumulation[36].

CONCLUSIONS

Up to now, environmental factors, including precursor feeding, elicitation culture and *in situ* separation, have been proved to be efficient to increase the production of paclitaxel

The only obstacle to improve Taxol biosynthesis by genetic modification is the difficulty of *Taxus* transformation. And we will envisage the explosion of metabolic engineering study of paclitaxel by transgenic method when a routine transformation of Taxus is developed in the future.

REFERENCES

[1] Ó.Besumbes, S.Sauret-Güeto, M.A.Phillips; Biotechnol Bioeng, **88**, 168-175 **(2004)**.

[2] V.Bringi, P.G.Kadkade, C.L.Prince; US Patent No. 5,407,816 **(1995)**.

[3] A.N.Buch, R.Waksman; Am.J.Cardiol, **99(3)**, 424-7 **(2007)**.

[4] R.Croteau, R.E.B.Ketchum, R.M.Long; Phytochemistry Rev., **5(1)**, 75-97 **(2006)**.

[5] R.M.Cusido, J.Palazon, M.Bonfill; Biotechnol Prog, **18(3)**, 418-23 **(2002)**.

[6] Y.Dai, Q.Qin, D.Dai; Biotechnol Lett. **31(11)**, 1801-1805 **(2009)**.

[7] J.M.Dejong, Y.Liu, A.P.Bollon; Biotechnol and Bioeng, **93**, 212-224 **(2006)**.

[8] A.G.Fett-Neto, F.DiCosmo, W.F.Reynolds; Biotechnology, **10(12)**, 1572-1575 **(1992)**.

[9] A.G.Fett-Neto, S.J.Melanson, K.Sakata; Biotechnology, **11(6)**, 731-734 **(1993)**.
[10] K.H.Han, P.Fleming, K.Walker; Plant Sci 95:187–196 **(1994)**.
[11] Q.Huang, C.A.Roessner, R.Croteau; Bioorg Med Chem, **9**, 2237-2242 **(2001)**.
[12] S.Jennewein, H.Park, J.M.DeJong; Proc.Natl Acad Sci USA, **98**, 13595-13600 **(2001)**.
[13] S.Jennewein, M.R.Wildung, M.Chau; Proc.Natl Acad Sci. USA, **101(24)**, 9149-54 **(2004)**.
[14] R.Ketchum, C.D.Rithner, D.Qiu; Photochem, **62**, 901-909 **(2003)**.
[15] R.E.Ketchum, L.Wherland, R.B.Croteau; Plant Cell Rep., **26(7)**, 1025-33 **(2007)**.
[16] C.H.Kim, K.I.Kim, I.S.Chung; J.Micro Biotech, **10**, 91–94 **(2000)**.
[17] K.Kovacs, L.Zhang; R.S.Linforth; Transgenic Res., **16**, 121-126 **(2007)**.
[18] I.C.Kwon, Y.J.Yoo, J.H.Lee; Process Biochem, **33(7)**, 701-707 **(1998)**.
[19] X.G.Mei, A.S.Wang, X.Yang; Life Sci Res, **6(1)**, 46-50 **(2002)**.
[20] Z.Q.Miao, Z.J.Wei, Y.J.Yuan; Sheng Wu Gong Cheng Xue Bao, **16(4)**, 106-110 **(2000a)**.
[21] Z.Q.Miao, Z.J.Wei, Y.J.Yuan; Acta Biophysica Sinic, **16(2)**, 204-211 **(2000b)**.
[22] M.McCoy; Chem.Eng.News, **82(35)**, 12–14 **(2004)**.
[23] E.Nims, C.P.Dubois, S.C.Roberts; Metab Eng., **8(5)**, 385-94 **(2006)**.
[24] E.B.K.Raymond, W.Lea, B.C.Rodney; Plant Cell Rep., **26**, 1025–1033 **(2007)**.
[25] V.Srinivasan, V.Ciddi, V.Bringi; Biotechnol Prog; **12(4)**, 457-65 **(1996)**.
[26] H.N.Trick, J.J.Finer; Plant Cell Rep., **17**, 482–488 **(1998)**.
[27] K.Vongpaseuth, E.Nims, M.St Amand; Biotechnol Prog., **23(5)**, 1180-5 **(2007)**.
[28] M.C.Wani, H.L.Taylor, M.E.Wall; J.Am.Chem Soc., **93(9)**, 2325-23277 **(1971)**.
[29] M.R.Wildung, R.A.Croteau; J.Biol Chem, **271(16)**, 9201–9204 **(1996)**.
[30] J.M.Won, S.Y.Byung, L.K.Dong; Biotech Tech, **12(1)**, 79–81 **(1998)**.
[31] L.X.Yang, H.J.Wang, R.A.Holton; Int.J.Radiat Oncol Biol.Phys., **46(1)**, 159-163 **(2000)**.
[32] L.J.Yu, W.Z.Lan, W.M.Qin; Process Biochem, 37: 477-482 **(2001)**.
[33] Y.J.Yuan, C.Li, Z.D.Hu; Process Biochem, **8**, 193-198 **(2002b)**.
[34] Y.J.Yuan, C.Li, Z.D.Hu; Enzyme Microb Tech, 30: 774-778 **(2002a)**.
[35] Y.J.Yuan, Z.J.Wei, Z.Q.Miao; Biochem Eng J, **10**, 77-83 **(2002b)**.
[36] Y.J.Yuan, Z.J.Wei, Z.L.Wu; Biotech Lett, **23**, 1659-1662 **(2001)**.
[37] C.Zhang, P.S.Fevereiro; Biotechnol Bioeng., **96(3)**, 506-14 **(2007)**.