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Mahavadi, S., Rao, R.S.S.K. and Murthy, K.S. (2007). Cross-regulation of VAPC2 receptor internalization by m2 receptors via c-Src-mediated phosphorylation of GRK2. Regulatory Peptides, 139: 109-114.

Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4^{th} edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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In Utero Low-Protein Diet Alters Vascular Estrogen Receptors, Endothelial Nitric Oxide Synthase and Angiotensin 11 Subtype 1 Receptors in adult male and female rat offspring

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Abstract

Background/Aims: We investigated whether: 1) *in utero* low-protein diet (LPD) induced adult hypertension alters vascular estrogen receptor (ER)-related mechanism in both male and female vascular tissues; and whether 2) flutamide, a specific, non-steroidal competitive antagonist of the androgen receptor, improved this system in female offspring.

Methods: Pregnant rats were fed either with 20% protein (control), or 6% protein (LPD) from day 1 (LPD-1) or day 12 (LPD-2) of gestation. Male and female hypertensive offspring were treated when adult with flutamide (10 mg/day/kg body weight, subcutaneous) for four days. Aortas (vascular) were isolated from control, LPD-1 female, LPD-2 male and flutamide treated female LPD-1 offspring. ER-alpha (ER-α), ER-beta (ER-β), endothelial nitric oxide synthase (eNOS), and the angiotensin 11 subtype 1 receptor 1 (AT_1-R) protein levels were analyzed using western immunoblotting.

Results and Conclusion: In female LPD-1 offspring aortas there was a decrease in ER-α, ER-β, eNOS and an increase in AT_1-R protein expression and flutamide treatment increased ER-β, eNOS and decreased AT_1-R protein expression. In male LPD-2 offspring aortas, ER-β was increased with no changes in ER-α and eNOS proteins. Changes in vascular ER-mediated pathway may cause hypertension in female LPD offspring and flutamide treatment reverses this effect.

Key Words: Low-protein diet, estrogen receptors, nitric oxide, angiotensin receptor, flutamide.

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Introduction

Epidemiological observations suggest that low birth weight is associated with an increased risk of developing cardiovascular disease, non-insulin dependent diabetes (1) and hypertension in adulthood (1-6). Studies in rats employing maternal low-protein diet (LPD) restriction results in the offspring developing high blood pressure (BP) and diabetes (7). Several mechanisms including alteration in maternal glucocorticoids (8), deficiency in placental 11β-hydroxy-steroid dehydrogenase activity (8,9), changes in kidney renin-angiotensin system either in newborns or adults (10), deficit in total nephron number (11), upregulation of renin aldosterone axis (11) and inappropriate renal Na+ retention leading to increased extracellular volume (11) may be responsible in developing hypertension in adult offspring. However, none of these studies have shown the involvement of

estrogen receptor (ER) as a possible mechanism for developing hypertension that was induced by *in utero* low-protein in adult offspring.

Recent observations from our laboratory demonstrated that exposure to a LPD throughout pregnancy (LPD-1) reduced feto-placental weights and increased mortality of the pups (12). In addition, these studies demonstrated that both systolic and mean arterial BP were elevated in adult male and female offspring exposed to maternal LPD-1. On the other hand, *in utero* LPD from day 12 of pregnancy (LPD-2) developed BP in adult male, but not in female offspring beginning from five months old. These data suggest that male offspring appeared to have a greater risk of developing hypertension exposed to *in utero* LPD either from day 1 or day 12 of gestation. Interestingly, the circulatory levels of estradiol decreased in female, but not in male offspring (12). Furthermore, flutamide, an androgen receptor antagonist, significantly decreased BP and increased serum estrogen levels in female offspring (12). However, flutamide failed to show an effect in lowering BP in male hypertensive rats (13, 14). These data suggest that altered estrogen levels in circulation may be responsible in developing hypertension in adult female offspring exposed to LPD in utero. However, the underlying mechanisms of estrogen actions remain uncertain in this study.

Estrogens have been shown to lower plasma lipoproteins (15), influence the renin-angiotensin system (16-19), exert anti-oxidative properties (20), and may act as calcium blocking agents (21). In addition, estrogens exert direct effects on the vessel wall, such as an increase of vascular nitric oxide (NO) production and modulation of expression of endothelial NO synthase (eNOS) (22-24). Studies of Nickening (25) demonstrated that estrogen treatment caused angiotensin subtype 1 receptor (AT_1-R) down-regulation through NO-dependent pathway in vascular smooth muscle cells. These studies further suggest that the beneficial effects of estrogens appeared to be mediated by estrogen receptors (ER) (25). Knowing the role of AT_1-R (T2) (18,19) in the pathogenesis of hypertension, these studies suggest that estrogens play an important role in lowering BP through NO-AT1-R pathway through activation of estrogen receptors (ER-α and ER-β).

The objectives of the present study are to investigate whether *in utero* LPD alters: 1) vascular ER-α, ER-β, eNOS and AT_1-R expression in both male and female adult offspring; and whether 2) flutamide treatment reverses this effect in female LPD-1 offspring.

Materials and Methods

Adult, non-pregnant (180g – 220g body weight [bw]) rats were purchased from Harlan Sprague Dawley (Houston, TX) and maintained on a 12L : 12D schedule. Animals received ad libitum supply of rat chow and water. All procedures were approved by the Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas. Virgin female rats were mated, and the day of observation of a vaginal plug with the presence of sperm was designated day 1 of gestation. Timed pregnant rats were fed a normal protein diet (20% casein, control) or a low-protein diet (6% casein) from day 1 (LPD-1) or day 12 (LPD-2) gestation. The dams delivered spontaneously at term and were then immediately switched back to the standard rat chow. The offspring were nursed by their mothers until weaned (21 days old) to regular chow. The isocaloric synthetic low-protein and normal-protein diets were obtained from Harlan Tekland (Madison, WI). The composition of the diets for two groups, except the protein content, was identical as described previously (11). Both diets also contained equal amounts of standard vitamins and minerals.

Effect of maternal LPD in developing hypertension in adult offspring has been demonstrated and published recently from our laboratory [12]. We have reported that male offspring exposed to either LPD-1 or LPD-2 *in utero* develop BP beginning from 2 or 5 month old respectively (12). On the other hand, LPD-1, but not LPD-2, caused BP elevation in female offspring. These studies further suggest that the magnitude of developing BP are greater in LPD-1 adult males compared to LPD-1 female rats. In addition, these studies suggest that flutamide treatment (10 mg/day/4 days, subcutaneous injections) decreases the BP in female hypertensive LPD-1 but not in male hypertensive (LPD-1 or LPD-2) offspring (12). To assess the mechanisms involved in elevated BP due to maternal LPD and the BP lowering effects of flutamide, we have collected thoracic aorta (vascular) tissues from control, female hypertensive LPD-1, male hypertensive LPD-2 offspring and biochemical analysis were performed. Since, the BP values are comparable between LPD-2 males versus LPD-1 females, the current studies were performed in these groups.

All animals were killed in a CO_2 inhalation chamber. Thoracic aortas were removed immediately, quickly frozen in liquid nitrogen and stored at -70°C until used. Tissues were thawed on ice and homogenized in 50mM TRIS buffer (pH 7.4) containing 2 mM EGTA (Ethyleneglycol-bis-[b-aminoethylaster] N, N, N^1, N^1-Tetraacetic acid), 2mM b-mercaptoethanol, 1mM PMSF (phenylmethyl-sulfony fluoride) (Sigma, St. Louis, MO). Complete protease inhibitor tablets were used as per the recommendation of the supplier (protease inhibitor cocktail- Roche, IN). The homogenates was centrifuged at 1000g for 15 minutes to remove tissue chunks and unbroken cells. Protein concentrations were determined by the Pierce BCA protein assay kit according to manufacturer's protocol.

Determination of ER-α, ER-β and AT_1-R and eNOS protein expression in thoracic aortas

Western blotting: Equal amounts of total protein (20μg each) from each preparation are resolved on an 8% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane by electroelution. The membranes were incubated in 5% non-fat milk Tris buffered saline (TBS) – Tween -20 at 22°C for 1 hr and incubated in the antibody solution at 4°C overnight. TBS-Tween -20 contained the following (in mmol/L): 80 Na_2HPO_4, 20 NaH_2PO_4, 100 Nacl, and 0.05% Tween -20. Monoclonal anti-ER-b (1:1000, Upstate Biotechnolgoy, NY), polyclonal anti-ER-a (1:1000, Affinity Bioreagents, CO), monoclonal anti-eNOS antibody (1:1000, Transduction Laboratory) and monoclonal anti-AT_1–R antibody (1:1000, Santa Cruz) were used. Nitrocellulose membranes were washed three times with TTBS (20mM Tris – [hydroxymethyl] aminomethane-HCl [ph 7.6], 0.05% Tween 20, 10mM NaCl), and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:2000, 1:5000 respectively) for 1.5 hrs. The blots were developed using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol. To verify equal loading of sample protein, the immunoblots were stripped in stripping solution (100 mM glycine pH 2-3 for 45 min) and reprobed with monoclonal anti-α-actin antibody (Sigma, 1:5000). Densitometric analysis was performed in the linear range using a Flourchem Analysis System (Alpha Innotech; San Leandro, CA) and the amount of ER, eNOS and AT_1–R were normalized to the α-actin signal.

Statistical Analysis

Results are expressed as the mean ± SEM. Data were analyzed for statistical differences with the Student t-test or one-way ANOVA followed

by the Bonferroni t-test to verify differences between individual groups. Differences were considered to be significant if $p < 0.05$ ($n = 3$-5).

Results

1. Effect of in utero LPD exposure and flutamide on vascular ER protein expression in adult female offspring: Figure 1 illustrates the changes in ER receptor-protein concentrations of vascular homogenates obtained from female offspring exposed to *in utero* either 20% casein (control), 6% casein throughout the pregnancy (LPD-1) or LPD-1 female offspring when adult treated with flutamide for four days prior to tissue collection. Initial studies from our laboratory (12) confirmed that female adult offspring exposed in utero to LPD-1 developed hypertension and decreased circulatory estrogen levels. Furthermore, flutamide treatment reversed both hypertension and decreased estrogen levels in circulation (12). As shown in Figures 1A and 1B, prominent bands at 60 and 55 kDa, corresponding to ER-α and ER-β protein were obtained from vascular tissues respectively. Densitometric analysis of both ER-á and ER-â protein followed by normalizing with â-actin signal from each group showed that both ER-a and ER-b protein expression are decreased significantly ($p < 0.05$) in LPD-1 rats compared to control group. Flutamide treatment restored the decreased levels of ER-β (Figure 1B), but not ER-α in LPD-1 females.

2. Effect of in utero LPD exposure on vascular ER protein expression in adult male offspring: Results from our laboratory demonstrated [12] that rats with low-protein diet either from day1 (LPD-1) or day 12 (LPD-2) of pregnancy caused an increase in BP in male adult offspring. In contrast to female offspring,

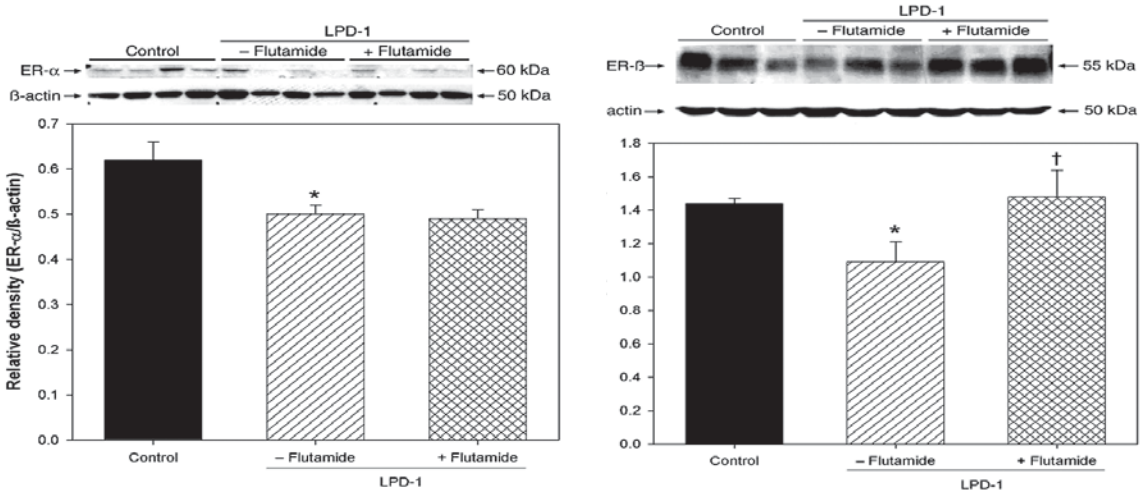

Fig. 1. Expression of protein for estrogen receptors (ER) in thoracic aortas in female rat offspring exposed to intrauterine LPD during pregnancy. The LPD was given *in utero* from day 1 (LPD-1) of pregnancy until delivery. The control group received 20% protein. Aortic tissue homogenates were prepared for Western blot analysis. **A (top):** Representative Immunoblot of rat thoracic aortas showing the expression of ER-α (60 kDa) and β-actin (50 kDa) from control, LPD-1, and LPD-1 + Flutamide (10mg/day/4 days; subcutaneous injections). **A (bottom):** Densitometric analysis was performed and the ratio of ER-α to β-actin are calculated. **B (top):** Representative immunoblot analysis of ER-β and β-actin from four animals per group. **B (bottom):** Densitometric analysis are performed and the ratio of ER-β (55 kDa) to β-actin are calculated. The bars represent the mean ± SEM. Significant differences between control versus LPD are noted. * $p < .05$ for LPD-1 compared to control group; † $p < .05$ for LPD-1 + flutamide compared to LPD-1 group.

flutamide did not show an effect in attenuating hypertension in both LPD-1 and LPD-2 male offspring. In this study, we investigated whether *in utero* LPD during second half of pregnancy (LPD-2) alters the vascular ER protein expression in male offspring. As shown in Figure 2A, the protein expression for ER-a were unchanged in LPD-2 male offspring compared to control group. However, significant ($p < .05$) increases in vascular ER-a protein expression was observed (Figure 2B) in LPD-2 male rats.

3. Effect of in utero LPD and flutamide on vascular eNOS protein expression in adult offspring: Because ER's expression is decreased in adult female LPD-1 offspring and restored by flutamide treatment in aortic homogenates, we investigated whether eNOS protein expression was also altered in vascular tissues. As shown in Figures 3A and 3B, a single band of eNOS protein was obtained with a predicted size of 140 kDa from both female LPD-1 (3A) and male LPD-2 (3B) rat offspring. Densitometric analysis of the eNOS protein from aorta homogenates in each group showed that this protein in LPD-1 adult female rats were substantially decreased and this effect was significantly ($p < 0.05$) attenuated by flutamide treatment (Figure 3A). However, no change in the vascular eNOS protein expression in adult male LPD-2 offspring was noticed (Figure 3B).

4. Effect of in utero LPD on vascular AT_1-R protein expression in female and male adult offspring: Next, we assessed whether AT_1-R protein expression was altered in LPD-1 adult females and if so, does flutamide treatment reverses this effect. We also examined whether the protein expression for AT_1-R is altered in male LPD-2 rat offspring. Total protein was isolated from aortic homogenates, and Western analysis was used to quantify AT_1-R protein. As shown in Figure 4A and 4B, a protein band was obtained with a predicted size of 50 kDa from vascular homogenates. Densitometric analysis revealed

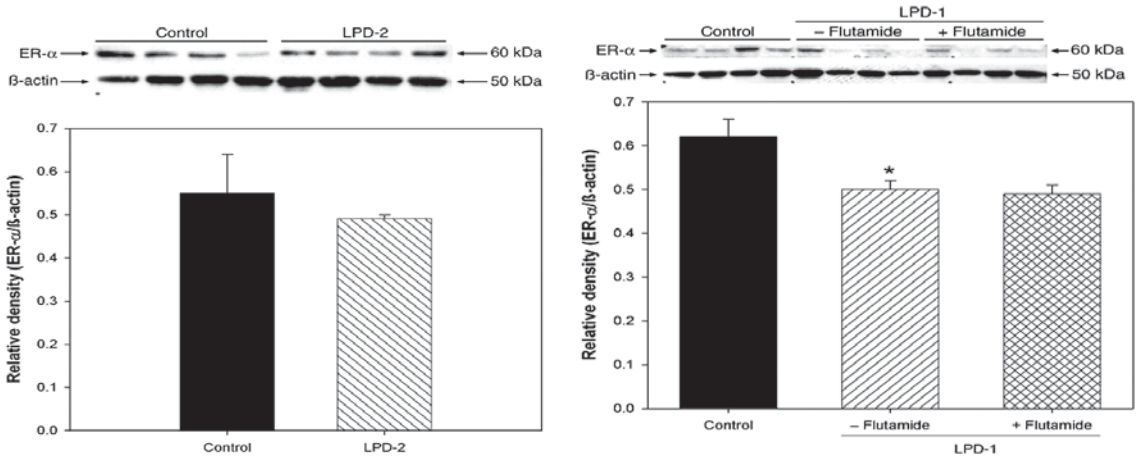

Fig. 2. Expression of protein for estrogen receptors (ER) in thoracic aortas in male rat offspring exposed to intrauterine LPD during pregnancy. The LPD was given *in utero* from day 12 (LPD-2) of pregnancy until delivery. The control group received 20% protein. Aortic tissues homogenates were prepared for Western blot analysis. **A (top):** Representative immunoblot of rat thoracic aortas showing the expression of ER-α (60 kDa) and β-actin (50 kDa) from control and LPD-2. **A (bottom):** Densitometric analysis were performed and the ratio of ER-α to β-actin are calculated. **B (top):** Representative immunoblot analysis of ER-β and β-actin from five animals per group. **B (bottom):** Densitometric analysis was performed and the ratio of ER-β (55 kDa) to β-actin are calculated. Values are mean ± SEM. * $p < .05$ compared with control group.

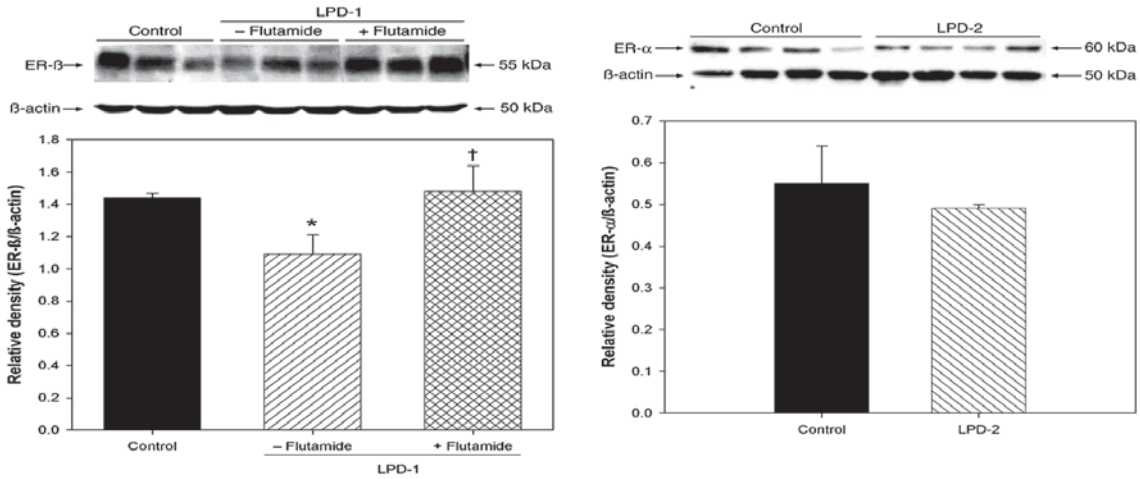

Fig. 3. Expression of protein for endothelial nitric oxide synthase (eNOS) in thoracic aortas in rat offspring exposed to intrauterine LPD during pregnancy. The LPD was given *in utero* either from day 1 (A; LPD-1) or day 12 (B; LPD-2) of pregnancy until delivery. The control group received 20% protein. **A (top):** Representative immunoblot of female rat thoracic aortas showing the expression of eNOS (140 kDa) and β-actin (50 kDa) from control, LPD-1 and LPD-1 + flutamide (10mg/day/4 days; subcutaneous injections). **A (bottom):** Densitometric analysis were performed and the ratio of eNOS to β-actin are calculated. **B (top):** Representative immunoblot analysis of eNOS and β-actin from male rat offspring. **B (bottom):** Densitometric analysis was performed and the ratio of eNOS to β-actin are calculated. The bars represent the mean ± SEM. Significant differences between control versus LPD offspring are noted. $p < 0.05$ for LPD-1 compared to control group; † $p < .05$ for LPD-1 + flutamide compared to LPD-1 group.

that the expression of AT_1-R receptor protein was significantly ($p < .05$) elevated in adult female LPD-1 offspring and this was attenuated by the flutamide treatment (Figure 4A). Similarly, a significant ($p < 0.05$) increase in AT_1-R protein expression in vascular homogenates were observed in male LPD-2 rat offspring (Figure 4B).

Discussion

In the present study, for the first time, we demonstrated that *in utero* LPD throughout pregnancy (LPD-1) caused a decrease in the protein expression of ER-α, ER-β, eNOS and an increase in AT_1-R concentrations in adult female offspring thoracic aortas. Flutamide treatment significantly ($p < .05$) attenuated the altered protein expression for ER-β, eNOS and AT_1-R in vascular homogenates in female adult LPD-1 group. We also observed that an increase in vascular protein expression of ER-β and AT_1-R in adult males exposed to *in utero* LPD during second half of pregnancy (LPD-2). Results from our laboratory demonstrated that *in utero* LPD elevated BP in both male and female offspring (12). Moreover, theses studies demonstrated that flutamide treatment lowers BP in female but not in male hypertensive offspring. The current study, together with our previous findings (12) suggest that in female offspring, decreases in circulatory estrogens (E_2) and vascular ER may down-regulate eNOS and perhaps elevate AT_1-R protein expression and this may provide a plausible mechanisms for developing hypertension reported in these animals. Flutamide treatment may increase the circulatory E_2 levels (12), as well as, ER protein concentrations in vasculature leading to an elevation of eNOS and down-regulation of AT_1-R protein expression and therefore, restoring normal BP. However, in adult male LPD-2 offspring, an increase in vascular ER-â and/or

AT_1-R protein expression may be involved in elevated BP.

Estrogens are known to exert beneficial effects on the vascular wall. Results from some clinical observations suggest that estrogen therapy (ET) lowers BP in hypertensive post-menopausal women (19, 26-28). The hypotensive effects of ET has been reported in spontaneous hypertensive rats (SHR) (29), high-fat diet induced females (30), rats with deoxycorticosterone salt-induced hypertension and non-pregnant ovariectomized rats with [31]and without psychological stress (32). Studies from our laboratory demonstrated that in hypertensive female LPD-1 offspring, circulatory E_2 but not testosterone levels are significantly decreased (12). For the first time, in this study, we have shown that both ER-a and ER-b protein concentrations in thoracic aortas are significantly ($p < .05$) lower in LPD-1 adult females (Figure 1A and 1B). We suggest that *in utero* LPD-1 during pregnancy may decrease serum E_2 levels and ER concentrations in the vasculature, thereby leading to the development of hypertension in female offspring**.** Further studies are warranted to address the direct effects of ER-α and ER-β agonists or antagonists on BP regulation in female *in utero* LPD offspring.

Recently, we have reported that flutamide lowers BP and as well as elevated serum E_2 levels in females but not in male adult offspring exposed to *in utero* LPD-1 (12). In the present study, we found that flutamide treatment in females restored the decreased ER-α but not ER-β protein concentrations in the vascular (Figure 1A and 1B) tissues. The hypotensive effects of flutamide has been demonstrated in spontaneous male hypertensive rats, male rats lacking a functional androgen receptor (testicular feminization, TFM) (13) suggesting the androgen dependent and – independent effects of this agent in regulating BP. Previous studies demonstrated the presence of aromatase (estrogen synthase), estradiol-17 β-hydroxysteroid dehydrogenase and 17-Ketoreductase enzyme activities in rat and human vascular smooth muscle cells [33]. It is possible that flutamide treatment may stimulate aromatase enzyme complex in both vascular and renal tissues, increases estrogen synthesis and its receptors, therefore lowering BP in LPD-1 adult female offspring. Additional work will be required to better define the underlying mechanism(s) involved on the action of flutamide on ER regulation.

In the present study, we observed that the protein expression of vascular eNOS is reduced in LPD-1 female but not in LPD-2 male offspring, and this was restored by flutamide treatment (Figure 3A and 3B). Previous studies have demonstrated the dysfunction of endothelium in the adult rats received low-protein diet in utero (28). However, the mechanism(s) responsible for this disorder is not well documented. It is well established that E_2 is known to improve the vascular dysfunction through up-regulation of endothelial cell genes, such as eNOS [34-36]. Furthermore, E_2 has rapid non-genomic effects on the vascular endothelium, including activation of nitric oxide (NO) synthesis (22-24). Positive cardiovascular effects, and especially the increase in NO production by E_2, have been reported to involve ER-α as well as ER-β (27, 35, 37). In addition, it has been demonstrated that selective ER-α agonist Cpd 1471, significantly improved the endothelial dysfunction in ovariectomized SHR rats. Taken together, these data suggest that decreases in both ER's perhaps may down-regulate eNOS protein expression in vasculature, therefore increases BP in adult female offspring. Iliescu et al [38]studies postulated that flutamide dose-dependently relaxes aorta via NO-cyclic guanosine monophosphate (cGMP) pathway in both male and female Sprague Dawley rats lacking a functional androgen receptor. Collectively, the above data indicate that the up-regulation of ER-β expression by flutamide

treatment may enhance the eNOS protein expression in the vasculature, therefore lower the BP in hypertensive adult female offspring.

In the present study, we further show that vascular AT_1-R expression was significantly ($p < .05$) elevated in adult female LPD-1 (Figure 4A). Furthermore, flutamide treatment decreased the elevated levels of AT_1-R protein concentration in this blood vessel obtained from adult female LPD-1 offspring (Figure 4A). The AT_1-R mediates many biological functions of renin-angiotensin system including vasoconstriction (39). Previous studies demonstrated that E_2 deficiency has been shown to increase AT_1-R expression, as well as the efficacy of angiotensins on vasoconstriction, whereas E_2 replacement therapy in ovariectomized rats reversed the overexpression of AT_1-R concentration (39-40). Moreover, studies of Nickenig et al (25) using aortic vascular smooth muscle cells demonstrated that E_2 caused down-regulation of AT_1-R mRNA expression through NO-dependent pathway and this effect was mediated through activation of ER's. These studies together with our current findings suggest that a deficiency in circulatory E_2 and vascular ER may down-regulate the eNOS and elevate AT_1-R protein expression and therefore increase the BP in female LPD-1 offspring. These studies further suggest that flutamide treatment reduces the elevated BP by altering E_2-dependent pathway in LPD-1 adult female offspring.

In LPD-2 adult male offspring, the vascular protein expression for ER-β but not ER-α appeared to be elevated (Figure 2A and 2B). Furthermore, no changes in vascular eNOS protein expression were noticed in this setting (Figure 3B). However, a significant increase in vascular AT_1-R protein expression was observed in adult male LPD-2 offspring (Figure 4B). At

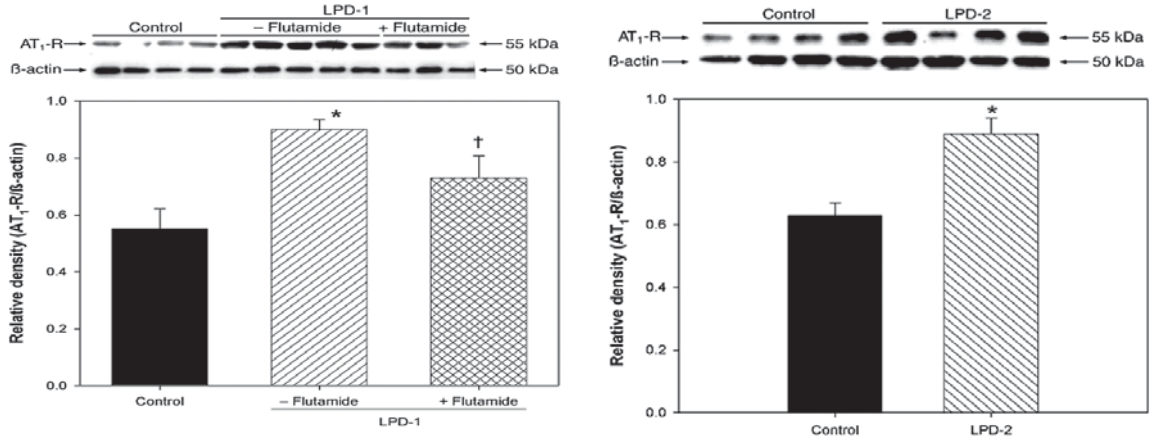

Fig. 4. Expression of protein for subtype 1 angiotensin 11-receptor 1 (AT_1-R) in thoracic aortas in rat offspring exposed to intrauterine LPD during pregnancy. The LPD was given *in utero* either from day 1 (A; LPD-1) or day 12 (B; LPD-2) of pregnancy until delivery. The control group received 20% protein. **A (top):** Representative immunoblot analysis of female rat thoracic aortas showing the expression of AT_1-R (55 kDa) and β-actin (50 kDa) from control, LPD-1 and LPD-1 + flutamide (10mg/day/4 days; subcutaneous injections). **A (bottom):** Densitometric analysis was performed and the ratio of AT_1-R to β-actin are calculated. **B (top):** Representative Immunoblot analysis of AT_1-R and β-actin from male rat offspring. **B (bottom):** Densitometric analysis was performed and the ratio of AT_1-R to β-actin are calculated. The bars represent mean ± SEM. Significant differences from control versus LPD offspring are noted. * $p < .05$ for LPD-1 compared to control; † $p < .05$ for LPD-1 + flutamide compared to LPD-1.

the present time, it is uncertain whether increases in AT_1-R protein expression in LPD-2 male adult offspring are due to alterations occurred in ER-β or independent of E_2 receptors. We speculate that in LPD-2 males, increased levels of vascular ER-β protein expression may play a compensatory vasodilator role to attenuate the elevated BP. We also speculate that AT_1-R but not NO pathway may be involved in elevating BP in LPD-2 adult male offspring. Further studies are warranted to address the direct effects of ER's in BP regulation using agonists and/or antagonist of these receptors in *in utero* programmed adult hypertension.

In summary, these results suggest that maternal LPD down-regulates E_2 levels in circulation and ER's in vasculature in female offspring. Alterations in ER expression may down-regulate eNOS and up-regulate AT_1-R concentrations and therefore develop hypertension in adult female LPD-1 offspring. Flutamide treatment may restore serum E_2, as well as vascular ER, which may lead to increased expression of eNOS and down-regulation of AT_1-R protein expression and lower the BP in female LPD-1 animals. The increased expression of ER-β and AT_1-R protein in male LPD-2 vasculature suggests that involvement of these receptors in the elevated BP. Therefore, we conclude the E_2 and ER-mediated pathway may be responsible for the BP-lowering effects of flutamide in female hypertensive offspring exposed to *in utero* LPD during pregnancy. Future investigations are warranted to address the effects of maternal LPD and flutamide on ER signaling pathway in adult female aortic vasculature.

Acknowledgement

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Inhibitory action of hydroxytyrosol from glucose-induced Insulin deficient and pancreas and liver toxicity *in vitro*

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Abstract

The present study aims to investigate the effect of hydroxytyrosol in insulin secretion and the antioxidant activity in liver slices in vitro. For this, pancreas and liver slices were incubated in presence of 1g/l or 4g/l glucose (± hydroxytyrosol (HT)) during 40 minutes. We interest to evaluate the action of HT in insulin secretion, antioxidant enzymes activities (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)), reduced glutathione (GSH), lipid peroxidation, lactate dehydrogenase (LDH) and histological changes in pancreas and liver slices. For the first time, our results show that hydroxytyrosol significantly induces insulin secretion in pancreas incubation. Besides, the present work prove that HT has a good antioxidant activity by preventing the decrease of superoxide dismutase (SOD), catalase (CAT) and gluthation peroxidase (GPX) activities and the reducing glutathione content (GSH) in hepatic slices incubated in high glucose concentration (HG) (4g/l). En parallel, a significant decrease in lipid peroxidation rate and lactate dehydrogenase (LDH) activity are observed after hydroxytyrosol administration in liver slices. Theses beneficial actions of HT are confirmed by histological changes in hepatic and pancreatic tissues. Conclusion**:** HT supplementation in diabetic can induces insulin secretion and prevents glucose toxicity in pancreas and liver.

Keywords: Hydroxytyrosol, Pancreas, Glucose toxicity, Insulin, Antioxidant, Liver slices.

Introduction

Diabetes mellitus (DM) is a major cause of disability and hospitalization that presents a significant burden on societies worldwide (1,2). Higher glucose level in vitro as well in vivo is the principal sources of reactive oxygen species (ROS) which are play a key role in the apparition of many diseases and disorders (3,4). The generation of ROS has been implicated in the pathogenesis of several forms of acute cell injury, where the oxidative stress process plays a central role (5,6). Oxidative stress is now recognized to be associated with more than 200 diseases (7). Dietary intake of foods rich in antioxidants, as phenolic compounds, is associated with the prevention of cardiovascular disease and reduces risk of liver dysfunction (5) and neuroprotective and cardioprotective actions (8-10). There is a continuing interest to define the preventive effects of phenols against reactive oxygen species mediated degenerative diseases. Phenolic compounds are important bioactive bio-molecules that are of increasingly interest for their ability to exert antioxidant actions. A significant number of reports exist in the literature indicating that hydroxytyrosol, a natural exist in olive oil, exerts antioxidative actions which are effective in preventing or reducing the deleterious effects of oxygen-derived free radicals associated with

many diseases (11, 12). In fact hydroxytyrosol has various biological activities, such as, preventing human erythrocytes from oxidative damage induced by hydrogen peroxide in rats (13), and human (14), inhibiting LDL oxidation in vitro, and anti-inflammatory (15-17).

Materials and Methods

Chromatographic purification of hydroxytyrosol

Fresh olive mill wastewaters (OMW) were supplied by discontinuous three-phase olive processing mill from a cooperative in Sfax (Tunisia). This sample was generated from *Chemlali* olive variety. Hydroxytyrosol was purified from OMW as described previously (18). Briefly, the polyphenolic fraction was extracted from OMW using ethyl acetate as solvent. The organic extract was evaporated under reduced pressure below 45 °C. an aliquot (1 g) of the obtained residue was chromatographed on a C-18 silica gel (liChroprep RP-18; 25–40 µm) column (2.5 x 70 cm) under medium pressure. Phenolic compound elution was carried out with a mixture of water/acetonitrile (8:2, V:V) . The flow rate was adjusted to 0.3 ml/min and 4.5 ml fractions were collected. These fractions were measured by optical density at 280 nm and the chromatogram (optical density versus fraction number) was represented (data not shown). The first separated peak corresponds to pure hydroxytyrosol.

Preparation of Liver Slices

Male adult Wister rat, weighing 280–200 g was taken and dissected after cervical dislocation. The liver and pancreas were removed, transferred and then incubated in KRB buffer (pH 7.4): 118 mM NaCl, 4 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 1.25 g/l BSA and 40 g/l dextran T70). The perfusate was continuously gassed with a mixture of O2:CO2 (95:5). Liver was sliced into small pieces (4–6 mg) of about 0.5∞ 0.5∞0.5 mm using a prep blade. Slices were divided into small portions (20–22 slices) of 100–120 mg wet weight. 1 g liver slices and 1 g pancreas were incubated for 40 min in 5 ml KRB medium at 37 °C equilibrated with 95% O_2 + 5%CO_2 gas.

Experiment Design

1g liver slices and 1g pancreas were divided in to three sets: set 1: control, tissues incubated in KRB medium a final concentration of glucose 1g/l; set 2: tissues incubated in KRB medium a final concentration of glucose 4g/l; set 3: slices incubated in KRB medium a final concentration of glucose 4g/l + 50 µg/ml Hydroxytyrosol. Triplicate cultures were set up for each concentration to minimize the errors. Effluent was fractionally collected every 10 minute, and its insulin concentration was measured by a radioimmunoassay kit for Bi-insulin RIA Diagnostic, Pasteur, Paris, France.

After the end of incubation, liver slices were homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.4) and centrifuged at 4,000∞g for 15 min at 4 °C. Liver marker enzyme lactate dehydrogenase (LDH) and antioxidant enzymes SOD, CAT, and glutathione peroxidase (GPx) were estimated in the supernatant. Lipid peroxidation and glutathione (GSH) contents were also measured. For histological studies, pieces of pancreas and liver were fixed in a Bouin Hollande solution for 24 hours, and then embedded in paraffin. Sections of 5µm thickness were stained with hematoxylin-eosin and examined under the Olympus CX41 light microscope.

Biochemical assays

Lipid peroxidation was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) using the methods of Buege and Aust (19). In brief, 0.1 ml of liver slices was treated with 2 ml of TBA–

trichloroacetic acid–HCl reagent (0.37%TBA, 0.25MHCl and 15%TCA, 1:1:1 ratio), placed for 15 min in a water bath and then cooled and centrifuged at 3500 ∞g for 10 min at room temperature. The absorbance of supernatant was measured at 535 nm against a reference blank. Values were expressed as mM/mg protein. Catalase (CAT) was estimated by the method of Aebi (20). The reaction mixture contained 1.0 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml sperm medium and 0.4 ml of 50M H2O2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). Then the absorbance was readden at 620 nm; CAT activity was expressed as µM of H2O2 consumed/min/mg protein. SOD was assayed according to the technique of Marklund and Marklund (21) based on the inhibition of the formation of nicotinamide adenine dinucleotide, phenazine methosulfate, and amino blue tetrazolium formazan. A single unit of enzyme was expressed as 50% inhibition of nitroblue tetrazolium reduction per min/mg protein. GPx activity was measured by the method described by Pagila and Valentine (22). Briefly, the reaction mixture contained 0.2 mL of 0.4 M phosphate buffer (pH 7.0), 0.1 mL of 10 mM sodium azide, 0.2 mL of sperm medium (supernatant; homogenized in 0.4 M phosphatebuffer, pH 7.0), 0.2 mL of GSH, and 0.1 mL of 0.2 mM hydrogen peroxide. The contents were incubated at 37°C for 10 min. The reaction was stopped by adding 0.4 mL of 10% TCA and centrifuged. The GSH content was estimated in the liver slices by the method of Ellman (23). Liver pieces were lysed with 0.1% EDTA solution and precipitating reagent which contains 0.16% metaphosphoric acid, 0.02% EDTA and 3% NaCl. After mixing, the solution was allowed to stand for 5 min before being filtered. Two milliliter of filtrate was added to 4 ml of disodium hydrogen phosphate (0.1 M, pH 8.0) and 1 ml of DTNB reagent. A blank was prepared from 1.2 ml of precipitating reagent, 0.8 ml of EDTA solution, 4 ml of disodium hydrogen phosphate and 1 ml of DTNB reagent. The color was immediately readden at 412 nm with the help of spectrophotometer. The amount of proteins was determined by the method of Lowry *et al* (24) using bovine serum albumin as the standard at 660 nm. The activity of lactate dehydrogenase (LDH) and Glycogen content were assayed using commercial kits from Biomaghreb, Tunis, Tunisia. For histological studies, pieces of pancreas and liver were fixed in a bouine's solution for 24 hrs and then embedded in a paraffin section of 5µm thickness were stained with hematoxylen, eosin and examined under olumpus CX41 light Microscope.

Statistical analysis

Data are presented as means ± SD. The determinations were performed from 6 animals per group and the differences were examined by the one-way analysis of variance (ANOVA) followed by the Fisher test (Stat View) and the significance was accepted at $p<0.05$.

Results

Antioxidant activity *in vitro*

In vitro, the antioxidant activity of HT was evaluated by its ability to scavenge DPPH free radicals. HT showed a scavenging activity with a percentage decrease, versus the absorbance of DPPH standard solution of 96% at a concentration of 40 µg/ml (Fig. 1).

Hydroxytyrosol, insulin and pancreas *in vitro*

Fig. 2 shows that higher glucose concentration causes a decline in insulin secretion after 40 min of pancreas incubation. However, in pancreas incubated at same time with HG concentration and hydroxytyrosol, we investigated in the first time a significant increase in the insulin secretion.

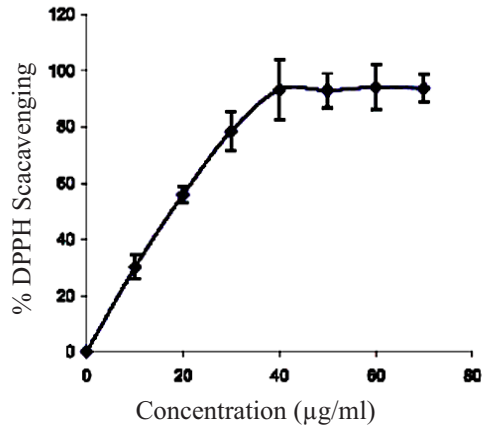

Fig. 1. A free-radical activity of hydroxytyrosol is measured using the DPPH assay. N=3

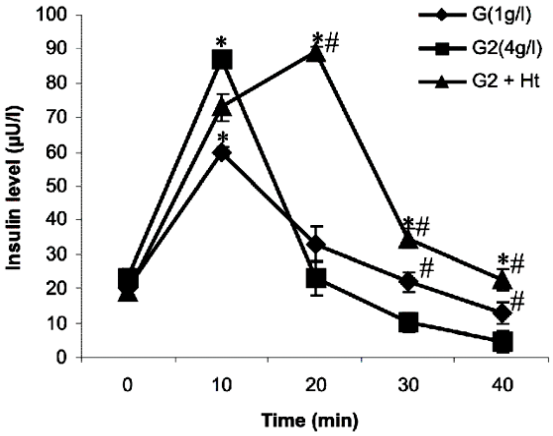

Fig. 2. Influence of hydroxytyrosol (HT) on the time course of glucose-induced insulin decline. Data are mean ± SD (n = 3). *P < 0.05 vs 1g/l glucose #P < 0.05 vs 4g/l glucose

Hydroxytyrosol, antioxidant enzymes and liver *in vitro*

Fig. 3 shows that HT exert a good antioxidant activity. In fact, this study shows a decrease in the SOD, CAT and GPX activities incubated in higher glucose level (4g/l). Moreover, glucose reduces the GSH contents in hepatic slices. However, in slices liver co-incubated with glucose (4g/l) and HT (50 µg/ml), a good antioxidant action is observed. Hydroxytyrosol in slices liver increase significantly the SOD, CAT and GPX activities and GSH level (Fig3).

Hydroxytyrosol, lipid peroxidation level and lactate deshydrogenase activity and liver *in vitro*

The effects of HT on the LDH activity and lipid peroxidation level in liver slices homogenate are presented in Fig. 4. LDH and TBARs contents were significantly increased in the liver slice treated with glucose (4g/l) compared to control (1g/l).However, HT administration to incubation medium decrease significantly the two indices contents in liver slices.

Hydroxytyrosol, histological changes and pancreas

Fig.5 reveals the toxicity of glucose at (4g/l) in incubated pancreas. β cells of Pancreas incubated with glucose (4g/l) during 40 min show damage evidences by intense DNA stained indicator of apoptosis. However, the incubation of pancreas at same time with HT and Glucose, a clearly protective action was observed.

Discussion

Higher glucose concentration in vivo as in vitro favourites the formation of advanced glycation endproducts (AGEs) (25). The chemical modification of sugars (glycation of proteins) in liver slices causes alteration in the structure and function of tissue proteins such as SOD, CAT, GPX and GSH leads to decrease in the antioxidant capacity. Moreover, the increase of glucose level in culture medium favourites glucose auto-oxidation reaction and this induces free radical generation en agreement with others works (26) lead to increase in LDH and TBARS rates. Both the disturbance in the antioxidant capacity and the increase in free radicals by glucose auto-oxidation are associated with excessive concentrations of reactive oxygen species (ROS) which attack many tissues, particularly pancreas; tissue characterised by low

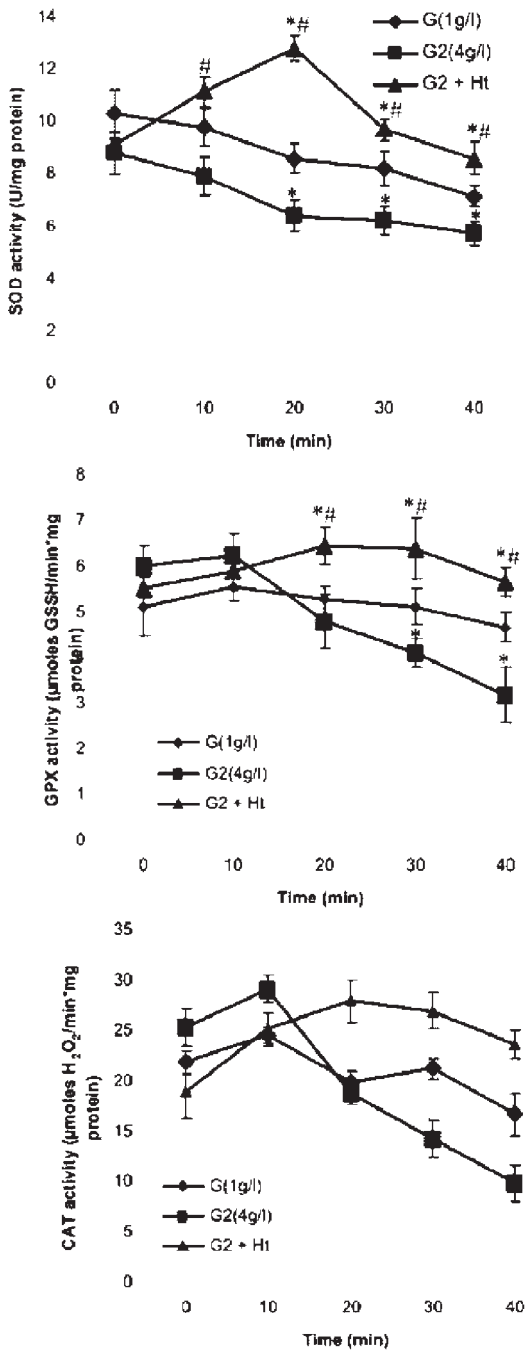

Fig. 3. Effect of hydroxytyrosol and glucose (1g/l or 4g/l) on SOD, CAT and GPX activities in liver slices in vitro. Statistical analysis as figure1.

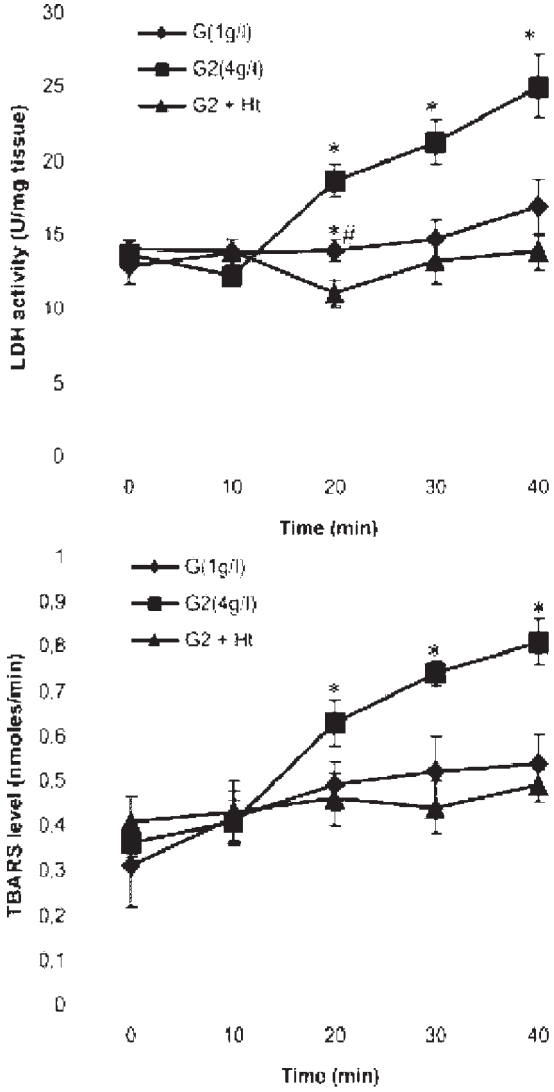

Fig. 4. Time course of thiobarbituric acid reactive substances (TBARS) level and LDH activity in liver slices incubated in medium contain glucose (1g/l); glucose (4g/l) or at same time glucose (4g/l) + hydroxytyrosol (50µg/ml). Statistical analysis as fig. 1.

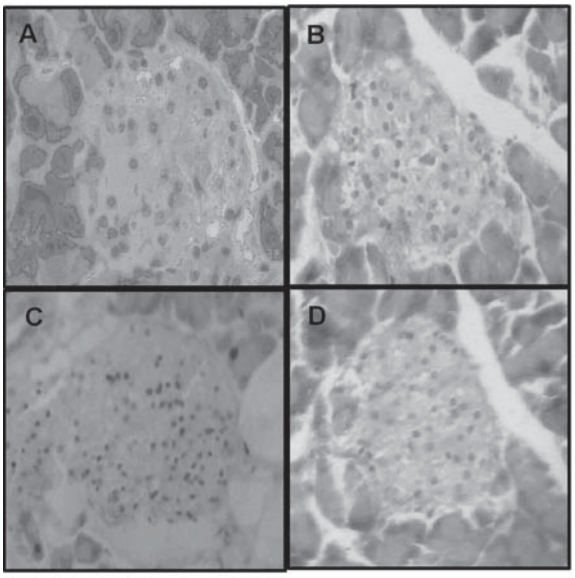

Fig. 5. Effect of glucose (1g/l or 4 g/l) and hydroxytyrosol on the histological changes of incubated pancreas by HE staining (100X).
A: at the beginning of the incubation (Time = 0 min). Normal control rats showed normal α-cells. **B**: pancreas incubated in 1g/l glucose; at the end of the incubation, no histological changes showed. **C**: However, in pancreas incubated with 4g/l glucose, damage like apoptosis appeared; in shows an intense DNA stained indicator of apoptosis. **D**: in pancreas, incubated at same time, with glucose (4g/l) and hydroxytyrosol (50 µg/ml) a clearly protective action of pancreatic cells was observed and the cells appear similar to these incubated in glucose (1g/l)

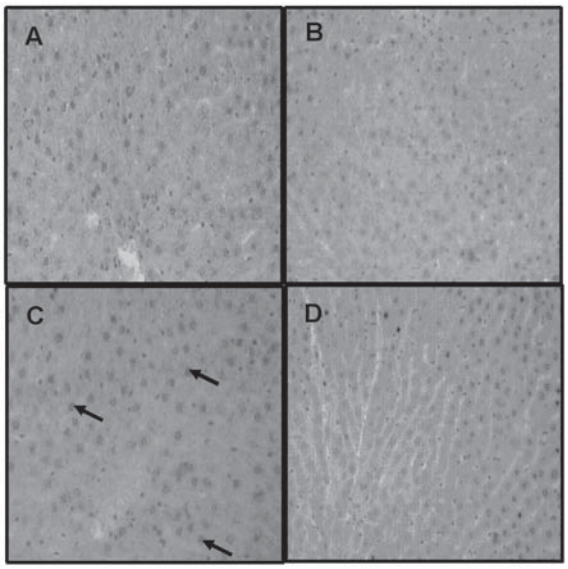

Fig. 6 The effect of glucose and hydroxytyrosol in liver slices. Section of liver slices before the beginning of treatment (A) or after the end of the incubation with (1g/l) glucose (B) and with (4g/l) glucose + 50µg/ml hydroxytyrosol (D) showing a normal histological appearance. However, in liver slices incubated only with (4g/l) glucose showing classic inflammation appearance (C).

content in antioxidant enzymes. This enhancement of ROS production by HG leads to damage, apoptosis and death in pancreatic βcells followed by a decrease in insulin secretion (Fig. 1).

However, the administration of hydroxytyrosol increases insulin secretion in the non-diabetic rat pancreas at dose 50µg/ml. The enhancing of insulin secretion by HT is explained by many mechanisms: i) âcells incubated in HG show a condense nucleus sign of cells damage and death in agreement with Chai *et al* (27) conversely, the cells incubated at the same time with HG and hydroxytyrosol appear normal and similar to cells incubated with 1g/l glucose. This result illustrates the good role of HT in the protection of pancreatic cells from glucose induces damage and death (Fig. 4); ii) like *others herbs* extract, HT can enhance insulin secretion by its insulinotropic effects: HT inhibits KATP channels and increases the voltage-dependent calcium channel which plays a key role in insulin secretion (28) iii) the three hydroxyl groups of HT inhibited some intestinal enzymes such as aldose reductase and disaccharidases and this lowered glucose absorption in intestine (29). Besides, in this study, an antioxidant effect of HT was observed in hepatic slices culture. In fact a protective effect of HT against glycation of protein and/or auoxidation of glucose is evidenced by significant increase of SOD, CAT and GPX activities in liver slices after HT supplementation. These results are in agreement with several studies (30-32) which have

demonstrated that HT possesses a clear antioxidant property in vitro as in human. HT plays the role of chain breakers or radical scavengers depending on its chemical structure. In fact the antioxidant activity of HT depends on the position of three hydroxyl groups in the molecule. These groups make it of a potential scavenging free radicals activity. This is confirmed by our observation that HT neutralised the DPPH free radicals a lower dose. Moreover, our results show that HT counteracted the oxidative modifications by its capacity of decreasing the TBARs level in hepatic cells in agreement with others (30, 33).

The present study presents interesting results from the time-course determination of GSH and Glucose concentration. In hepatic slices treated only with higher glucose concentration, GSH concentration decreases probably due to both increase of ROS production and the contemporary inactivation of GSH-related enzymes, especially GPX. However, HT administration to slices medium protects from GSH level decrease and this is probably the result of the protection of GPX activity, enzyme able to preserve the functionality of GSH cycle after 40 min. The preservation of GPX activity consequently maintains of the normal content of GSH. Theses results are in accordance with many literature data indicating the potent 'in vitro' antioxidant activity of hydroxytyrosol (34,35). This antioxidant activity of HT protects hepatic cells from death and damage by lower contents in LDH, TBARs level and inhibits histological changes compared to liver slices treated with only HG.

In conclusion, this study demonstrated the beneficial effect of HT as an effective hypoglycemic and antioxidant agent in alleviating oxidative stress and free radicals as well as in enhancing insulin secretion and both enzymatic and nonenzymatic defenses diabetes.

Acknowledgements

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Influence of Environmental Factors on siderophore production by *Streptomyces fulvissimus* ATCC 27431

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Abstract

Streptomyces fulvissimus ATCC 27431 was studied for siderophore production. Biosynthesis of siderophore was found to be influenced by environmental factors. Out of various media tried, chemically defined low iron medium gave maximum siderophore production (94 % siderophore units). A number of carbon and nitrogen compounds were tested for their effect on growth of *Streptomyces fulvissimus* and siderophore production. Glycerol, sucrose and glucose were good carbon sources while ammonium chloride, sodium nitrate and urea were good nitrogen sources. Moreover, 0.05 - 0.3 M of Carbon, 0.05 - 0.1 M of Nitrogen sources and 20 - 30 mM of phosphate was required for maximum siderophore production (92 % siderophore units). Increase in Fe (III) concentration > 5 μM had a negative effect on siderophore production. Optimum pH for siderophore production was 8 while temperatures below 20 ºC and above 40 ºC were not suitable for culture growth and siderophore production. To the best of our knowledge this is the first report on effects of environmental factors on siderophore production by *S. fulvissimus*.

Key Words: Siderophore, *Streptomyces fulvissimus*, Carbon source, pH.

Introduction

Most of the living organisms require iron for metabolic activities and growth. Aerobic metabolism of microbes requires iron for variety of functions including the electron transport chain, in deoxyribonucleotide synthesis, in the synthesis of heme and for incorporation in the proteins involved in nitrogen fixation (1, 2). Iron is the fourth most abundant element in the earth's crust (3). Although iron is abundant in the environment, it is not readily available since it exists mostly in ferric Fe (III) state. Reduced form iron Fe (II) is soluble and biologically available that can be easily taken into the living cell using various mechanisms under aerobic conditions, ferrous is oxidized to ferric form existing as insoluble oxyhydroxide polymers (4) and hence such complexes are not easily assimilated by microorganisms. Acquisition of iron thus becomes a challenge. To solve this bioavailability problem, microorganisms synthesize and excrete selective and strong Fe (III) binding low molecular weight molecules known as siderophores (3).

Streptomyces, Gram-positive soil bacteria are well known for contributing majority of antibiotics. Apart from this typical character, the only commercially available siderophore,

Desferrioxamine B, called Desferal is produced by *Streptomyces pilosus* which is used to treat iron overload in humans (5). *Streptomyces pilosus* produces a group of desferrioxamines (A1, A2, B, D1, D2, E, F, G, H) wherein B and E are predominantly present (6), *S. coelicolor* A 3(2) is known to produce coelichelin (7), coelibactin, DFO G1 and DFO E (8). *S. antibioticus* produces desferrithiocin (9) and *S. griseoflavus* produces ferrimycins (6). Shimi and Dewedar (10) reported production of gluconimycin by *Streptomyces* A S 9. Enterobactin is produced by *S. tendae* Tu 901/8c (11) and oxachelin by *Streptomyces* sp.GW9/1258 (12).

Streptomyces fulvissimus is known for production of an antibiotic, valinomycin (13), but siderophore production by *Streptomyces fulvissimus* has not been studied earlier. Attributes like production of spores, antibiotic and siderophore may be helpful in exploitation of this organism in plant nutrition and biocontrol of plant diseases. But as the biosynthesis and secretion of siderophores is strictly regulated by environmental factors (14), the aim of this study was to find out the suitable parameters for maximum siderophore production.

Materials and Methods

Microorganism and growth conditions

Streptomyces fulvissimus was used for this study which was maintained by frequent transfer on medium composed of (gl^{-1}): Yeast extract 4, malt extract 4, and glucose 10, agar 20, and pH 7 $\pm$ 0.2. For siderophore production, Chemically Defined Low Iron Medium (CDLIM) consisting of (gl^{-1}): K_2SO_4 2, K_2HPO_4 3, NaCl 1, and NH_4Cl 5 was used. To this solution, 2 mg thiamine and the following elements were added (mgl^{-1}): $CaCl_2.2H_2O$ 100, $MgSO_4.7H_2O$ 80, $ZnSO_4.7H_2O$ 2, and $MnSO_4$ 0.0035, $CuSO_4$ 0.005 and 2.5 % glycerol as an energy source (**15**). The culture was inoculated in CDLIM and incubated at 28 ºC for 120 h with shaking at 220 rpm.

Factors affecting siderophore production growth medium

Various media were used to study the siderophore secretion CDLIM Glycerol Aspergin Medium [(gl^{-1}): Glycerol 10 , $K_2HPO_4$1, L-Aspragine 1, trace salt solution 1, pH 7.4 (Pridham and Gotttlieb trace salt solution (gl^{-1}): $CuSO_4.5H_2O$ 0.64, $FeSO_4.7H_2O$ 0.1, $MnCl_2$ 0.79, and $ZnSO_4$ 0.15)], Synthetic Medium [(gl^{-1}): K_2HPO_4 1, $MgSO_4.7H_2O$ 0.5, KCl 0.5, $NaNO_3$ 2, Glycerol 30, pH 7.2], Casein Starch Medium [(gl^{-1}): Casein 0.3, Starch 10, NaCl 2, KNO_3 2, K_2HPO_4 2, $MgSO_4$ 0.09, $CaCO_3$ 0.02 , pH 7]. Siderophore production was checked after completion of 120 h of incubation at 28 °C, on a rotary shaker (Remi, Mumbai) at 220 rpm. Unless and otherwise specified in the text through out the experimentation cultivation conditions were incubation period of 120 h at 28 °C temperature and 220 rpm shaking speed.

Carbon source

The effect of different carbon sources such as glucose, sucrose, glycerol, arabinose, maltose, sodium acetate, lactose was studied on siderophore production by *Streptomyces fulvissimus*. All carbon sources were added at concentration 2.5 % w/v. Also experiments were carried out by varying the concentrations of sucrose, glucose and glycerol in the range of 0.05 – 0.4 M.

Nitrogen source

Effect of urea, sodium nitrate, ammonium chloride was studied at concentration 0.5 % w/v and varied in the concentration range of 0.04 – 0.5 M. CDLIM was fortified with different nitrogen sources.

Phosphate

In CDLIM, K_2HPO_4 is the source of phosphate. Different concentrations of K_2HPO_4 in the range of 0.005 – 0.05 M were used to study the effect of phosphate on siderophore production.

Iron

Iron content of CDLIM was varied by the addition of ferric chloride. Effect of iron was studied in the range of 0 - 60 µM concentration.

pH

The effect of pH on siderophore production was studied by varying the pH of CDLIM in the range of 5 – 10.

Temperature

To check the influence of different incubation temperatures on siderophore production, culture grown in CDLIM was incubated at different temperatures in the range of 20 - 44 ºC for 120 h at 220 rpm.

Aeration

The culture *Streptomyces fulvissimus* was inoculated in CDLIM and one was incubated on rotary shaker at 220 rpm and 28 °C and another set was incubated at stationary conditions and at 28 ºC for 120 h.

Siderophore detection and partial characterization

Qualitatively siderophore production was detected by Universal chemical assay (16) and comparative account of siderophore production was done in terms of percent siderophore units (17). After incubation of 120 h at 28 ºC at 220 rpm, siderophore production by *Streptomyces fulvissimus* was determined. Centrifuged (10,000 rpm for 15 min) cell free supernatant was subjected to estimation of siderophore as per Chrome Azurol S (CAS) liquid assay of Payne (17). Briefly, 0.5 ml of culture supernatant was mixed with 0.5 ml of CAS assay solution. Un-inoculated medium was used as reference. The optical density (OD) at 630 nm was measured for loss of blue colour resulting from siderophore production. Siderophore produced was calculated by using following formula,

$$\% \text{ Siderophore Units} = \frac{Ar - As}{Ar} \times 100$$

Where,
Ar - Absorbance of Reference
As - Absorbance of Sample at 630 nm

Hydroxamate type of siderophore was determined by Csaky's Assay (18). After growth, cell mass was separated by centrifugation at 10,000 rpm at 4 ºC for 15 min. Siderophore was extracted by using amberlite XAD 4 column chromatography. Thin layer chromatography of the purified siderophore was carried out. Desferrioxamine mesylate (Desferal, Novartis) was used as standard.

HPLC of siderophores

Analytical HPLC of purified siderophores was carried out on chromeleon (c) Dionex version 6.60 SP8 build 1544 (Switzerland) using REFTEK, pinnacle II C_{18} reverse phase column (250 ∞ 4.6 mm, 5µM integrated pre column) as stationary phase with detector (UV-Visible, PDA 100) and methanol: water (8:2 v/v) as mobile phase at flow rate of 1 ml min^{-1} at 25 ºC and at 220 nm.

Results and Discussion

Siderophore production by *S. fulvissimus* was studied by cultivating it in chemically defined low iron medium and detection by Universal Chemical Assay for siderophores developed by Schwyn and Neilands (1987). Further factors affecting siderophore production were also studied including carbon source, nitrogen source, iron, inorganic phosphate, pH, temperature and aeration.

Siderophore detection and partial characterization

The Chrome Azurol S (CAS) assay is based on the principle of higher affinity of siderophores towards Fe (III) than CAS reagent. Siderophores acquire iron from its complex with weak chelator in the reagent due to which it undergoes

decolorization. For this, the pH of the culture supernatant was adjusted to neutrality and equal amount of CAS reagent was added to it. Colour change from blue to orange was observed at λ_{max} 630 nm which indicated siderophore production. The type of siderophore was detected by Csaky's test where formation of red coloration indicated the presence of hydroxamate group in the siderophore.

Cell free supernatant was concentrated under rotary vacuum evaporator (Buchi, Switzerland) and subsequently subjected to amberlite XAD 4 column chromatography. CAS test positive fractions were pooled together dried and weighed. About 90 – 100 $mg.l^{-1}$ of purified siderophore powder was obtainable and subjected to HPLC. Thin layer chromatography of the purified siderophore showed R_f value 0.65 in butanol: acetic acid: water (9:1:1) which matched with desferrioxamine mesylate (Desferal, Novartis).

HPLC analysis of purified siderophores

The HPLC of desferrioxamine showed the peak at 6.60 min while the partially purified sample showed three peaks among which one peak had similar retention time to standard desferrioxamine (Fig. 1a, 1b), which clearly suggested the presence of desferrioxamine in the sample.

Influence of media on siderophore production

The influence of medium on siderophore production was studied in four different media such as CDLIM, GASPM, SM, and CSM. Optimum production of siderophores relies on performance of producer organism, the choice of medium, available iron content of the medium used and the iron requirement level of the organism (19). As depicted in Fig. 2, CDLIM was found to be the best medium among all others compared since it gave maximum yield of siderophores (94 % siderophore units); where as Glycerol - L - Asparagine medium gave 89.14 % siderophore units. In case of casein starch medium, siderophore production was lower with 83 % siderophore units, followed by synthetic medium for actinomycetes. Hence for further experiments CDLIM was used.

Influence of different carbon sources on siderophore production

Streptomyces species is reported for the utilization of simple sugars (20, 21), alcohol and some organic acids (22). Pridham and Gottlieb (23) characterized actinomycetes according to the utilization of different carbon sources. In antibiotic novobiocin production, the producer organism *S. niveus* favored citrate over glucose (24) while *S. kanamyceticus* M 27 can utilize dextrose excellently for kanamycin production (25). Hence, *Streptomyces fulvissimus* was grown in CDLIM fortified with different carbon sources for 120 h at 28 ºC at 220 rpm. Sucrose followed by glucose and glycerol were proved to be the best carbon sources (Fig. 3) achieving maximum siderophore production i.e. 93.08, 92.05, 91.61 % siderophore units, respectively within 0.07 – 0.2 M range of carbon source concentration. While sodium acetate and lactose, did not support siderophore production.

Glucose, sucrose and glycerol gave higher siderophore production. Concentration of these three carbon sources was varied in a range of 0.05 – 0.4 M. Glucose did not support siderophore production above 0.2 M concentration (Fig. 4) with about 91 % siderophore units in range of 0.05 – 0.1 M concentration. In case of Sucrose (Fig. 4), the siderophore production was highest (93.25 % siderophore units) at 0.05 M concentration and thereafter it declined gradually. When glycerol (Fig. 4) was used as carbon source over a range of 0.05 to 0.4 M concentration, the siderophore production remained constant in between concentration range 0.1 – 0.3 M *i.e.* 91

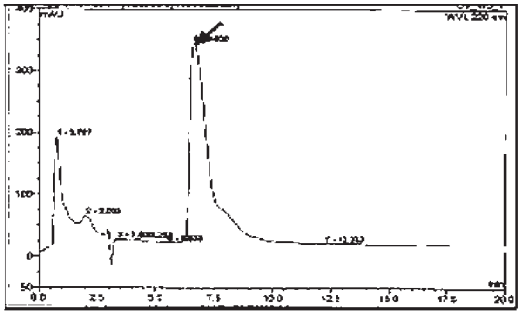

Fig. 1a. HPLC of standard (Desferal)

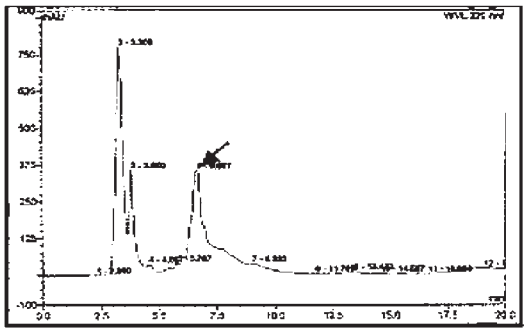

Fig. 1b. HPLC of sample

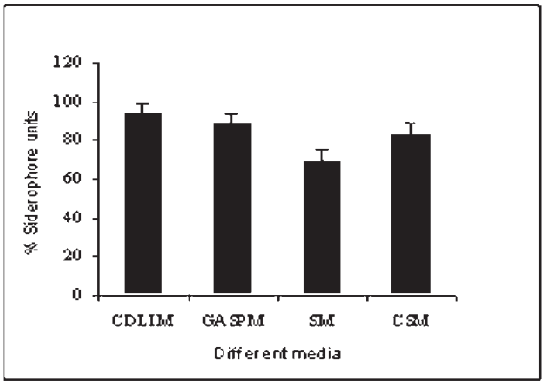

Fig. 2. Optinal medium for siderophore production.

(CDLIM : Chemically Defined Low Iron Medium, GASPM : Glycerol Aspergin Medium S.M. : Synthetic Medium, CSM : Casein Starch Medium

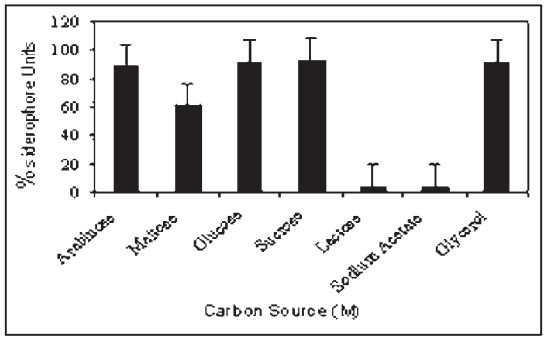

Fig. 3. Influence of different carbon sources on siderophore production

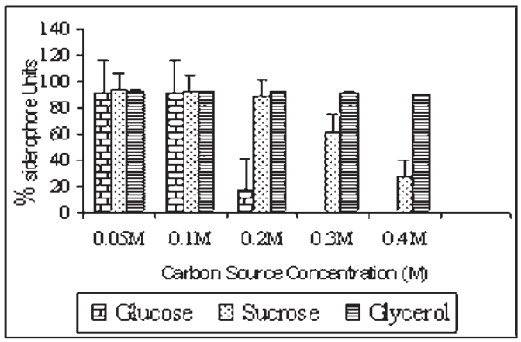

Fig. 4. Effect of different carbon sources on sider ophore production

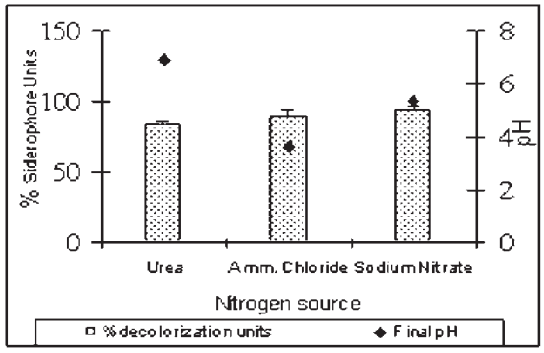

Fig. 5. Influence of different nitrogen sources on siderophore production

%. The results indicated that glycerol can achieve the maximum siderophore production over a wide range of concentrations.

Influence of different nitrogen sources on siderophore production

The production of secondary metabolites is influenced by the availability of nutrients. The availability of nitrogen and its source can affect the production of secondary metabolites (26, 27). In this study, the regulation of siderophore production by nitrogen using different nitrogen sources and synthetic culture medium was investigated. Wherein urea, ammonium chloride, sodium nitrate were used. Each nitrogen source was studied at the concentration of 0.5 % w/v (Fig. 5). When sodium nitrate was used as nitrogen source, highest siderophore production *i.e.* 93.12 % siderophore units was obtained. Urea as nitrogen source was observed to give 83.3 % siderophore units and ammonium chloride gave 90.05 % siderophore units. Urea at concentration at 0.05 M showed 92 % siderophore units (Fig. 6) on 120 h of incubation at 28 ºC. Siderophore production gradually decreased to 23 % siderophore units as concentration was increased to 0.4 M. Ammonium chloride gave maximum % siderophore units (91 %) at concentration range of 0.05 - 0.1 M (Fig. 6) which gradually decreased to 70 % siderophore units at 0.5 M concentration. Ammonium salts are the principal nitrogen sources which have been reported to interfere with antibiotic production. Gonzalez *et al.,* reported an increase in the growth and specific production of gentamicin, proportional to the amount of ammonium present in the culture medium for ammonium concentration ranging from 20 - 150 mM (28). In case of sodium nitrate, siderophore production was 93.19 % siderophore units at 0.05 M concentration and declined with the increase in concentration. At 0.5 M concentration it was 30 % siderophore units (Fig. 6).

Influence of inorganic phosphate on siderophore production

Phosphate concentration is one of the important factors that affect siderophore production (26). Effect of phosphate concentration in the range of 0.005 – 0.05 M on siderophore secretion by *Streptomyces fulvissimus* was studied. As depicted in Fig. 7, the range of 0.02 – 0.03 M phosphate concentration was found to be optimum for siderophore production, since within this range maximum (92 % siderophore units) siderophore production was recorded which declined at higher concentrations. Very high or low phosphate concentration did not support siderophore production. It needs to have specific concentration of phosphates which is in agreement with results obtained by Barbhaiya and Rao (22).

Influence of iron concentration on siderophore production

Among environmental factors, iron concentration is the most important that mainly regulates the biosynthesis and secretion of siderophore (29, 30). Taking into account this factor, the influence of extraneously added Fe (III) in increasing order to the CDLIM on the siderophore production was observed. Although cell growth reached maximum at value above 5 µM of added Fe (III), (Fig. 8) siderophore production was lowered at this concentration. In the absence of exogenous iron / less than 1 µM *Streptomyces fulvissimus* produced maximum siderophores while in iron containing media, siderophore production was decreased proportionally with the increase in iron concentration. Concentration > 5 µM of iron could not induce siderophore production. Thus indicating that 5 µM of iron showed the iron sufficiency which lead to negligible amount of siderophore production. Iron more than 5 µM enhanced the growth of *S. fulvissimus* but

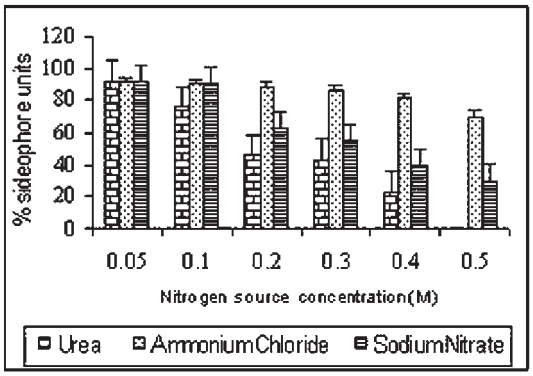

Fig. 6. Influence of different nitrogen sources on Siderophore Production

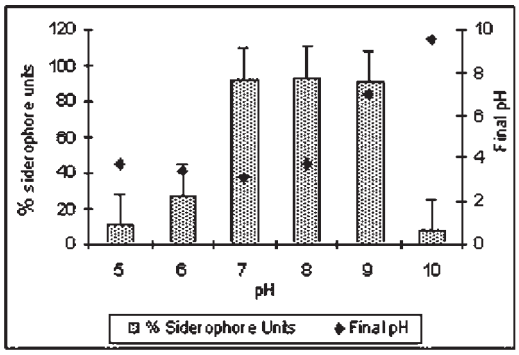

Fig. 9. Influence of pH on siderophore production

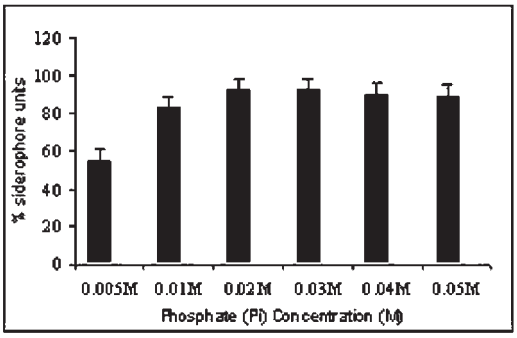

Fig. 7. Influence of inorganic phosphate (Pi) on siderophore production

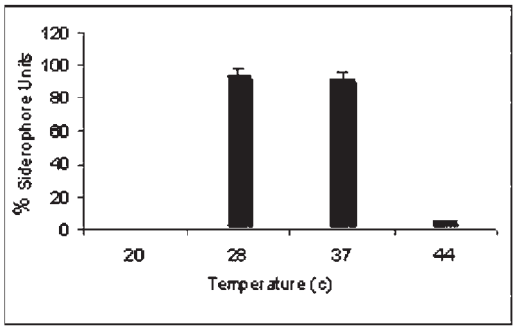

Fig. 10. Influence of temperature on siderophore production

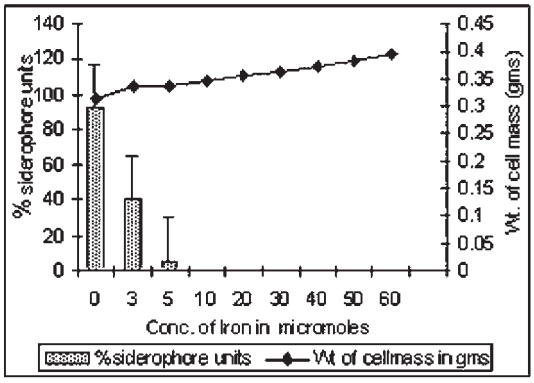

Fig. 8. Influence of Iron on siderophore production

responsible for repression of siderophore production. Similar results have been reported by Yang *et al.* (31), for siderophore production by *P. pseudomallei* U7.

Influence of pH on siderophore production

The pH plays vital role in the solubility of iron in production medium and thereby siderophore production. Iron is insoluble at neutral to alkaline pH but solubility increases at acidic pH values (32). So at the acidic conditions iron is available to microorganisms which in turn reduce the siderophore production.

For *Streptomyces fulvissimus*, pH 8 was found to be optimum for siderophore secretion (Fig. 9). At pH 8, highest siderophore production (93 % siderophore units) was seen, while very sharp decrease at pH 10. At alkaline pH, solubility of iron is highly affected therefore theoretically siderophore yield should increase as the available iron is less. But poor growth of *Streptomyces fulvissimus* would be the main reason for sharp decrease in siderophore production at high alkaline pH (above 9). Very poor growth and the higher alkaline conditions did not support siderophore production. In contrast, at acidic pH, iron is in soluble form, can repress the siderophore production and so the yield was negligible.

Influence of temperature on siderophore production

To check the influence of temperature on siderophore production, *S. fulvissimus* was grown at various temperatures (Fig. 10). At 28 ºC, maximum siderophore production was observed *i.e.* 93.47 % siderophore units which was little bit lowered at 37 ºC but no siderophore production was induced at 20 ºC and at 44 ºC when grown in CDLIM.

This may be because at these temperatures, *S. fulvissimus* was not able to grow. Similar results have been reported earlier. Worsham and Konisky (33) have reported decreased siderophore production by *S. typhimurium* at elevated temperatures, Garibaldi (34), and Kochan (35), have also reported that in *E. coli* and *S. typhimurium,* enterochehin biosynthesis was inhibited at temperatures greater than 40 ºC. The temperature range of 28 ºC to 37 ºC was suitable for optimum siderophore production by *S. fulvissimus*.

Influence of aeration on siderophore production

When incubation was carried out at stationary conditions, siderophore production (Fig. 11) either did not take place or was too negligible. Incubation with aeration at 220 rpm had achieved high production *i.e.* 90 % siderophore units. *Pseudomonas stutzeri,* strain CCUG 36651, a facultative anaerobe was shown to produce siderophore when grown under aerobic conditions. In contrast no siderophores were observed from anaerobically grown *P. stutzeri* (36).

Conclusion

Streptomyces fulvissimus was found to produce siderophores under iron depleted conditions. Environmental factors such as temperature, pH, iron concentration, growth medium, carbon, nitrogen and phosphate influence siderophore biosynthesis. As siderophores have wide application in biocontrol of plant diseases and plant nutrition, these attributes are very important.

Acknowledgement

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An integrated approach for production of cephamycin C using *Streptomyces clavuligerus* NT4: Sequential optimization of production medium and effect of amino acids

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Abstract

The present work reports on an integrated approach of media optimization and amino acid supplementation for production of cephamycin C by submerged culture fermentation of *Streptomyces clavuligerus* NT4. The fermentation was carried out for 144 h at 28 ± 2 ^{o}C on an orbital shaker rotating at 180 rpm. In the first step, the effects of media constituents such as carbon and nitrogen sources were investigated followed by optimization using response surface methodology (RSM). The yield of cephamycin C in the optimized medium increased from 5.5 mg/ml to 10.45 ± 0.34 mg/ml. Effect of amino acids on cephamycin C production was further studied by using RSM. An optimized combination of L-lysine, L-valine, L-cysteine and DL-methionine further increased the yield to 13.64 ± 0.23 mg/ml of cephamycin C.

Key words: Cephamycin C, Response surface methodology, Amino acids, Submerged fermentation, *Streptomyces clavuligerus.*

Introduction

Cephamycin C (Fig. 1) is an extracellular broad spectrum β-lactam antibiotic produced by *Streptomyces cattleya, Streptomyces clavuligerus* and *Nocardia lactamdurans*. The broad spectrum of activity and resistance to β-lactamases make cephamycin C more effective in treating many cephalosporin resistant isolates such as *E. coli, Klebsiella, Proteus, Serratia,* and *Bacteriodes.* Improved derivatives of cephamycin C such as cefoxitin, cefotetan, cefametazole, and temocillin are produced from cephamycin C, and are used in preventing post-operative infections caused by drug resistant bacteria.

Cephamycin C can be produced by submerged as well as solid-state fermentation (SSF) (1, 2). Submerged fermentation (SmF) has been widely used for the production of cephamycin C because of easier process control, although it is associated with higher power consumption and generation of huge amount of wastewater.

Medium optimization by employing one factor at-a-time method involves changing one

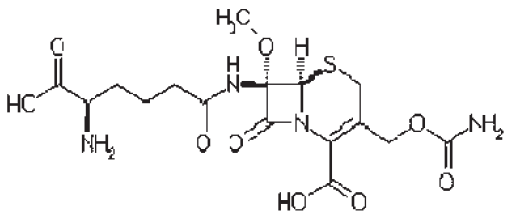

Fig. 1. Chemical structure of cephamycin C

independent variable (nutrient, pH, temperature etc.) while fixing all the others at a certain level. This single dimensional approach is laborious and time consuming, especially for large number of variables, and frequently does not guarantee the determination of optimal conditions. An alternative to this method is provided by the RSM, which can be employed to study the relationship between the medium components and their effects on microbial growth and production. In RSM, several factors are simultaneously varied. The application of these design techniques in fermentation process results in improved product yields, reduced process variability, closer confirmation of the output response (product yield or productivity) to nominal and target requirements and reduced development, and overall costs (3).

To the best of our knowledge, there is scarcity of literature on nutritional and environmental conditions for submerged culture *S. clavuligerus* for cephamycin C production. Previous work in our laboratory on a mutant strain of *S. clavuligerus* (*Streptomyces clavuligerus* MTCC 1142 was exposed to 1 mg/ml nitroso-guanidine, a chemical mutagen for 60 min) showed it to totally inhibit production of clavulanic acid, but produce unprecedented amounts of cephamycin C. This mutant is referred to as *S. clavuligerus* NT4. In the present study, a preliminary optimization of carbon and nitrogen sources for maximum production of cephamycin C using *S. clavuligerus* NT4 was followed by optimization using RSM, wherein the concentrations of selected media components were optimized. The amino acids were also optimized for maximum production of cephamycin C by using RSM.

Materials and Methods

Glucose, yeast extract, agar, malt extract, soybean meal, mycological peptone, ammonium sulphate, ammonium chloride, *p*-dimethyl aminobenzaldehyde, urea, sodium carbonate, NaOH and corn steep liquor were procured from Himedia Ltd, Mumbai. Magnesium sulphate, potassium di-hydrogen phosphate, sodium chloride, zinc chloride, manganese chloride and solvents like acetonitrile, methanol, ethyl acetate, butyl acetate, acetone, ethanol, concentrated HCl and concentrated H_2SO_4 were purchased from Merck India Ltd. Mumbai. All solvents used were of AR grade, except acetonitrile which was of HPLC grade. Standard cephamycin C (authentic sample) was obtained as a gift sample through the kind courtesy of Merck Research Laboratories, USA.

Microorganism

The strain *Streptomyces clavuligerus* MTCC 1142 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. An NTG mutant of the strain that gave enhanced production of cephamycin C was used in the study. This mutant strain was named as *S. clavuligerus* NT4 and maintained on a medium containing (in g/l) yeast extract 4, malt extract 1, dextrose 4, and agar 20 at pH 7.2-7.4; the slants were subcultured every 15 days.

Fermentation

In the present study, medium developed by Saudagar and Singhal (4) was used for optimization of cephamycin C production by *S. clavuligerus* NT4. The production medium contained (in g/l) glycerol 15.0, sucrose 20.0, arginine 17.4, $CaCl_2$ 0.4, K_2HPO_4 2.0, $MnCl_2$ 0.05, $ZnCl_2$ 0.05, NaCl 5.0, $MgSO_4$1.0, L-glutamic acid 16.3 with pH 6.5 After autoclaving at 121^{o}C for 15 min, the media was inoculated with 3 ml of spore suspension (2 x 10^6 spores/ml) and incubated in an orbital incubator shaker at room temperature (28 ± 2°C) for 6 days. All experiments were carried out in triplicate.

Optimization of fermentation medium using one factor-at-a-time method

Sucrose in the basal media was substituted with other carbon sources such as glucose, maltose, fructose, galactose, soybean oil, palm oil and sesame oil at 20 g/l, and L-glutamic acid with yeast extract, biopeptone, beef extract, malt extract, ammonium acetate, urea, NH_4Cl, NH_4SO_4, $NaNO_3$ and KNO_3 to check their suitability for cephamycin C production. Here, organic nitrogen sources were screened at 16.3 g/l and inorganic nitrogen sources were used at 0.111 M.

Optimization of media by RSM

Optimization of concentrations of the selected medium components by RSM

A CCRD for four independent variables was used to obtain the combination of values that optimizes the response within the region of three dimensional observation spaces, which allows one to design a minimal number of experiments. The experiments were designed using the software, Design Expert Version 6.0.10 trial version (State ease, Minneapolis, MN). Regression analysis was performed on the data obtained from the design experiments.

Coding of the variables was done according to the following equation

$$x_i = (Xi - Xcp) / \Delta Xi \quad i = 1, 2, 3, \ldots, k \qquad (1)$$

Where: x_i, dimensionless value of an independent variable; *Xi*, real value of an independent variable; *X*cp, real value of an independent variable at the center point; and *_Xi*, step change of real value of the variable *i* corresponding to a variation of a unit for the dimensionless value of the variable *i*.

Four independent variables selected for evaluating the combined effect on cephamycin C production were glycerol, fructose, K_2HPO_4 and yeast extract. The media optimized by one factor at-a-time method was used where sucrose and L-glutamic acid were substituted with fructose and yeast extract, respectively.

A central composite factorial design of 16 plus 5 center points leading to 21 experiments were performed. Each factor was varied at five levels. The coded and actual values of independent variables are given in (Table. 1). The experiments were carried out in triplicate. Replicates at the centre of the domain in three blocks permit the checking of the absence of bias between several sets of experiments. The relationship of the independent variables and the response was calculated by the second order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i X_j + \sum_i \sum_{i<j} \beta_{ij} X_i X_j \qquad (2)$$

Where: Y is the predicted response; β_0 a constant; β_i the linear coefficient; β_{ii} the squared coefficient; and β_{ij} the cross-product coefficient, k is number of factors.

The second order polynomial coefficients were calculated using the software package Design Expert Version 6.0.10 to estimate the responses of the dependent variable. Response surface plots were also obtained using Design Expert Version 6.0.10.

The effect of metabolic precursors on cephamycin C production by RSM

Based on the biosynthetic pathway of cephamycin C in *S. clavuligerus* (5), and our earlier work on the effect of four individual amino acids (L-lysine hydrochloride, L-valine, L-cysteine and DL-methionine) on cephamycin biosynthesis by *Nocardia lactamdurans* NRRL 3802 (6), the combined effect of four different amino acids on cephamycin C production was examined in the media optimized by RSM. A central composite factorial design of 16 plus 5

centre points leading to 21 experiments were performed.

Analytical determinations

Culture was extracted with 0.1 % (V/V) Tween-80 in distilled water, and the total was made to 50 ml. It was shaken at room temperature (28 ± 2 °C) on an orbital shaker at 180 rpm for 2 h. The extract was centrifuged at 10, 000 g and 1 ml of supernatant extracted with 9 ml of methanol to remove proteins. This mixture was filtered using Whatman filter paper (No.1) and then using Pall 0.2 µm membrane filter (Ultipor® N_{66} ® Nylon 6,6 membrane), and cephamycin C was estimated as described earlier (2). Jasko HPLC system fitted with a reverse phase column Waters Sperisorb® ODS (C_{18} octadecyl silane, 250 X 4.6 mm ID) and UV detector was used for cephamycin C analysis. The mobile phase was 0.05 M KH_2PO_4 adjusted to pH=3.0 with concentrated phosphoric acid. A 60 % 0.05 M KH_2PO_4 (pH=3.0) and 40 % acetonitrile mixture was used for fine resolution of peaks. Cephamycin C was detected at 253 nm.

Results and Discussion

Optimization of fermentation medium using one factor at-a-time method

Among the various carbon sources screened, fructose was found to support maximum production of 6.04 ± 0.08 mg/ml of cephamycin C after 6 days of fermentation (Fig. 2). Of the evaluated nitrogen sources, yeast extract gave a maximum yield of 6.29 ± 0.06 mg/ml of cephamycin C and potassium nitrate gave the least (1.66 ± 0.03 mg/ml) (Fig. 3).

Optimization of production of cephamycin C by *S. clavuligerus* NT4 using RSM

The components (independent variables) selected for the optimization were A: glycerol (g/l); B: fructose (g/l); C: K_2HPO_4 (g/l) and D: yeast extract (g/l). Each factor was varied at five levels.

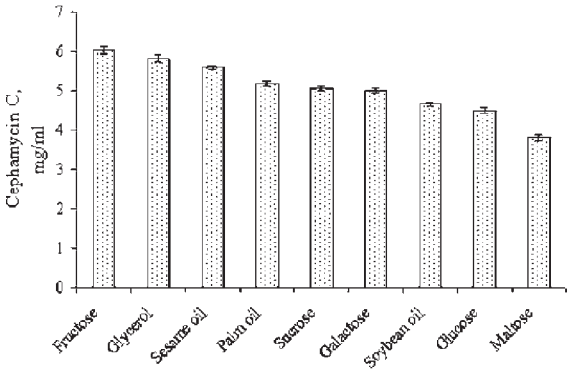

Fig. 2. Evaluation of different carbon sources for cephamycin C production by *S. clavuligerus* NT4

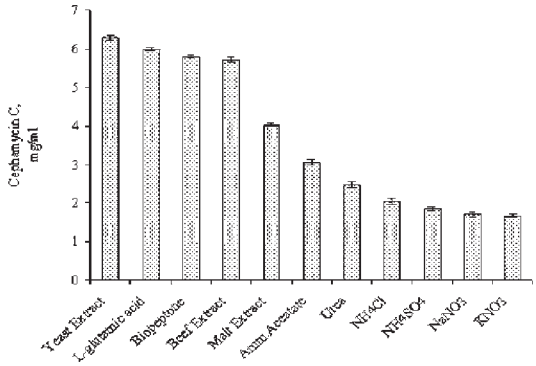

Fig. 3. Evaluation of different nitrogen sources on cephamycin C production by *S. clavuligerus* NT4

Regression analysis was performed on the data obtained from the design experiments. The experimental and predicted values of yields of cephamycin C are given in (Table. 1)**.** The results were analyzed by using ANOVA i.e. analysis of variance suitable for the experimental design used, and cited in (Table. 2). The Model F-value of 146.11 implied the model to be significant Model P-value (Prob > F) was very low (< 0.0001). This again signifie the model to be significant. The *F* value and the corresponding *P* values, along with the coefficient estimate, are given in Table 2. The smaller the magnitude of the *P*, the more significant is the corresponding coefficient. Values of *P* less than 0.0500 indicate model terms to be significant. The coefficient estimates and the corresponding *P* values

suggested that among the test variables used in the study, A, B, C, D, A^2, C^2, D^2, AB, AC, AD, BC, BD and CD (where A: glycerol (g/l); B: fructose (g/l); C: K_2HPO_4 (%) and D: yeast extract (g/l)) were significant model terms. A, B, D, AC & BD ($P< 0.0001$) had the largest effect on cephamycin C production, followed by C, D^2, AD, C^2, BC, A^2, CD & AB.

The corresponding second-order response model for Eq. (2) that was found after analysis for the regression was

Yield of cephamycin C (mg/ml) =

$$4.390 + (1.725 \times A) + (0.613 \times B) - (0.372 \times C) + (1.085 \times D) + (0.179 \times A^2) + (0.011 \times B^2) + (0.211 \times C^2) - (0.276 \times D^2) + (0.236 \times A\,B) - (0.669 \times AC) - (0.631 \times AD) + (0.361 \times BC) + (1.661 \times BD) + (0.256 \times DC) \quad \text{---------- Eq. (3)}$$

$R^2 = 0.997$ suggest the prediction of experimental data to be quite satisfactory. The "Pred R-squared" of 0.82 was found to be in reasonable agreement with the "Adj R-Squared" of 0.99. Here, Adeq Precision of 48.79 (>4) indicates an adequate signal.

The predicted values by "contour plot generation" and "point prediction" were experimentally verified. The maximum production of cephamycin C obtained using the optimized medium was 10.45 ± 0.34 (mg/ml). Table 3 documents the yields of cephamycin C by various predicted media combination. The final media optimized by RSM contained (in g/l) glycerol 23.45, fructose 19.35, arginine 17.4, $CaCl_2$ 0.4, K_2HPO_4 0.89, $MnCl_2$ 0.05, $ZnCl_2$ 0.05, NaCl 5.0, $MgSO_4$ 1.0 and yeast extract 21.1. This was further optimized for supplementation of four amino acids.

Here, glycerol (A) plays a very significant role ($p< 0.0001$). Glycerol supports antibiotic production, and fructose supports the growth. This concept of dual carbon source utilization appears to be the basis for published media, which support commercial antibiotic synthesis. As the residual concentration of glycerol is increased, antibiotic accumulation increases rapidly. In the present study, glycerol at 23.45 g/l was found to be optimum. Fructose (B), ($p< 0.0001$) also played an important role in determining the product yield in antibiotic fermentations. When the concentration of fructose was low, cephamycin C production was relatively low. As the residual concentration of fructose increased, antibiotic accumulation increased rapidly. In the present study, the optimum fructose concentration was found to be 19.35 g/l. Yeast extract (D) as the nitrogen source ($p< 0.0001$) also played an important role in determining the product yield in antibiotic fermentations. Cephamycin C production was relatively low at lower concentrations of yeast extract, and its accumulation increased with an increase in the residual level of yeast extract. In the present study, the optimum fructose concentration was found to be 21.1 (g/l). K_2HPO_4 (C), ($p = 0.0002$) had a great significance in cephamycin C fermentation. The required concentration of K_2HPO_4 is governed mainly by the glycerol concentration. Even though an increase in phosphate concentration does increase the cephamycin C production, at higher concentrations of glycerol the required concentration of K_2HPO_4 is comparatively low. In the present study, the optimum K_2HPO_4 concentration was found to be 0.89 %, as the glycerol concentration was higher (23.45 g/l).

Maltose and glucose as carbon source did not have any major effect on cephamycin C production. *Streptomyces clavuligerus* is a rare actinomycete unable to grow on glucose (7). Aharonowitz and Demain (8) have observed a strong suppressive effect of ammonia on antibiotic production in the cephamycin producer *S. clavuligerus*. The nature of this regulation appears to be similar to that observed with rapidly metabolisable carbon sources. Khaoua et al. (9) reported that the production of cephamycin C by *Streptomyces cattleya* varies with the use of asparagines, glutamine or ammonium as nitrogen

sources. Bussari et al. (10) reported the use of ammonium oxalate and casein peptone as additional nitrogen sources to increase the production of cephamycin C in solid state fermentation. Devi and Sridhar (11) immobilized *S. clavuligerus* on sponge and alginate beads and evaluated it in a production media containing starch as carbon source and corn steep liquor as nitrogen source.

The effect of metabolic precursors on cephamycin C production by RSM

In *S. clavuligerus*, the first step in the biosynthesis of cephamycin C is the condensation of three amino acid precursors: α-aminoadipate, L-cysteine and L-valine. The α-aminoadipate is generated from L-lysine by two sequential enzymatic steps. The first step involves a lysine-6-aminotransferase activity (LAT), considered to be one of the rate-limiting steps for antibiotic biosynthesis. According to Omstead et al. (5) L-lysine hydrochloride supplemented cultures showed higher titers of cephamycin C. The methyl group at 7α position in all cephamycins is derived from methyl group of methionine (5). Hence these four amino acids could serve as metabolic precursors of cephamycin. Kagliwal et al. (6) reported an increased production of cephamycin C in solid state fermentation when amino acids were added in combination rather than individually. A similar result was obtained in submerged fermentation (Kagliwal et al., unpublished data). Hence, attempts were made to evaluate amino acid combinations for production of cephamycin in the present work.

The components (independent variables) selected for the optimization were four amino acids (A′=L-lysine hydrochloride (%), B′ = L-valine (%), C′ = L-cysteine (%) and D′ = DL-methionine (%). Each amino acid was varied at five levels. Regression analysis was performed on the data obtained from the design experiments. The experimental and predicted values of yields of cephamycin C are given in (Table. 4).

The results were analyzed by using ANOVA i.e. analysis of variance suitable for the experimental design used, and cited in Table 5. The Model F-value of 127.27 implied the model to be significant Model P-value (Prob > F) was very low (< 0.0001), again signifying the model to be significant. The *F* value and the corresponding *P* values, along with the coefficient estimate are given in Table 5. The coefficient estimates and the corresponding *P* values suggested that among the test variables used in the study, B′, C′, D′, A′^{2}, B′^{2}, C′^{2}, D′^{2}, A′B′, A′C′, A′D′ & B′C′ were significant model terms. C′, D′, B′^{2}, C′^{2}, D′^{2}, A′B′, A′C′ & B′C′ (P< 0.0001) had the largest effect on cephamycin C production, followed by A′^{2}, B′ & A′D′. Only one variable, A′ was found to be insignificant.

The corresponding second-order response model for Eq. (2) that was found after analysis for the regression was

Yield of cephamycin C (mg/ml) =

13.324 – (0.022 x A′) + (0.057 x B′) – (0.192 x C′) + (0.280 x D′) – (0.057 x A′^{2}) – (0.069 x B′^{2}) + (0.179 x C′^{2}) – (0.161x D′^{2}) + (0.204 x A′B′) + (0.159 x A′C′) – (0.081 x A′D′) + (0.184 x B′C′) ---------- Eq. (4)

R^2 = 0.995 showed the prediction of experimental data to be quite satisfactory. The "Pred R-Squared" of 0.957 was in reasonable agreement with the "Adj R-Squared" of 0.986. Here, Adeq Precision of 33.79 (>4) indicates an adequate signal.

The predicted values by "contour plot generation" and "point prediction" were experimentally verified. The maximum production of cephamycin C obtained using the optimized medium (in %, L-lysine 1.07, L-valine 0.339, L-cysteine 0.12 and DL-methionine 1.06) was 13.64 ± 0.23 (mg/ml). Table 6 documents the yields of cephamycin C by various predicted media combination. Bussari et al. (10) reported

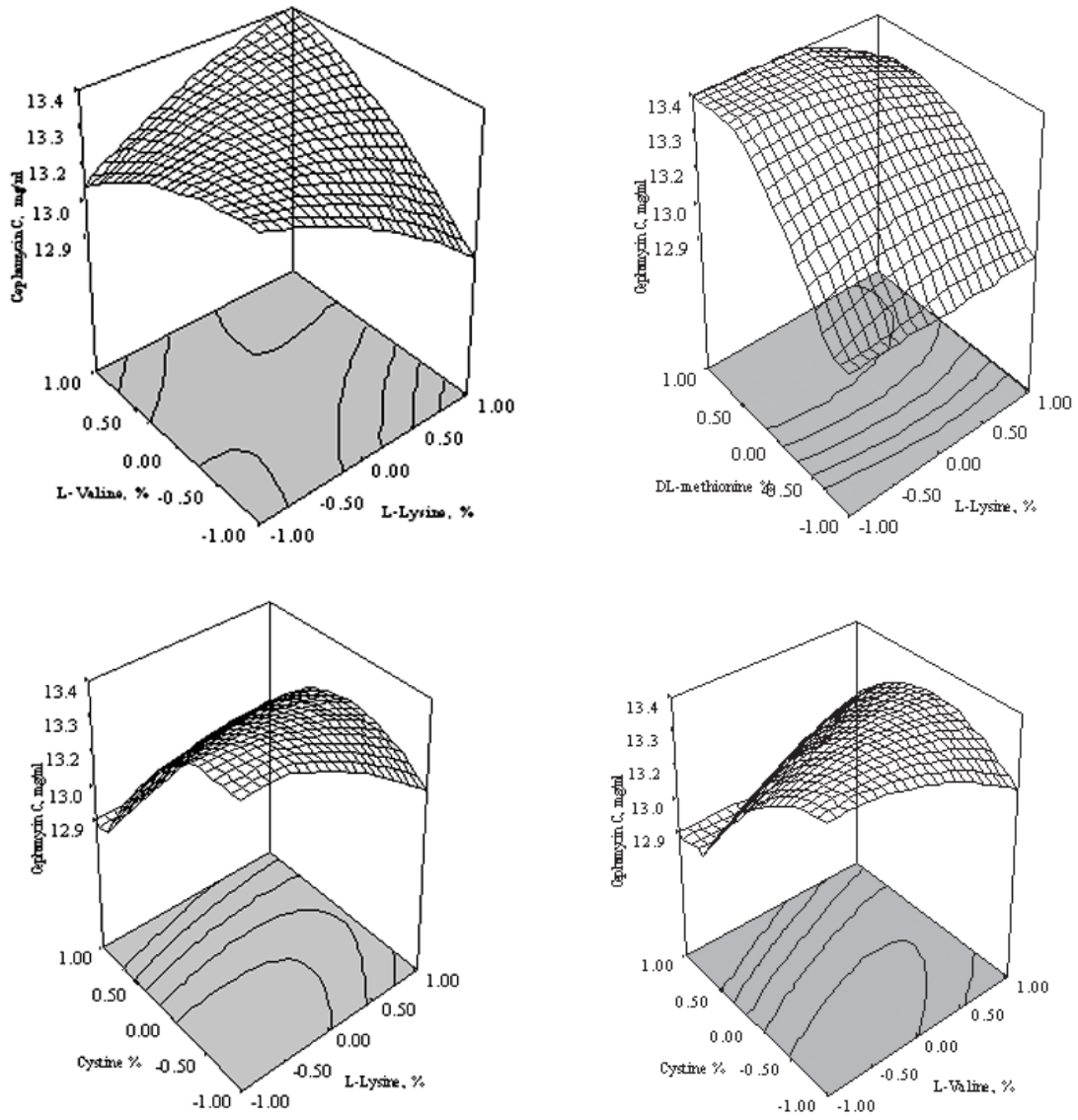

Fig. 4. 3D-surface plots for cephamycin C production A. Effect of L-lysine hydrochloride and L-valine when other variables are held at zero level; B. Effect of L-Lysine hydrochloride and L-cystine when other variables are held at zero level; C. Effect of L-lysine hydrochloride and DL-methionine when other variables are held at zero level; D. Effect of L-cystine and L-valine when other variables are held at zero level

on optimization of cephamycin C production using solid state fermentation. They examined the combined effect of four different amino acids on cephamycin C production, using RSM and found the optimum L-lysine concentration to be 3.86 %, valine 0.84 %, L-cysteine 0.1 % and DL-methionine 1.5 %. Accordingly, three-dimensional graphs were generated for the pair-wise combination of the five factors, while keeping the other three at their center point levels. Graphs are given here to highlight the roles played by various factors (Fig. 4).

Mendelowitz and Aharonowitz (12) reported that addition of L-lysine to the culture of *S. clavuligerus* grown in a chemically defined medium increased specific antibiotic production by 75 %. Fang et al. (13) observed 100 mM L-lysine to increase the production of β-lactam antibiotic approximately by 500 % with only minor effects on growth. Leitão et al. (14) reported the effect of exogenous lysine on cephamycin C production by *N. lactamdurans* MA4213. Lysine-supplemented cultures showed higher titers of cephamycin C, and the effect was found to be more significant in the early staes of fermentation. It should be noted that all these reports were using only exogenous L-lysine (without any other additional amino acid simultaneously). In the present study, the optimum L-lysine hydrochloride was found to be 1.07 %, which also considers the effect of other amino acids. From ANOVA (Table. 5) it was concluded that L-lysine hydrochloride ($p=0.0891>0.05$) had an insignificant role, when all other three amino acids were present in the media.

L-cysteine (C`) and DL-methionine (D`), ($p<0.00001$) had a significant role in cephamycin C production. L-cysteine, DL-methionine and inorganic sulphate & thiosulphate are the major sources of sulphur for cephamycin C production. To incorporate sulphur into β-lactam, one way is the conversion of methionine in to cysteine prior to formation of the Arnstein tripeptide. L-cysteine is incorporated into the Arnstein tripeptide. Contour plot and 3D-surface plot for L-cysteine with other amino acids, predicts that a maximum cephamycin C production in the presence of L-cysteine at in the range 0 to 0.5 %. An increase in L-cysteine concentration from 0.5 to 2 % decreased the production of cephamycin C drastically. In this study, the optimum L-cysteine was found to be 0.12 %. Contour plot and 3D-surface plot for DL-methionine with other amino acids predicts that an increase in DL-methionine from 0 to 1.2 % increased the production of cephamycin C. DL-methionine beyond 1.2 % decreased the production of cephamycin C. In present study, the optimum DL-methionine was found to be 1.06 %.

L-valine (B`) also played a very significant role ($p=0.007$) in production of cephamycin C. L-valine is incorporated into the Arnstein tripeptide. The inclusion of L-valine, in addition to other three amino acids stated above, stimulated antibiotic production by *S. clavuligerus* NT4. Contour plot and 3D-surface plot for L-valine with other amino acids predicted an increase in cephamycin C production with an increase in concentration of L-valine. In the present study, 0.339 % L-valine was found to be optimum, which also considered the effect of other amino acids.

Conclusion

Fructose and yeast extract were found to be suitable carbon and nitrogen source for the production of cephamycin C. Supplementation of the RSM-optimized fermentation medium with a combination of L-lysine hydrochloride, L-valine, L-cysteine and DL-methionine increased the cephamycin C production from 5.5 mg/ml to 13.64 mg/ml. The work is significant in view of the cost of derivatives of cephamycin C in the world market.

Table 1. The CCRD matrix of independent variables in coded and uncoded form with their corresponding experimental and predicted yields of cephamycin C

Run	Variables, g/l [b]				Cephamycin C, mg/ml	
	Glycerol	Fructose	K_2HPO_4	Yeast Extract	Experimental[a]	Predicted
1	1 (20)	1 (20)	1 (2.5)	-1 (10)	4.02 ± 0.05	4.03
2	1 (20)	1 (20)	-1 (1.0)	-1 (10)	6.06 ± 0.09	5.91
3	1 (20)	-1 (10)	1 (2.5)	1 (20)	3.03 ± 0.06	3.04
4	-1 (10)	1 (20)	-1 (1.0)	1 (20)	5.78 ± 0.05	5.63
5	1 (20)	-1 (10)	-1 (1.0)	1 (20)	5.48 ± 0.04	5.32
6	-1 (10)	-1 (10)	1 (2.5)	-1 (10)	2.02 ± 0.06	2.03
7	-1 (10)	1 (20)	1 (2.5)	1 (20)	7.42 ± 0.04	7.45
8	-1 (10)	-1 (10)	-1 (1.0)	-1 (10)	2.83 ± 0.13	2.67
9	-2 (5)	0 (15)	0 (2.0)	0 (15)	1.61 ± 0.16	1.65
10	2 (25)	0 (15)	0 (2.0)	0 (15)	8.51 ± 0.11	8.55
11	0 (15)	-2 (5)	0 (2.0)	0 (15)	3.15 ± 0.02	3.21
12	0 (15)	2 (25)	0 (2.0)	0 (15)	5.60 ± 0.13	5.65
13	0 (15)	0 (15)	-2 (0.5)	0 (15)	5.73 ± 0.09	5.97
14	0 (15)	0 (15)	2 (2.5)	0 (15)	4.62 ± 0.07	4.48
15	0 (15)	0 (15)	0 (2.0)	-2 (5)	1.06 ± 0.02	1.12
16	0 (15)	0 (15)	0 (2.0)	2 (25)	5.41 ± 0.07	5.45
17	0 (15)	0 (15)	0 (2.0)	0 (15)	4.39 ± 0.05	4.39
18	0 (15)	0 (15)	0 (2.0)	0 (15)	4.40 ±0.06	4.39
19	0 (15)	0 (15)	0 (2.0)	0 (15)	4.38 ± 0.08	4.39
20	0 (15)	0 (15)	0 (2.0)	0 (15)	4.41 ± 0.05	4.39
21	0 (15)	0 (15)	0 (2.0)	0 (15)	4.37 ± 0.02	4.39

[a] results are mean ± SD of three determinations
[b] Values in parentheses are uncoded variables

Table 2. Analysis of variance (ANOVA) for the experimental results of the central-composite design (Quadratic Model)

Factor[a]	Coefficient Estimate	Sum of squares	Standard Error	DF[b] value	F	p[c] (Prob >F)
Model (or Intercept)	4.390	66.57	0.081	14	146.11	< 0.0001*
A	1.725	23.81	0.064	1	731.47	< 0.0001*
B	0.613	3.00	0.064	1	92.22	< 0.0001*
C	-0.372	2.21	0.045	1	67.99	0.0002*
D	1.085	9.41	0.064	1	289.39	< 0.0001*
A^2	0.179	0.81	0.036	1	24.77	0.0025*
B^2	0.011	0.002	0.036	1	0.08	0.7810†
C^2	0.211	1.11	0.036	1	34.16	0.0011*
D^2	-0.276	1.91	0.036	1	58.65	0.0003*
AB	0.236	0.22	0.090	1	6.86	0.0396*
AC	-0.669	3.58	0.063	1	109.93	< 0.0001*
AD	-0.631	1.59	0.090	1	48.97	0.0004*
BC	0.361	1.04	0.063	1	32.08	0.0013*
BD	1.661	11.04	0.090	1	339.20	< 0.0001*
CD	0.256	0.52	0.063	1	16.14	0.0070*

A: Glycerol (g/l); **B:** Fructose (g/l); **C:** K_2HPO_4 (g/l); **D:** Yeast Extract (g/l);
[b] Degree of freedom; [c] † $p > 0.05$, not significant; * $p < 0.05$, significant; $R^2 = 0.997$

Table 3. Validation of the model for fermentative production of cephamycin C by *S. clavuligerus* NT4 in submerged media

Std Run Order	Glycerol (g/l)	Fructose (g/l)	K_2HPO_4 (g/l)	Yeast Extract (g/l)	Cephamycin C (mg/ml)	
					Experimental[a]	Predicted
1	23.45	19.35	0.89	21.1	10.45 ± 0.34	10.52
2	19.15	6.55	0.735	5.55	10.25 ± 0.25	10.34
3	11.6	24.15	2.895	19.7	10.05 ± 0.24	10.25
4	15.9	21.55	2.04	24.85	9.55 ± 0.35	9.95

[a] results are mean ± SD of three determinations

Table 4. The CCRD matrix of independent variables in coded form with their corresponding response from experiments and predicted

Std run	Amino acids, %				Cephamycin C (mg/ml)	
	L-lysine	L-valine	L-cysteine	DL-methionine	Experimental [a]	Predicted
1	1 (3.0)	1 (0.9)	1 (1.5)	-1 (0.4)	13.06 ± 0.21	13.05
2	1 (3.0)	1 (0.9)	-1 (0.5)	-1 (0.4)	12.76 ± 0.32	12.75
3	1 (3.0)	-1 (0.3)	1 (1.5)	1 (1.2)	12.61 ± 0.01	12.55
4	-1 (1.0)	1 (0.9)	-1 (0.5)	1 (1.2)	13.21 ± 0.21	13.26
5	1 (3.0)	-1 (0.3)	-1 (0.5)	1 (1.2)	12.96 ± 0.54	12.99
6	-1 (1.0)	-1 (0.3)	1 (1.5)	-1 (0.4)	12.12 ± 0.26	12.13
7	-1 (1.0)	1 (0.9)	1 (1.5)	1(1.2)	12.96 ± 0.25	12.92
8	-1 (1.0)	-1 (0.3)	-1 (0.5)	-1 (0.4)	13.19 ± 0.31	13.19
9	-2 (0.0)	0 (0.6)	0 (1.0)	0 (0.8)	13.16 ± 0.15	13.14
10	2 (4.0)	0 (0.6)	0 (1.0)	0 (0.8)	13.03 ± 0.24	13.05
11	0 (2.0)	-2 (0.0)	0 (1.0)	0 (0.8)	12.93 ± 0.34	12.93
12	0 (2.0)	2 (1.2)	0 (1.0)	0 (0.8)	13.16 ± 0.51	13.16
13	0 (2.0)	0 (0.6)	-2 (0.0)	0 (0.8)	13.03 ± 0.16	12.99
14	0 (2.0)	0 (0.6)	2 (2.0)	0 (0.8)	12.18 ± 0.15	12.22
15	0 (2.0)	0 (0.6)	0 (1.0)	-2 (0.0)	12.12 ± 0.18	12.12
16	0 (2.0)	0 (0.6)	0 (1.0)	2 (1.6)	13.24 ± 0.27	13.24
17	0 (2.0)	0 (0.6)	0 (1.0)	0 (0.8)	13.32 ± 0.21	13.32
18	0 (2.0)	0 (0.6)	0 (1.0)	0 (0.8)	13.37 ± 0.53	13.32
19	0 (2.0)	0 (0.6)	0 (1.0)	0 (0.8)	13.32 ± 0.14	13.32
20	0 (2.0)	0 (0.6)	0 (1.0)	0 (0.8)	13.28 ± 0.16	13.32
21	0 (2.0)	0 (0.6)	0 (1.0)	0 (0.8)	13.33 ± 0.42	13.32

[a] results are mean ± SD of three determinations
[b] Values in parentheses are uncoded variables

Table 5. Analysis of variance (ANOVA) for the experimental results of the central-composite design (Quadratic Model)

Factor[a]	Coefficient Estimate	Sum of squares	Standard Error	DF[b]	F value	p[c] (Prob >F)
Model (or Intercept)	13.324	3.13	0.019	12	122.51	< 0.0001*
A	-0.032	0.008	0.015	1	4.62	0.0749†
B	0.057	0.026	0.015	1	14.49	0.0089*
C	-0.191	0.58	0.010	1	322.73	< 0.0001*
D	0.28	0.62	0.015	1	343.63	< 0.0001*
A^2	-0.056	0.081	0.008	1	44.57	0.0005*
B^2	-0.069	0.121	0.008	1	66.30	0.0002*
C^2	-0.179	0.808	0.008	1	442.74	< 0.0001*
D^2	-0.160	0.648	0.008	1	355.05	< 0.0001*
AB	0.203	0.166	0.021	1	90.97	< 0.0001*
AC	0.158	0.201	0.015	1	110.46	< 0.0001*
AD	-0.081	0.026	0.021	1	14.46	0.0089*
BC	0.183	0.270	0.015	1	147.99	< 0.0001*

[a] A=L-Lysine hydrochloride (%), B = L-valine (%), C = L-cystine (%) and D = DL-Methionine (%)
[b] Degree of freedom [c] † p >0.05, not significant; * p < 0.05, significant; $R^2 = 0.995$

Table 6. Validation of the model explaining the effect of amino acids on the fermentative production of cephamycin C by *S. clavuligerus* NT4 in submerged media

Sr. No.	L-lysine hydro-chloride (%)	L-valine (%)	L-cysteine (%)	DL-methionine (%)	Cephamycin C (mg/ml)	
					Experimental[a]	Predicted
1	0.59	0.387	0.785	0.956	13.31 ± 0.35	13.70
2	0.18	0.285	0.13	0.54	13.26 ± 0.41	13.71
3	1.07	0.339	0.12	1.06	13.64 ± 0.23	13.84
4	0.27	0.564	0.165	1.052	13.45 ± 0.32	13.75

[a] results are mean ± SD of three determinations

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Genotype and Allele Frequencies of *DGAT* 1 Gene in Indian Holstein Bulls

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Abstract

A Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) test was performed to investigate the allele frequencies of the diacylglycerol o-acyltransferase 1 gene (*DGAT* 1) in Indian Holstein Friesian (HF) bulls. Recent studies indicated that the *DGAT* 1 gene is responsible for fat production and found on centromeric region of bovine chromosome 14. Because of polymorphism, the *DGAT* 1 gene has two major genetic variants (K and A) which occurred due to an amino acid substitution in the candidate gene. The allele (K) encoding the lysine proved to be more efficient with regard to the milk fat synthesis is obviously useful markers for milk fat traits on which bull can be evaluated and selected for future breeding programmes. DNA samples were extracted from blood samples of 281 HF bulls stationed at various sperm stations across the country. A 411 bp fragment of *DGAT* 1 was amplified and digested with *Cfr*1 restriction enzymes. Two types of alleles K and A and three types of genotypes KK, AA and KA were observed in the study. The results indicated allelic frequency of K-allele (0.59) was higher compared to A-allele (0.41) for *DGAT* 1 in Indian HF population.

Key words: *DGAT*1, HF, Milk fat, QTL, PCR-RFLP, Genotype.

Introduction

Milk fat is composed primarily of triglycerides or triacylglycerides which accounts for 98% of the total fat by weight. The synthesis of triglycerides is catalysed by acyl CoA diacylglycerol acyltransferase (*DGAT*). A quantitative trait locus (QTL) affecting milk fat percentage has been mapped to the centromere region of the bovine chromosome 14 (1). This genomic area includes the *DGAT* 1 gene, which encodes acyl CoA diacylglycerol acyltransferase 1. The enzyme participates in a fundamental role in metabolism of cellular diacylglycerol and is important in higher eukaryotes for physiologic processes involving triacylglycerol metabolism, such as intestinal fat absorption, lipoprotein assembly, adipose tissue formation, and lactation (2). It was shown that QTL variation is most likely caused by a nonconservative two bases substitution (AA→CG) in the candidate gene *DGAT* 1 changing lysine to alanine (K232A) in the enzyme acyl CoA diacylglycerol acyltransferase (1,3). The allele encoding the lysine 232 variant proved to be more efficient with regard to milk fat synthesis and other milk characteristics in the Dutch, Poland, New Zealand, German, Israeli Holstein cattle (1, 4, 5, 6 and 7). The nucleotide variation underlying the K232A substitution can be determined by PCR-RFLP assay. The objective of the present study was to characterize the *DGAT* 1 variants and to

estimate the allele frequency of *DGAT* 1 gene in Indian Holstein Friesian (HF) bulls used for artificial insemination (AI) programmes.

Materials and Methods

Blood samples were collected from 281 HF bulls stationed at different semen collection centres across the country. The DNA was extracted from blood cells by phenol chloroform method. The quality and quantity of DNA was estimated using spectrophotometer and agarose gel electrophoresis. As described by Winter et al. (3), the 411 bp DNA fragment was amplified by Polymerase chain reaction (PCR), which was set by adding sense primer: 5' GCA CCA TCC TCT TCC TCA AG 3' and antisense primer: 5' GGA AGC GCT TTC GGA TG 3'). The PCR mix contained 1X PCR buffer, 0.4 mM dNTPs, 1 Unit of *Taq* DNA Polymerase, 0.4 pM each of sense and antisense primer, 50 ng genomic DNA and sterilized distilled water to make a final volume of 25 µl. The PCR reaction included the following steps: Predenaturation for 1 minute at 94°C followed by 30 cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds and final extension for 10 minutes at 72°C. The PCR product of 411 bp was seen on 2.5% agarose gel. The amplified PCR product was digested by using *Cfr*I restriction enzyme and 1X reaction buffer at 37 °C for overnight. The digested product was loaded and visualized on 2.5% agarose gel after staining with ethidium bromide. The genotype and allele frequencies were estimated by direct counting.

Results and Discussion

The restriction digestion analysis of 411 bp PCR product of *DGAT* 1 indicates the presence of three restriction patterns. In the first pattern, one fragment (uncut) of 411 bp was observed while in the second pattern two fragments 208 and 203 bp was observed. The third pattern produced three fragments 411, 208, 203 bp, which was the coupling of first and second pattern, in other words it is a heterozygote (Fig. 1). Consistent with Pareek et al. (4), the first pattern

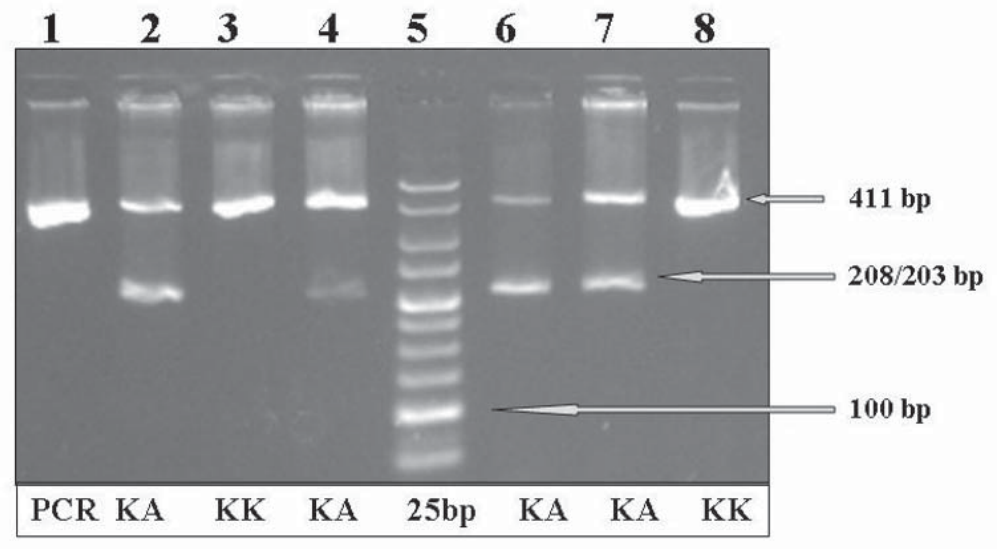

Fig. 1. Electrophoretogram of *Cfr* I digested PCR product generated by amplification of genomic DNA, using DGAT specific primers. Lane #1:PCR product of 411 bp. Lane # 2, 4, 6 & 7: 411, 208, and 203 bp for KA variant. Lane # 3 & 8: 411 bp (uncut) for KK variant. Lane # 5: 25 bp DNA ladder.

was assigned as genotype KK (lysine variant); second pattern as genotype AA (alanine variant) and the third as genotype KA (heterozygous).

The results mentioned in the table reveal that KK genotype frequency (0.21) for *DGAT* 1 was higher than AA (0.03) but lower than KA genotype (0.76). The similar observation was also recoded in American Holstein (8). In present study, the allelic frequency of *DGAT* 1 K-allele (0.59) was higher than A-allele (0.41), indicating that more than fifty percent Holstein bulls in India have K-allele for higher fat (Table. 1). The similar observation (0.60) was recoded in Poland black and white AI sires (4), whereas they observed higher allele frequency in young bulls (0.68) and low in cows (0.48). In the Holstein cattle, allele frequencies reported for lysine variant range from 0.30 in New Zealand to 0.63 in Dutch populations (9). The *DGAT* 1 allelic frequency in Holstein cattle may be greatly influenced due to origin of the genetic material. Lot of Holstein cattle and their frozen semen were imported in past from various countries mainly to enhance milk production through crossbreeding with local breeds of India, which could be possible reason of polymorphism in *DGAT* 1 gene in Indian Holstein. In India, where milk is priced by the fat and SNF contents and bulls are selected based on their dams' milk and fat yield, *DGAT* 1 lysine variant may serve as a criterion to asses the breeding value of bull. Hence, it is also likely to increase allele frequency of lysine variant in Holstein population of India in future. Recent studies indicated that *DGAT* 1 locus is represented by more than one QTL regions and multiple QTL alleles on chromosomes 14 affecting fat content of milk in cattle (10,11) may also be taken under consideration while assessing breeding value of a bull for fat content.

Table 1. Genotypes, genotype frequency and allelic frequency of *DGAT* 1 gene

HF bulls	Genotype		
281	KK	AA	KA
Total genotype	59	8	214
Genotype frequency	0.21	0.03	0.76
Allele frequency	0.59	0.41	

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Purification and characterization of Human Intestinal alkaline phosphatase and its role in the colonization of *Helicobacter pylori* in the duodenum

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Abstract

Intestinal alkaline phosphatase (IAP) from normal duodenum was concentrated by 0-35% ammonium sulphate and was fractionated in DEAE cellulose column and thus, partially purified IAP was purified by passing through Sephadex G-75 column in all the steps the purification was monitored through enzyme activity. The purity of the IAP was established on C-18 RPHPLC which gave single peak at a retention time of 15 minutes and SDS-PAGE analysis showed single band with molecular weight of 66 KD. Further, PAS staining confirmed the glycoprotein nature of IAP. It is very well known that IAP dephosphorylates lipid-A moiety of lipopolysaccharide layer present in the gram-negative bacteria and protects from gram negative sepsis. However, in the present study it was observed that colonisation of *H. pylori* in the duodenum showed decrease in the intestinal alkaline phosphatase activity and increased Km (IAP from normal tissue 0.98 µM of PNP/ml/min and Km =0.4µM. IAP from *H. pylori* infected tissue and Km = 0.48 µM of PNP/ml/min and Km 1 µM). The reverse-transcript PCR results indicated that, the expression of IAP was normal in both *H. pylori* infected tissue and normal intestinal tissue. Therefore, it can be concluded that colonisation of *H. pylori* in the intestine resulted in the lowering of IAP activity.

Keywords: *H. pylori,* Intestinal alkaline phosphatase, Sepsis, Colonisation.

Introduction

Alkaline phosphatase activity is found essentially in all tissues. In almost all mammals, the most abundant isozyme of alkaline phosphatase is the one found in liver, bone and kidney called non tissue specific alkaline phosphatase (AP). A second isozyme found in greatest abundance in the intestine (IAP) of all mammals in particular human beings. In humans and higher primates a third type of isozyme is present in the placenta called placental alkaline phosphatase (PLAP). Human IAP has been cloned and sequenced. The tissue non-specific AP is located on the chromosome 1 and IAP is present on chromosome 2q.37.1, (1, 2).

The levels of AP vary in ABH secretors and non-secretors. ABH non-secretors have low levels of IAP and are therefore, more prone to duodenal and peptic ulcers (3, 4). However, infections caused due to *Helicobacter pylori*, one of the most common bacterial pathogens of humans, are not dependent on ABH alleles (4). *H. pylori* colonize in the gastric mucosa, where it appears to persist throughout the host's life unless the patient is treated. Colonization induces chronic gastric inflammation which can progress

to variety of diseases ranging in severity from superficial gastritis and peptic ulcer to gastric cancer and mucosal associated lymphoma. Strain-specific genetic diversity has been proposed to involve in the organisms ability to cause different diseases in the infected host. *H. pylori* cause more than 90% of duodenal ulcers and up to 80% of gastric ulcers (3, 5-7).One of the major functions of IAP is protection of intestine and stomach from the infections of gram negative bacteria. One of the most life-threatening lipopolysaccharide (LPS) mediated diseases, Gram negative sepsis, is characterized by excessive production of pro-inflammatory cytokines, activation of proteolytic cascades, coagulation abnormalities (8), and hemodynamic responses, resulting in hypotension, poor tissue perfusion, and multi-organ failure (9,10). IAP dephosphorylates lipid A moiety of LPS layer of gram negative bacteria thereby, protecting the human beings from severe inflammatory responses generated due to this LPS (11). In spite of IAP clearing the infections caused due to gram negative bacteria, how *H. pylori* a gram negative bacteria overcomes the action of IAP and colonizes in the intestine, causing severe gastric and duodenal ulcers leading to cancerous condition? In order to address this question in the present study we have observed the expression of IAP in the *H. pylori* infected tissue whether the expression of IAP is down regulated or the activity of IAP is down regulated when compared with the normal IAP.

Material and Methods

Human duodenal tissue was obtained from Department of Pathology, SVIMS, and Tirupati

1. Histology: Duodenal biopsy specimens were fixed in 10% formalin. Paraffin sections were cut, Warthin Starry silver and haematoxylin and eosin stained and scored for *H. pylori* microscopically by adopting upgraded Sydney classification (12-14), were considered as the samples for the expressional studies of intestinal alkaline phosphatase (IAP).

2. Reverse-Transcript Polymerase chain reaction (RT-PCR): The total mRNA from both normal duodenal tissue and *H. pylori* infected duodenal tissue and first strand synthesis was carried using AMV-reverse transcriptase (Promega). Primers for this experiment were constructed from the cDNA clone of human IAP (2), Forward primer 5'-ACTTGGG TGGATC AGGACAC-'3 and Reverse primer 5'-TCTGAGTGGCTGTGACT TGG-'3. RT-PCR was performed in the (90^0C for 60s, 55^0C for 30s and 72^0C for 30s for 45 cycles) Eppendrof Mastercycler gradient and the obtained PCR product was analysed by running 1% agarose gel electrophoresis (15).

3. Purification and characterization of Human Intestinal Alkaline Phosphatase

3.1 Homogenization: 2gms of Human duodenal tissue was homogenized in 0.1 M Tris-HCl, pH 7.4 that contain 0.25 N Sucrose. The homogenate was centrifuged at 1200 rpm for 10 minutes at 4^0C in order to remove all the cell debris. The supernatant was used as a starting material for the isolation and purification of IAP and for expression studies (16).

3.2 Enzyme Assay

The 3ml reaction mixture contains 800µl of 0.1M Carbonate-bicarbonate buffer pH 10.0, 2 ml p-Nitrophenyl phosphate and mix thoroughly. At zero time, add 200µl of enzyme. The absorbance was measured at 405nm against blank. Enzyme activity was expressed as concentration of product (PNP) formed per minute per ml. In order to check the maximum velocity of the enzyme varying concentrations of substrate p-Nitrophenyl phosphate from 1mM to 10mM were taken and enzyme assay was performed (17,19).

Determination of K_M: Michaelis-Menton constant for IAP in the normal tissue and IAP in

H. pylori infected duodenal tissue were determined by Hanes – Woolf plot using [S] vs. [S]/ V(17, 19).

3.3 Purification of Human IAP

From the crude extract the enzyme were purified in the following manner

3.3.1 $(NH_4)_2SO_4$ Fractionation: 0-35% $(NH_4)_2SO_4$ was added to concentrate the IAP from the tissue extract and the solution was centrifuged at 10,000 rpm for 10 min. The pellet was suspended in 2ml of 0.1M Tris-HCl pH 7.2, and dialysed against the same buffer for overnight with intermittent changing of the buffer. The enzyme assay was performed as mentioned in 3.2. The enzyme was concentrated in speed vac concentrator.

3.3.2 DEAE Cellulose Chromatography: IAP was further fractionated on DEAE cellulose column. 35µg of 0-35% $(NH_4)_2SO_4$ concentrate was loaded on DEAE Cellulose column and IAP eluted with stepwise gradient of NaCl concentration prepared in 0.1M Tris-HCl pH 7.2. IAP was eluted using NaCl gradient 50mM to 200mM concentration. The peak fractions in each gradient were assayed using enzyme assay mentioned in 3.2. IAP was eluted in 100mM NaCl gradient and this was dialysed against 0.1M Tris-HCl pH 7.2. The dialysed peak fraction was concentrated using speed vac concentrator (1, 16, 23 and 26).

3.3.3 Gel filtration: 25µg IAP eluted at 100mM NaCl concentration from DEAE cellulose column was loaded on Sephadex G-75 (30cm x 1.5cm) which was swollen in 0.1M Tris-HCl pH 7.2 and the IAP was eluted with 0.1M Tris-HCl pH 7.2 containing 500mM NaCl. Each peak fractions were assayed using enzyme assay mentioned in 3.2 (1, 16, 23 and 26).

3.3.4 Reverse Phase HPLC purification of IAP: 5µg active fraction of IAP obtained from gel filtration was purified on reverse phase C-18 column (4.6 x 150x 5 microns) HPLC (Shimadzu) equilibrated with 0.1% trifluoroacetic acid (TFA) and elute with a linear gradient of acetonitrile containing 0.1% TFA. (1and16).

3.4 Characterization of IAP

Molecular weight determination of IAP

The molecular weight of purified IAP was determined by running 7.5% SDS-PAGE and the gel was stained with 0.125% Coomassie Brilliant blue R250. 2.5µg of purified IAP was applied, also 25µg protein from *H.pylori* infected human duodenal tissue and 25µg protein from normal human duodenal tissue was applied to assess the presence of IAP isozymes. Standard molecular weight markers of molecular weights 96, 66, 43, 29, 20, and 14 KD obtained from Bangalore Genei Pvt Ltd were used in the calculation of IAP molecular weight. The glycoprotein nature of IAP was detected by staining with Periodic acid Schiff (PAS) stain (18 and 23).

The protein concentrations in all steps were determined by Bradford, 1976 method (25)

Results and Discussions

It is being observed that alkaline phosphatase present in the intestine prevents several gram negative bacteria to colonize in the intestine thus, unable to establish its pathogenicity in the host organism. However, this scenario changes dramatically in the ABH non-secretors where lower levels of IAP have been observed and are therefore, not only prone to duodenal ulcers but are also more prone to infections caused due to gram negative bacteria (4). It has been demonstrated that calf intestinal alkaline phosphatase, has provided protection against various gram-negative bacteria by de-phosphorylating the lipid A moiety of LPS (20, 21). This has thrown the importance of this enzyme in clearing infections posed by gram negative bacteria (22). If high levels of IAP are expressed in the human intestine what happens to IAP activity during *H. pylori* infection? The

present study was aimed to identify this question. In the duodenal biopsy material *H. pylori* presence was confirmed by haematoxylin and eosin staining detected as spiral shaped organisms (13).

Expression levels of IAP in the *H. pylori* infected tissue was studied in two ways one through levels of IAP mRNA present in the normal and in the infected tissue and second by studying the IAP enzyme activity in the infected and in the normal tissue. Primers for the IAP detection were constructed from the IAP gene sequence (2).the reverse transcript polymerase chain reaction showed presence of 0.2 Kb products in both normal and infected tissue indicated the expression of IAP was normal in the infected tissue [Fig-1].

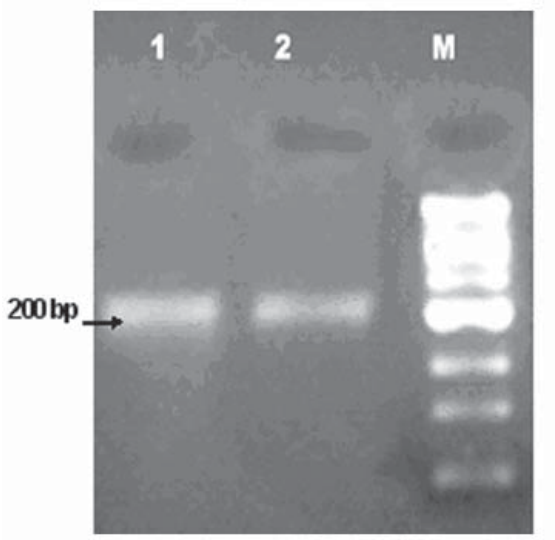

Fig. 1. Agarose gel electrophoresis showing RT-PCR amplification of intestinal alkaline phosphatase gene from *H. pylori* infected duodenal tissue. Lanes: M, 100bp molecular size ladder (Bangalore Genei); 1, RT-PCR product of IAP gene from normal duodenal tissue 2,RT-PCR product of IAP gene from *H. pylori* infected duodenal tissue

IAP was isolated and purified from normal duodenal tissue [Table-1 and Fig 2] and the purification of the enzyme was monitored through its activity. The 0-35% $NH_4(SO_4)_2$ concentrated IAP was purified by passing through DEAE cellulose column. IAP was eluted at 100mM NaCl concentration. IAP was further fractionated on Sephadex G-75 column and the first peak showed highly pure IAP. The purity of IAP was further confirmed on C-18 RP-HPLC where protein was eluted at a retention time of 15 minutes confirming highly hydrophilic nature of the protein. The molecular weight of the purified IAP was found to be 66 KD [Fig 3] and the glycoprotein nature was confirmed by the PAS stain (1, 2, 16, 23 and 26). Molecular mass of normal intestinal alkaline phosphatase (NIAP) is present in the serum of both secretors and non-secretors, regardless of ABO blood group. However, the high molecular mass intestinal alkaline phosphatase only appears in serum of Le (a-b+) blood group secretors and these persons are not prone to *H. pylori* infection (4). The SDS-PAGE analysis of IAP present in normal and *H. pylori* infected intestinal tissue was found to be same with no apparent change in the molecular weight of IAP this indicated that *H. pylori* infection did not affect the IAP and the same IAP was expressed in normal and in *H. pylori* infected intestinal tissue [Fig 3]. However, the enzyme activity of IAP in the *H. pylori* infected intestinal tissue was almost half of the normal IAP (IAP from normal tissue 0.98 µM of PNP/ml/min, IAP from *H. pylori* infected tissue 0.48 µM of PNP/ml/min). The K_M of IAP of normal tissue was found to be 0.4mM while IAP from the *H. pylori* infected intestinal tissue was found to 1mM (Table-2). This clearly indicated that the colonization of *H. pylori* in the duodenum has a profound effect on the activity of IAP. This observation can be corroborated with the fact that the patients infected with *H. pylori* when treated with Clarithromycin showed improved IAP activity (24). Therefore, it can be concluded that *H. pylori* colonisation in the duodenum resulted in the lowering of IAP activity in the intestine.

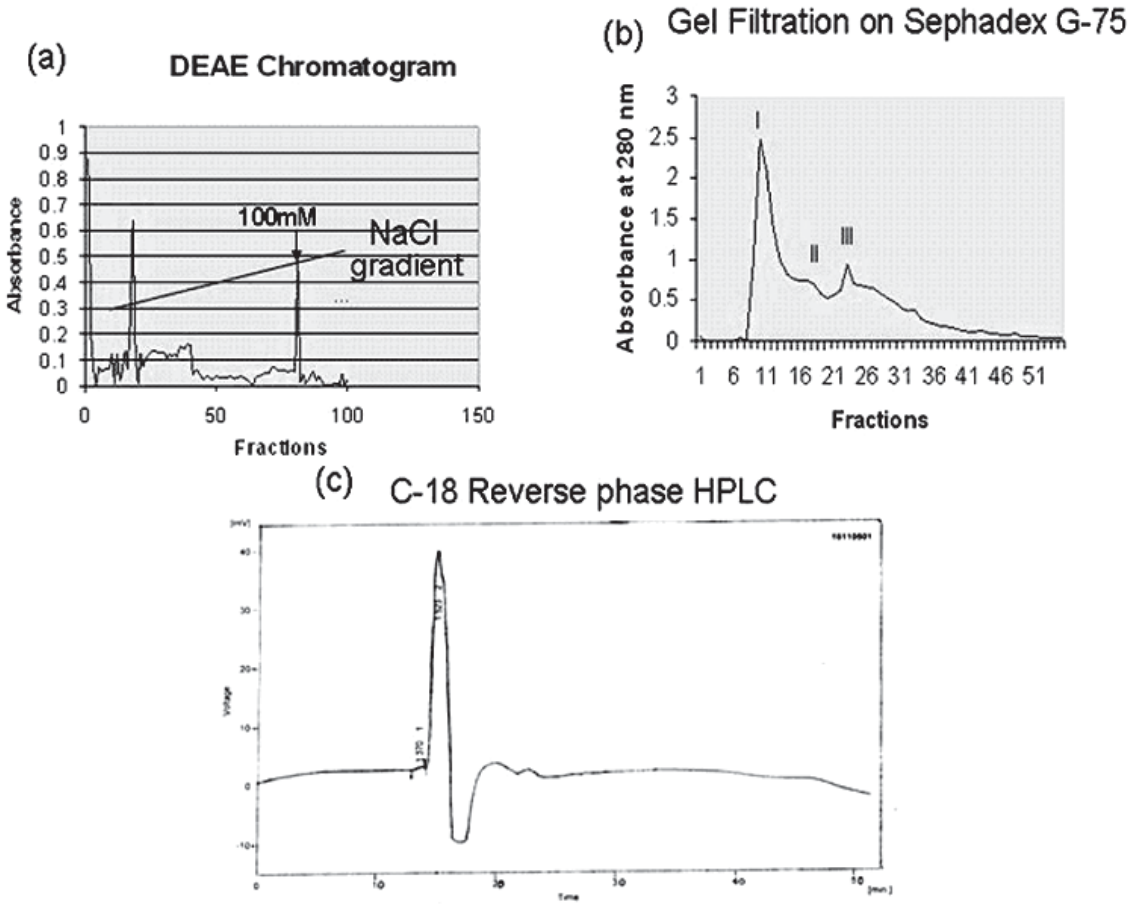

Fig. 2. (a) Anion exchange chromatogram (DEAE cellulose) and IAP was eluted at 100mM NaCl concentration. (b) Gel filtration on Sephadex G-75, I. IAP was eluted with 0.1M Tris-HCl pH 7.2 containing 500mM NaCl. (c) C-18 RP HPLC and IAP was eluted at a retention time of 15 minutes.

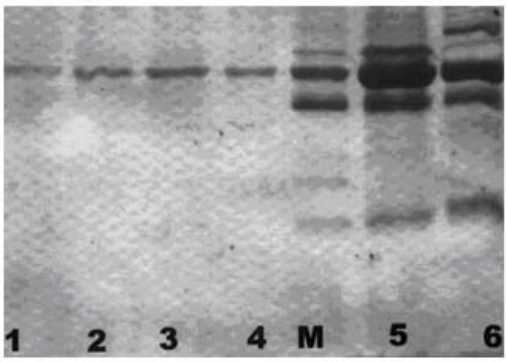

Fig. 3. Electrophoretogram showing the purification of human intestinal alkaline phosphatase 7.5% SDS-PAGE gel and the gel was stained with 0.125% coomassie brilliant blue R250. 2.5µg of purified protein was applied in 1-4 lanes, lane 5, 25µg protein from *H.pylori* infected human duodenal tissue, lane 6, 25µg protein from normal human duodenal tissue. Lane 1 IAP obtained from RP-HPLC column, lane 2 and 3 IAP obtained from gel filtration and lane 4 IAP obtained from DEAE cellulose column, lane M Molecular weight markers obtained from Bangalore Genei Pvt Ltd (96KD, 66KD, 45KD, 29KD, 21KD, 14KD).

Table 1.

S.No.	Sample	Protein concentration µg/ml	Enzyme Activity micromoles of PNP/ml/min.	Specific activity micromoles of PNP/min/mg	Folds purification	Percent recovery
1	Crude (intestinal Homogenate)	195.58	0.98	0.227	-	
2	0-35% $(NH_4)SO_4$	35	9.10	0.377	10	17
3.	DEAE cellulose purified fraction (IAP)	4.13	18.40	4.03	20	12
4	Sephadex G-75 (30cm x1.5cm)	3.0	27.60	4.87	30	12
5.	C-18 RP-HPLC (4.6 x 150x 5 microns)	4.0	45	5.27	45	80

Table 2.

Tissue	Activity µM of PNP/ml/min	K_M (mM of PNPP)
Normal duodenal tissue	0.98	0.4
H. pylori infected duodenal tissue	0.48	1

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Antiproliferative and antiangiogenic effects of partially purified *Luffa acutangula* fruit extracts on human lung adenocarcinoma epithelial cell line (A-549)

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Abstract

The present study was undertaken to evaluate the possibility of *Luffa acutangula (cucurbitaceae* family*)* fruit as a potential anticancer agent by examining antiproliferative and antiangiogenic activities. Fruit methanolic extract showed significant antiproliferative activity (IC-50, 131.63±2.31 µg/ml) on human lung adenocarcinoma epithelial cell line (A-549). The extract was partially purified by chromatography. Fraction F2-3 showed most potent antiproliferative activity (IC-50, 7.61±1.03 µg/ml) and was further evaluated for antiangiogenic activity by evaluating vascular endothelial growth factor (VEGF), matrix metalloproteinases-2 (MMP-2) and matrix metalloproteinases-2 (MMP-9) as in-*vitro*, chick chorioallantoic membrane (CAM) as an *in-vivo* model for VEGF. VEGF and both MMP protein expressions were significantly inhibited in F2-3 treated A-549 cells compared to control cells (VEGF: 4.36±0.47 and 14±0.75 pg/ml, MMP-2: 10.17±1.3 and 20.28±1.68, MMP-9: 12.93±1.70 and 21.12±2.12 ng/ml, respectively). Egg chorioallantoic membrane treated with F2-3 fraction (10 µg) showed clear avascular zones compared to phosphate buffered saline (PBS) treated eggs. In conclusion, our data provides a scientific proof for *Luffa acutangula* as a potential antitumor agent.

Keywords: Vascular endothelial growth factor (VEGF), Matrix metalloproteinases MMP), Extracellular matrix (ECM), Chick chorioallantoic membrane (CAM) model.

Introduction

A new blood vessel sprouting from nearby pre-existing capillary is generally called as angiogenesis (1). It plays a crucial role in both physiological (wound-healing, acute injury healing, healing of chronic ulceration of the gastrointestinal mucosa, in embryonic development, female reproductive cycle etc) and pathological (tumors, rheumatoid arthritis, retinopathy of prematurity, etc.) aspects (2-5). Angiogenesis is considered to be the crucial step for tumor proliferation, expansion and metastasis. During avascular stage (<0.5 mm size), tumor does not need any vessels to carryout the function like transport of O_2, CO_2, nutrients, etc. as these function are taken by means of diffusion. Beyond 0.5 mm, tumor needs vesicular vessels to carryout the transportation process for its survival (6, 7). In this avascular stage, tumor can not survive without vessels and gases cannot diffuse to the tumor.

Vascular endothelial growth factor (VEGF) is one of the first angiogenic factor and mostly regulates both normal and pathological

angiogenesis. Loss or inactivation of tumor suppressor gene and activation of oncogene are associated with increased levels of VEGF in cancer cells (8,9). Many tumors are reported to have mutations in the p53 gene and ultimately loss the cell cycle regulation associated with high level of VEGF (10,11).

Matrix metalloproteinases (MMP) are a large family of proteolytic enzymes and play important role in tumor metastasis and cell invansion by acting as pro-enzyme in normal physiological conditions secreted from a variety of cells. These proteins are over expressed and cause extracellular matrix (ECM) protein precipitation and also facilitate metastasis and invasion (12,13). Both MMP-2 and MMP-9 are over expressed in invasion prostate cancer and endothelial cells (14,15). MMP-9 (gelatinase-B) is the member of MMPs that denatures the collagenase IV which is the major component of the basement membrane (laminin). MMP-2 is structurally related to MMP-9 and cleaves ECM proteins such as collagen type I and IV. Angiogenesis in tumor progression became a new strategy for cancer therapy (16), but many angiogenic inhibitors are available which might act non-specifically causing various side effects. Some non-toxic phytochemicals viz., gastrodiaelata blume (17) and sedum sarmentosum bunge (18) were reported to have no affect on the normal cells and act as antiangiogenesis (indirectly antitumerogenic).

In this study, effect of fruit methanolic fractions of *Luffa acutangula* on angiogenesis activity was studied by measuring the VEGF, MMP-2 and MMP-9 as *in vitro* and CAM as *in vivo* model. *Luffa acutangula* belongs to *Cucurbitaceae* family which is having laxative and antidiuritic property used as traditional medicine in India. None of its therapeutic potentials are scientifically evaluated except ribosome inactivating activity (19).

Materials and Methods

Extraction

Around 300 g of fresh plant material (fruit) was washed with tap water, air dried and then chopped into small fragments which were shade dried and reduced to coarse powder with mortar and pestle. The powdered materials were extracted three times each with hexane (2.5 l), and methanol (2.5 l) followed by distilled and deionised water (1.0 l) at room temperature in a cycle of 48 h each on orbital shaker. The combined hexane and methanolic extracts were then concentrated in rotavapour at reduced pressure, below 40^{o}C and pooled water extracts were concentrated by lyophilization.

Partial purification

Since methanolic extract showed significant antiproliferative activity, this extract was further fractionated chromatographically. Initially, 15 g of extract was loaded on a silica column (column height and diameter were 24 and 2 inches, respectively) and was eluted with stepwise gradient elution of ethyl acetate-methanol (4:1?0:1, v/v). Four fractions were collected at regular intervals and named as F1, F2, F3 and F4. Antiproliferative activity of these fractions was evaluated in MTT assay (20) and it was observed that fraction F2 only was showing significant activity. Hence, this fraction was further fractionated with the solvent system methanol-water (4:1?0:1, v/v). Four fractions were collected at regular intervals and named as F2-1, F2-2, F2-3 and F2-4.

HPTLC fingerprinting

Fingerprinting of *Luffa acutangula* was performed on HPTLC plates (5x10 cm) coated with 0.25 mm layer of silica gel (60F254; Merck, Germany). The plates were washed with methanol and activated at 110^{0}C for 5 min before the samples were applied as 4 mm and 6 mm wide bands by using a Camag (Muttenz, Switzerland) and Linomat IV sample applicator equipped with

a 100 µl syringe. A constant application rate of 6 µl/sec was used. With mobile phase of benzene: acetone (60:40) along with vanillin and H_2SO_4 as reducing agent. Chromatograms were recorded at 600 nm.

Phytochemical screening

Partially purified extracts that showed significant cytotoxicity were screened for the presence of alkaloids, tannins, terpenoids, glycosides, flavonoids, saponins, anthraquinones and steroids. Chemical tests were carried out on the methanolic extracts using standard procedures to identify the constituents as described in the literature (21-23).

Cell culture conditions

Human lung cancer cell lines were brought from the Center for Cellular and Molecular Biology (CCMB), Hyderabad, India and were cultured in RPMI-1640 medium (Himedia, India). The media were supplemented with 10% fetal bovine serum (FBS), 10 mg/l penicillin–streptomycin (Sigma) and 2 mg/l gentamicin (Sigma) and maintained at 37^0C in 5% CO_2.

Antiproliferative effects of L. acutangula extracts

Antiproliferative activity of the extracts was evaluated according to the method described elsewhere (Reddy et al., 2008). The cells were seeded in clear 96-well microplate at a density of 20,000 cells per well and left overnight to recover from handling stress. After 12 h, the medium was replaced with fresh medium containing various concentrations of crude extracts (hexane, methanol and water) of *L. acutangula* fruit (hexane and methanol concentrations will be below 0.1%). Following incubation for 24 h, medium was removed and replaced with sterile MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] solution (50 µg/100 µl in DMEM), incubated for another 3 h and then the medium was discarded and replaced with 200 µl of DMSO. Formazan crystals formed were read at 570 nm using SPECTRAmax PLUS®, Molecular Devices, USA. IC-50 values (IC-50 values were taken from the formula Y= mx+C)) were plotted from the results. Since the methanolic extract showed significant antiproliferative activity, this crude extraction was fractionated as described under the section *Partial purification* and these fractions (F1, F2, F3 & F4) were screened for antiproliferative activity at a concentration range of 6.25-100 µg/ml as described above. Fraction F2 was active for antiproliferative activity and this fraction was further fractionated as described in the section *Partial purification* and these fractions (F2-1, F2-2, F2-3 & F2-4) were again subjected to MTT assay at a concentration range of 1.25 to 25 µg/ml.

Detection of VEGF protein in supernatant of L. acutangula treated cells

A-549 cells were seeded in 6-well plate at a density of 0.5 million/well and left overnight to recover from the stress. Medium was replaced with serum free medium and incubated at 37^oC for another 24 h. Wells were marked as control and experimental groups. Later, the groups were given starvation medium along with various extracts (hexane, methanol and water extracts at 75 µg/ml) or methanolic fractions (F1, F2, F3 & F4 were at 25 µg/ml and F2-1, F2-2, F2-3 & F2-4 were at 5 µg/ml) and the former groups were incubated with starving medium alone. Control and experimental groups were incubated for 3 days and the supernatant samples were collected and analyzed for the presence of VEGF according to the ELISA Kit manufacture's instructions (R&D System, USA). Samples were analyzed in triplicate and a graph was plotted with time against the concentration of VEGF present in the supernatant (Fig 3). Since, partially purified methanolic fraction F2-3 showed significant activity this fraction was further evaluated for time dependant inhibition

Chick chorioallantoic membrane (CAM) Assay

The CAM was prepared according to the method described by Blebea and coworkers (24). On day three a small puncture was made on the wider side of the egg to which 2 ml of albumin was aspirated and the hole was covered with a tape. On day eight, a circular window approximately 1.5–2.0 cm in diameter was opened aseptically on the egg shell with a sterile scissor and forceps, exposing the part of the CAM containing the central vein. Then, the window was sealed with sterile parafilm and the eggs were replaced in the incubator for 24 h. On day nine, the test substance was dissolved in phosphate buffered saline (PBS) and a 10 µl of PBS aliquot was taken in the center of cover slip. The drug loaded cover slip was kept at a sterile place and allowed to air dry. Previously prepared egg window was opened by cutting off the tape. The air-dried cover slip containing the corresponding sample was then opened on the top of the CAM with the sample in direct contact with the CAM. The eggs were covered with tape and returned to the incubator. On the day twelve, the tape was removed and score the angiogenesis captured through microscope.

Effect of L. acutangula on MMP-2 and MMP-9 release

A549 cells were seeded in 6-well plate at a concentration of 0.5 million/well and incubated overnight. In experimental groups, media was replaced with fresh media containing methanolic Fraction F2-3 at 3 µg/ml whereas, control cells were not challenged with extracts. Both the groups were incubated for 3 days and a sample of 400 µl was collected from the supernatant at a regular interval of 8, 12, 36, 48 and 72 h, respectively. Collected supernatant was assayed for the quantification of MMP-2 and MMP-9 according to the instructions provided by the Kit manufacturers. Each concentration was tested in triplicate and the results were plotted with time against the concentration of the MMP-2 and MMP-9.

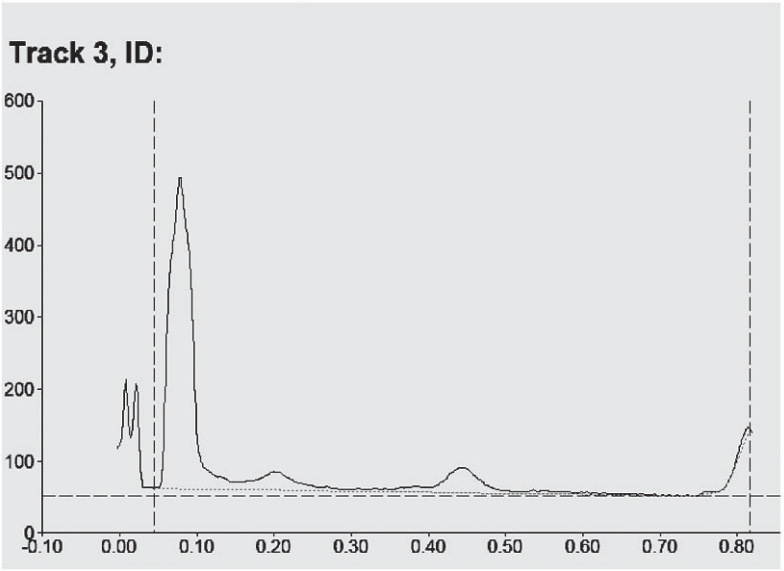


Fig.1. HPTLC chromatogram of fruit methanolic extract (mobile phase of benzene : acetone at (60:40); chromatogram was monitored at 600 nm; Y-axis legend, Rf; X-axis legend, AU).

Results

3.1. Extraction yield, fingerprinting and phytochemical studies

The extraction yield of hexane, methanol and aqueous extract of *L. acutangula* fruit was 32 %, 51.02 % and 16.98 %, respectively. HPTLC fingerprinting of *L. acutangula* fruit methanolic extract is presented in Fig 1. Methanolic extract of the fruit showed the qualitatively the presence of flavonoids, tannins, steroids and glycosides but the absence of alkaloids, anthraquinones and terpenoids. Chemical tests were carried out using standard procedures to identify the constituents as reported by (Sofowara, 1993; Trease and Evans, 1989; Harborne, 1973).

Antiproliferative effects of L. acutangula extracts

As a first step to examine whether the *L. acutangula* fruit extracts have the potential to be an anticancer agent, the antiproliferative activity of the extracts on Human lung adenocarcinoma epithelial cell line (A-549) was examined. The IC-50 values for hexane, methanol and water extracts of *L. acutangula* fruit against A-549 cell line was found to be 3071±12.22, 131.63±2.31 and 38934±50.34 µg/ml, respectively (Fig 2a). IC-50 values for methanolic extract fractions, F1, F2, F3, F4, F2-1, F2-2, F2-3 and F2-4 were found to be >1000, 75.1±3.5, >1000, >1000, >1000, >1000 >7.61±1.03 and 812.03±23.09 µg/ml, respectively (Fig 2b and 2c).

VEGF protein in supernatant of L. acutangula treated cells

To determine the antiangiogenic activity of the extracts, initially all the extracts were examined at a fixed concentration (75 µg/ml) for their inhibitory effects on VEGF secretion. The results showed that only methanolic extract, fraction 2

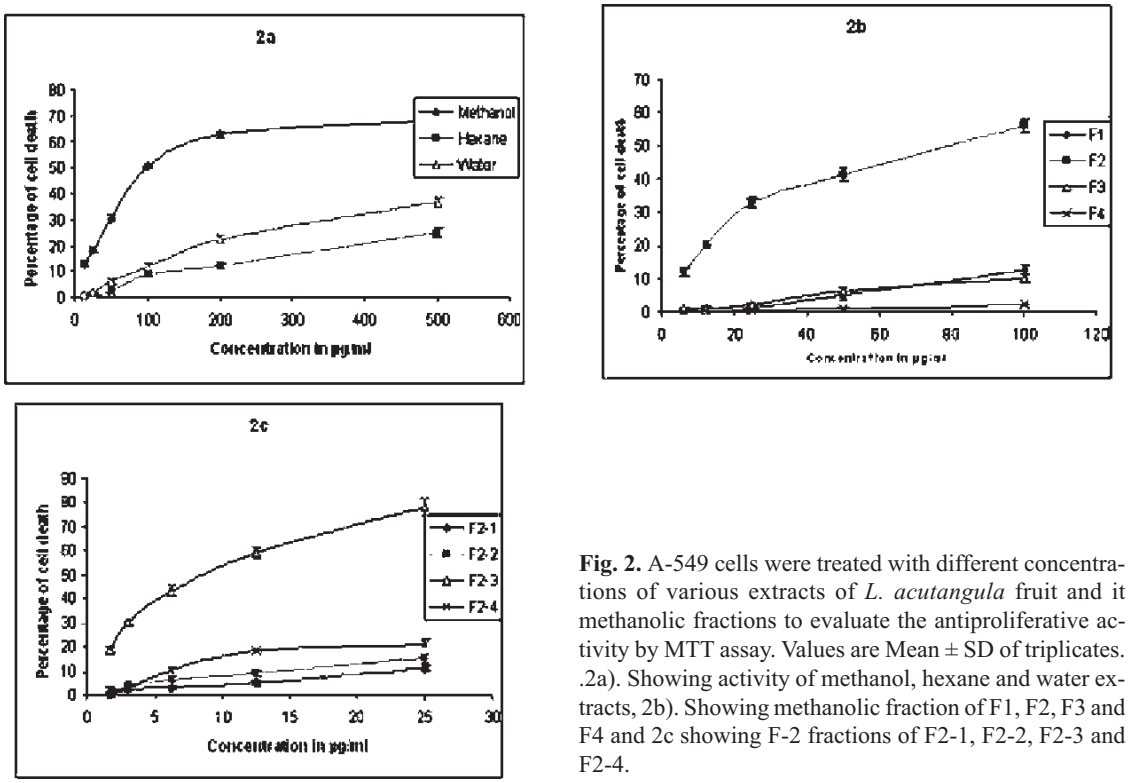
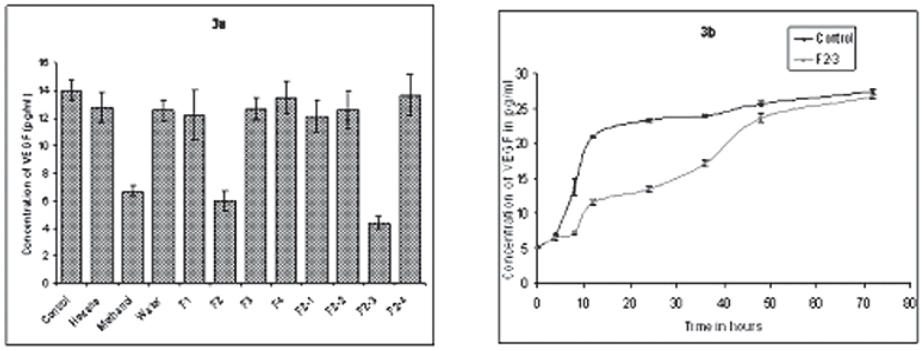


Fig. 2. A-549 cells were treated with different concentrations of various extracts of *L. acutangula* fruit and it methanolic fractions to evaluate the antiproliferative activity by MTT assay. Values are Mean ± SD of triplicates. .2a). Showing activity of methanol, hexane and water extracts, 2b). Showing methanolic fraction of F1, F2, F3 and F4 and 2c showing F-2 fractions of F2-1, F2-2, F2-3 and F2-4.

(F2) and fraction 2-3 (F2-3) showed significant decrease (Fig 3a) in the concentration of VEGF protein compared to control/untreated A-549 cells (6.64±0.40 pg/ml for methanolic extract, 5.98±0.69 for fraction F2, 4.36±0.47 for fraction F2-3 and 14.0±0.75 pg/ml for control, respectively). These fractions were further evaluated for their time dependant inhibitory activity. Results obtained for control were 5.22±0.18, 6.91±0.18, 13.74±1.15, 20.98±0.14, 23.32±0.21, 23.90±0.35, 25.67±0.41 and 27.38±0.37 and F2-3 fraction were 5.054±0.12, 6.46±0.34, 7.18±0.15, 11.63±0.46, 13.5±0.4, 17.16±0.48, 23.64±0.63, 26.65±0.29 at the time intervals of 0, 4, 8, 12, 24, 36, 48, and 72 h respectively (Fig 3b).

CAM Assay

None of the extract concentrations used in the experiment appeared to be toxic. Nearly all



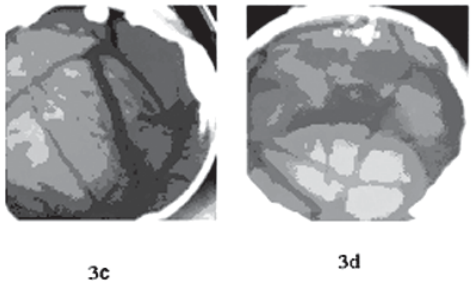


Fig. 3. All the crude extractions and fractions of methanolic extracts was subjected to VEGF inhibitory activity at a fixed concentration. Values are Mean ± SD of triplicate values. 3a) showing activity against the VEGF, fraction 2-3 is highly active in inhibiting the VEGF expression. 3b) indicates fraction F2-3 was subjected to VEGF inhibitory activity in a time dependant manner in A-549 cells. 3d showing Antiangiogenic activity of the fraction F2-3 was evaluated in egg CAM assay, 3c is control egg where vessels are prominent whereas 3d was treated with fraction F2-3.

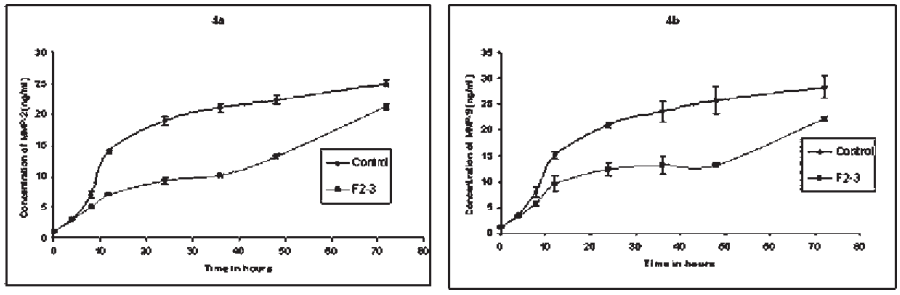


Fig. 4. Methanolic extract fraction F2-3 was subjected to MMP inhibitory activity on A-549 cells and results have shown that fraction F2-3 reducing the expression of MMP-2 and MMP-9 in a time dependent manner, Fig 4a and Fig 4b, respectively.

embryos survived for additional 48 h with test compound on CAMs. In comparison with the controls, application of the extracts showed significant inhibitory effect on the angiogenesis. Angiogenesis inhibition action of F2-3 at a concentration of 5 µg/egg substantially inhibited new blood vessel growth of chick embryos with a clearly avascular zone in CAM (Fig 3d), while no avascular zones were observed in the control embryos treated with phosphate buffered saline (Fig 3c).

Effect of L. acutangula on MMP-2 and MMP-9 release

MMP-2 and MMP-9 protein levels were significantly reduced compared to the control cells time dependently. Both, MMP-2 and MMP-9 (Fig 4a & 4b respectively) decreased in the supernatants of A-549 cells. Reduction in these protein levels started form 8 h and continued.

Discussion

An integrated approach for managing a patient with cancer should target the multiple biochemical and physiologic pathways that support tumour development and minimize normal-tissue toxicity. Natural health products contain a wide range of complex organic chemicals that may have synergistic activity. They may inhibit angiogenesis by interacting with multiple pathways and by acting in other ways that can affect cell signalling. Anti-angiogenic therapy is one of the new approaches to anticancer therapy. At the molecular level, antiangiogenic mechanisms include inhibition of MMP-2 and MMP-9 and VEGF along with other factors such as urokinase plasminogen activator and repression of AP-1, NFnB and STAT-1 transcription factor pathway (25).

Natural products are playing a key role in the fields of health management and prevention or cure of diseases. Currently 11 angiogenesis inhibitors out of 22 are under clinical trials which are from natural products indicating their importance (26). In this study, the medicinal plant, *Luffa acutangula*, was selected based on the literature available in the *Ayurveda*, an Indian system of medicine. The possibility of the use of this plant as an anti-tumor agent was examined by evaluating the two targets that is antiproliferative activity and anti-angiogenic activity.

Initially, all the crude extracts viz; hexane, methanol and aqueous extracts were evaluated for antiprolefarative activity in A-549 cells by MTT assay and it was found that only methanolic extract showed significant antiprolefarative activity with IC-50 value of 131.63 µg/ml. Methanolic extract was further fractionated by activity guided chromatography and it was observed that fractions F2 and F2-3 showed significant antiprolefarivie activity. Hence, fraction F2-3 was selected to evaluate the antiangiogenic activity. Since, VEGF and MMP play key role in the promotion of angiogenesis, these parameters were selected to evaluate the antiangiogenic activity and it was further confirmed in vivo by CAM assay.

VEGF is shown to be the most potent angiogenic factor (6). The availability of specific monoclonal antibodies capable of inhibiting VEGF-induced angiogenesis in vivo and in vitro made it possible to generate direct evidence for the role of VEGF in tumorgenesis (27). The results obtained in the present study demonstrated that the expression of VEGF was reduced in a time-dependent manner by fraction F2-3. Matrix metallo-proteinases (MMPs), a family of zinc-dependent endopeptidases, play a crucial role in ECM degradation associated with tissue repair, cancer cell invasion, metastasis and angiogenesis. Among members of the MMP family, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are particularly up-regulated in malignant tumors (mmp-2 pcr.pdf). *L .acutangula* methanolic

fraction F2-3 showed significant inhibition on MMP-2 and MMP-9 indicating the effective role of extract in the prevention of angiogenesis. Finally, antiangiogenic activity of the *L. actangula* was confirmed by CAM assay. Eggs treated with fraction F2-3 showed clear avascular zones compared to control or PBS treated eggs. All extractions and fractions showed angiogenesis inhibitory activity in dose and time dependent manner, after certain concentrations activity showed intact because it may be compounds present in the extractions and fractions acting against the activity.

Conclusion

L. acutangula methanolic extract fraction (F2-3) showed significant antiproliferative and antiangiogenic activity and further studies are required to elucidate the molecular mechanism of the fraction. Hence, this fraction may be further fractionated and purified to isolate and characterize the active compound.

Acknowledgments

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Gluco-oligosaccharides production from glucan of *Leuconostoc mesenteroides* NRRL B-742 by microwave assisted hydrolysis

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Abstract

The glucan composed of α -(1→6) linkages with α -(1→3) and α -(1→4) branched linkages points was produced from *Leuconostoc mesenteroides* NRRL B-742. The glucan was hydrolysed by acid using microwave oven for production of gluco-oligosaccharides for time intervals of 2 min and 3 min and the optimum time period for oligosaccharide production was determined. The yield of oligosaccharides production from glucan was 2.5% for 2 min of hydrolysis time. The hydrolysed glucan was run on Sephadex G-10 column using FPLC and oligosaccharides produced were eluted and analyzed by MALDI Q-TOF mass spectrometer in MS/MS mode. The glucan sample hydrolysed by microwave for 2 min yielded the peak at ~527 m/z value showing the presence of 3 glucose units (m/z ~180) corresponding to the glucooligosaccharide with degree of polymerisation 3.

Key words: Glucansucrase, *Leuconostoc mesenteroides*, Glucan, Gluco-oligosaccharide, MALDI-TOF.

Introduction

Lactic acid bacteria produce a wide variety of exopolysaccharides that have nutritional and health applications (1). In addition to heteropolysaccharides lactic acid bacteria produce homopolysaccharides which contain only one type of monosaccharide, fructose or glucose called fructans and glucans, respectively. Most of the glucans share the feature of being synthesized by extracellular glucansucrases using sucrose as the glucosyl or fructosyl donor. Glucan synthesis in lactic acid bacteria has been mainly studied from *Leuconostoc* spp. (2-4). Glucans are used as viscosifying, stabilizing, emulsifying, sweetening, gelling, or water-binding agents, in the food as well as in the non-food industries (5,6). The biological importance of oligosaccharides is increasing rapidly. Certain oligosaccharides (e.g. fructooligosaccharides, isomaltooligosaccharides and lactulose) and polysaccharides (e.g. fructans) are used as prebiotic foods (7,8). Prebiotics are nondigestible food ingredient that beneficially affects the host by selective stimulation of growth and activity of one or a number of bacteria including probiotic bacteria in the colon. Other applications for oligosaccharides such as an anticariogenic agent or a low-sweetness humectant have been explored (8). Prebiotic oligosaccharides are noncarcinogenic, nondigestible and low calorific compounds stimulating the growth and development of gastrointestinal microflora described as probiotic bacteria such as

Bifidobacteria and *Lactobacilli* (9). The oligosaccharides are used widely in various food products such as soft drinks, cookies, cereals, candies and dairy products (10). Gluco-oligosaccharides have been produced by enzymatic synthesis using glucosyl donor and an accepter molecule (11,12).

The microwave has become a useful tool for organic and inorganic synthesis, in analytical chemical laboratories and in many chemical manipulations and processes (13-16). It has been applied for methylation of plant polysaccharides (13), for synthesis of carbohydrates (15) and for hydrolysis of levan for oligo-fructan production (16). The heating effect is due mainly to dielectric polarization. In the present study a microwave oven was used to accelerate the glucan hydrolysis envisaging the production of gluco-oligosaccharides for possible commercial use. The glucan composed of α -(1→6) linkages with α -(1→3) and α -(1→4) branch points was produced from *Leuconostoc mesenteroides* NRRL B-742. The experiments were directed for studying the glucan hydrolysis under specific conditions of microwave oven potency for production of gluco-oligosaccharides. This report is first of the kind where microwave assisted hydrolysis of glucan is carried out for production of gluco-oligosaccharides with α -(1→6) linkages having α -(1→3) and α -(1→4) branching.

Materials and Methods

Production and purification of glucan

The glucan producing strain of *Leuconostoc mesenteroides* NRRL B-742 was obtained from the Agricultural Research Service Culture Collection, National Centre for Agricultural Utilization Research, Peoria, USA. The culture was maintained in modified MRS agar (17) as a stab at 4°C and sub cultured every 2 weeks. A loop of culture from an agar stab was transferred to 5 ml of sterile medium described by Tsuchiya *et al.* (18). The culture was grown at 28°C under static condition for 12-16h. 5% of the culture inoculum was used for the glucan production from *Leuconostoc mesenteroides* NRRL B-742. All the experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml medium of Tsuchiya *et al.* (18). The inoculated flasks were incubated under static conditions at 28°C for 48h. The culture supernatant was obtained by centrifugation of the broth at 10,000***g*** for 10 min at 4°C. The crude glucan was purified by precipitating it from the supernatant by the addition of 3 volumes of 95% (v/v) ethanol pre-chilled at 0°C and centrifuged at 13,000*g*. The process of precipitation was repeated to remove any trace impurities or free reducing sugars.

Hydrolysis of glucan

An aqueous solution of glucan (10 mg/ml) was prepared and the pH was adjusted to 2.5 using 0.1M hydrochloric acid. The hydrolysis was performed in an open 250 ml beaker at 1 atmospheric pressure in a microwave oven working at 580 Watts that corresponded to 60% of the normal potency. The working volume was 15 ml of glucan solution and the initial temperature was 30°C. The beaker was placed into another 600 ml beaker with 200 ml of water to avoid temperatures above 100°C and for safety reasons. The temperature during the hydrolysis was not measured. The samples were cooled and neutralized using dilute NaOH (2.5M, approximately 50 µl) solution to stop the reaction. To eliminate chloride content of the solution the carbohydrate content was resuspended in distilled water and precipitated twice by ethanol addition.

Size exclusion chromatography of glucan hydrolysates

The carbohydrate mixture containing oligosaccharides obtained after microwave hydrolysis was loaded onto a column (1cm x 25cm) containing Sephadex G-10 (Sigma

Chemical Co., St. Louis, USA). The hydrolysed fractions containing oligosaccharides were freeze dried and dissolved in 50 mM sodium phosphate buffer pH 7.0 and 500 µl sample each of 100 mg/ml were loaded on a Sephadex G-10 column. 1 ml fractions were eluted using the same buffer at a flow rate of 0.1 ml/min on FPLC (GE Healthcare, model AktaPrime). The fractions containing oligosaccharides were pooled. The total carbohydrate content of hydrolyzed was determined by phenol sulfuric acid method of Fox & Robyt, 1991 (19) using glucose as the standard. The pooled fractions were lyophilized for further analysis.

Estimation of glucan and glucooligosaccharides

The carbohydrate content before and after hydrolysis of glucan was determined by phenol-sulfuric acid method of Dubois *et al*., 1956 (20) in a micro titer plate as described by Fox & Robyt, 1991 (19). To 0.025 ml of sample on a microtitre plate, 0.025 ml of 5% (w/v) phenol was added. The plate was mixed at slow speed on a vortex mixer for 30s. The plate was then placed onto ice bath and 0.125 ml of concentrated sulfuric acid was added to each well containing the sample and phenol. The plate was again mixed using vortex mixer for 30 s and then incubated in water bath at 80°C for 30 min. It was then cooled and the absorbance was read at 490 nm on a microplate reader (BioRad, model 680). Standard curve was prepared using dextran (10 kDa, Amersham) in the concentration range 0.1-1 mg/ml.

The concentration of glucan and oligosaccharides was calculated as follows:

$$\text{Glucan concentration (mg/ml)} = \frac{\Delta A_{490} \times C}{v} = \text{(mg/ml)}$$

C = 1 OD equivalent of glucan from standard plot

ΔA_{490} = change in absorbance of the sample

v = volume of the sample

Oligosaccharide analysis by mass spectrometry

Mass spectrometry was performed on a MALDI Q-TOF Premier mass spectrometer (Waters). The mass spectrometer was operated in negative ion mode and prior to analysis was calibrated over the range 50–1000 *m*/*z* using glucose, sucrose and raffinose (Himedia, India). Mass spectra were acquired manually over the *m*/*z* range 100–1000. In MS/MS mode, precursor ions were isolated using MS (quadrupole) and fragmented in an argon filled collision cell (4×10^{-3} mbar). MS/MS data were acquired with the collision energy adjusted in such a way that the majority of the precursor ion was attenuated. Collision energies used were typically between 50eV and 100 eV.

Results and Discussion

Mirowave hydrolysis of glucan and size exclusion chromatography analysis

Leuconostoc mesenteroides NRRL B-742 was selected for the production of oligosaccharides as the glucan produced by this strain contains 50% main chain α (1→6) and rest 50% α (1→3) and α (1→4) branched linkages that are of single glucose residues (21). This gives a structure with glucose branch on every glucose residue in the α (1→6) linked main chains. This structure has been described as a bifurcated comb dextran. The α (1→6) linked glucose residues in the main chain are the back bone of the comb and α (1→3) linked single branched glucose residues are the teeth of the comb (21). The hydrolysis of this polymer would yield oligomers with α (1→3) or α (1→4) branches.

The glucan from *Leuconostoc mesenteroides* NRRL B-742 was produced and purified by alcohol precipitation. The purified glucan samples (10 mg/ml) were subjected to hydrolysis in a microwave oven for 2 and 3 min time intervals. The hydrolysed glucan samples were freeze dried and dissolved in 50 mM sodium

phosphate buffer pH 7.0 and 500 µl sample each of 100 mg/ml were loaded on Sephadex G-10 column connected to FPLC. The fractions (1 ml) were eluted using the same buffer at a flow rate of 0.1 ml/min. Fig. 1 shows the elution profile of the microwave hydrolysates obtained by after 2 and 3 min. The increase in hydrolysis time of glucan increased the fragmentation of the polysaccharide glucan and thus reduction in the carbohydrate content. The elution of carbohydrate fractions from column for the 3 min hydrolysed sample occurred later (Fig. 1B). The fractions 4 and 5 from 2 min hydrolysed sample and the fractions 6 and 7 from 3 min hydrolysed sample were pooled separately and the total carbohydrate content was determined using phenol-sulphuric acid method. The Fig. 2 shows the total carbohydrate content obtained after microwave hydrolysis of glucan for 2 min and 3 min. The 2 min hydrolysis fraction pool showed more carbohydrate content than the 3 min pooled fraction (Fig. 2). The 15 ml of glucan solution (10 mg/ml) was partially converted to oligosaccharides and the yield was 2.5% for 2 min of hydrolysis time. For 3 min hydrolysis the glucan might have been over hydrolysed to even glucose break down products or more elemental forms as shown by mass spectrometry.

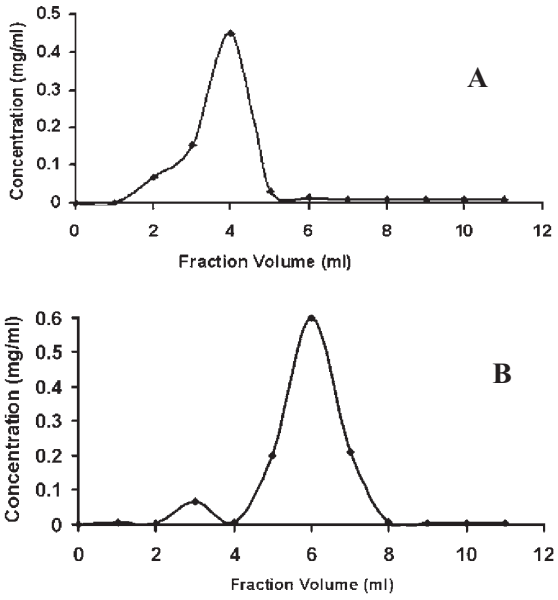

Fig. 1. Elution profiles of carbohydrates (concentration in mg/ml) from Sephadex G-10 column of glucan samples (10 mg/ml) run after microwave hydrolysis for a) 2 min; b) 3 min.

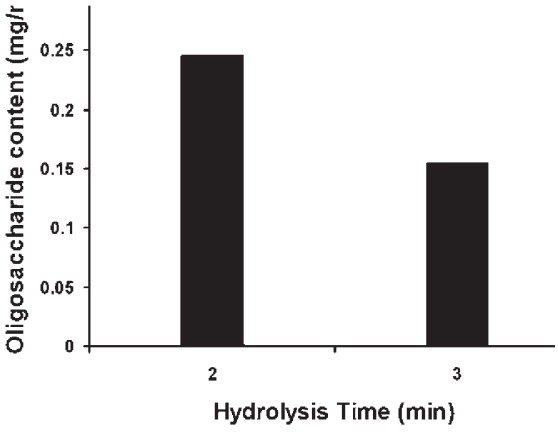

Fig. 2. Carbohydrate content (concentration in mg/ml) obtained after elution from Sephadex G-10 column after microwave hydrolysis of glucan samples for 2 and 3 min.

Oligosaccharide analysis by mass spectrometry

The pooled fractions from chromatography were analysed by mass spectrometer. The mass spectrometer was operated in negative ion mode and prior to analysis was calibrated over the *m*/*z* range 50-1000 using glucose, sucrose and raffinose (Fig. 3). The MALDI-TOF MS peaks for standards were obtained at following *m*/*z* values: glucose, 179.0149; sucrose, 341.0426; raffinose, 503.0623. The MALDI-TOF-MS of fraction 2 revealed $[M+Na]^+$ pseudomolecular ions at *m*/*z* 527.1527 corresponding to Hex_3 that was

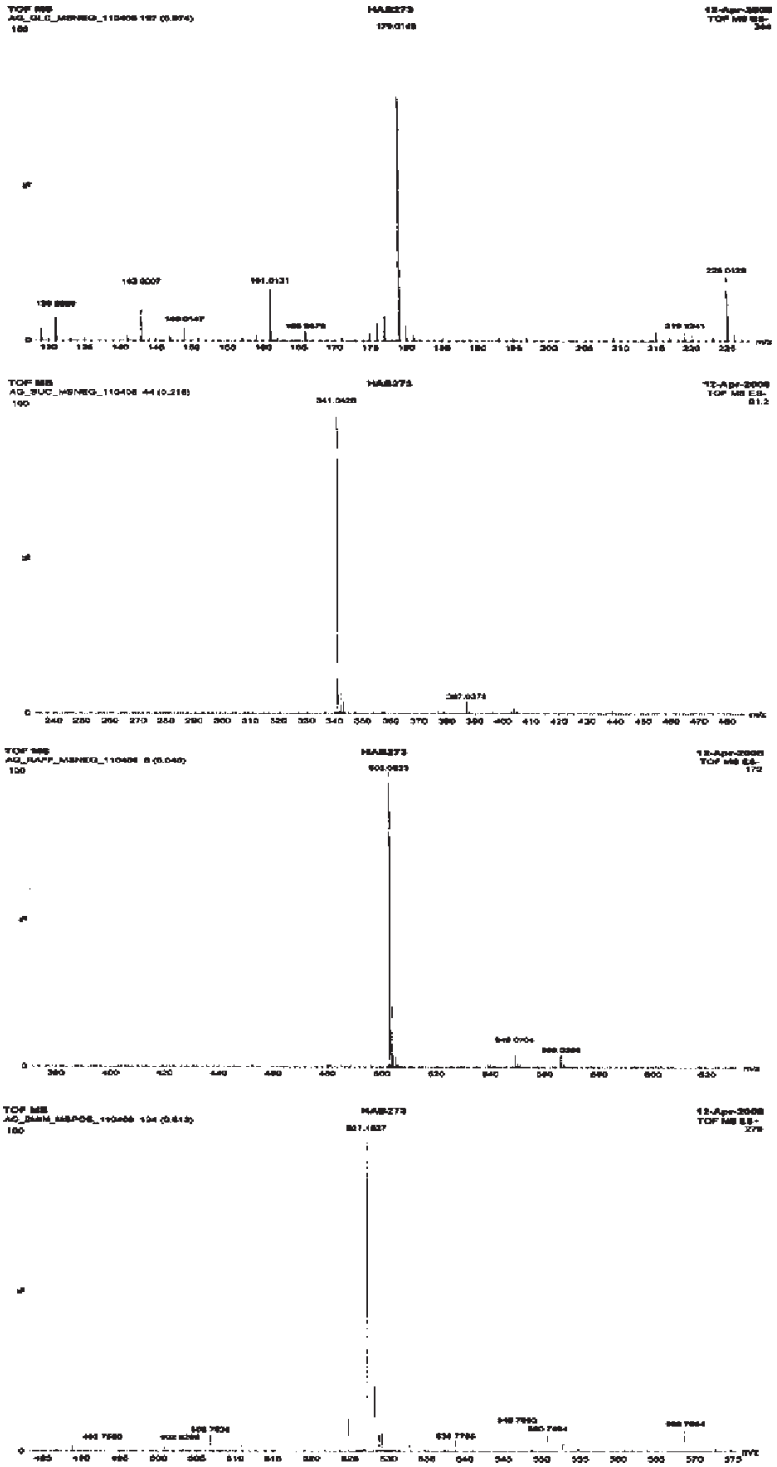

Fig. 3. MALDI-TOF-MS analysis of a) glucose, b) sucrose, c) raffinose and d) the glucan sample after microwave hydrolysis for 2 min.

comparable to molecular weight of raffinose or three times molecular weight of glucose (Fig. 3C). Thus the degree of polymerization (DP) of the 2 min hydrolysed glucan was 3. This also showed that glucan hydrolysed for 2 min gave only one type of gluco-oligosaccharides with DP3. In mass spectrum of the sample hydrolysed for 3 min, multiple peaks were obtained along with one prominent peak at 186 *m/z* value (data not shown) showing the breakdown of glucan to monomer glucose. No other conclusions for multiple peaks could be drawn as mass spectrum was not interpretable.

The peak was obtained at ~527 m/z value using MALDI-TOF-MS showed presence of 3 Glucose units of (m/z ~180). A degree of polymerisation of 3 (DP-3) for the oligosaccharide was obtained for the glucan sample hydrolysed for 2 min. Enzymatic synthesis of gluco-oligosacchairde is cumbersome and not feasible on industrial scale whereas the microwave is able to reduce the reaction times from hours to minutes and can be applied on large scale. The use of microwave oven in the glucan hydrolysis leads to considerable reduction in the hydrolysis time. The optimum time period for gluco-oligosaccharide from glucan was determined. The results allow one to infer that the procedure may be useful for oligosaccharide production from any polysaccharide. Further studies are in progress to find exactly the optimum time period for each oligomer and their identification and linkages. For the purpose of production of other gluco-oligosaccharides it is worthwhile to experiment with other hydrolysis time periods, effect of pH and microwave potency.

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Adsorption Studies of Congo Red and MethyleneBlue on the Surface of *Citrus lemonii*

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Abstract

The adsorption of organic dyes like congo red and methylene blue on *Citrus lemonii* using batch adsorption process has been investigated. The effect of certain parameters on adsorption has been studied. Applicability of Freundlich adsorption isotherm and Langmuir isotherm has also been tested.

Keywords: Adsorption, Congo red, Methylene blue, *Citrus lemonii*.

Introduction

Techniques for removal of colour from waste water include biological treatment, coagulation, floatation, oxidation, hyperfiltration and adsorption. Adsorption on to granulated activated carbon or powdered activated carbon is widely practiced, particularly for the organics, which are not easily broken down by biological treatment and by other methods. The technology for manufacturing good quality activated carbon is still cost-prohibitive and regeneration of the used carbon is often problematic. This has prompted the use of many novel materials as adsorbents with a two fold objective to replace activated carbon with cheaper alternatives and to utilize various waste products for the purpose (1,2). Plant materials are used as a cheap and low cost material for adsorption,for example, modified corn starch (3), modified onion skins (4), saw dust (5), phosphate treated saw dust (6),water lettuce (7), *Alterneuthera triandra* (8),*Citrus aurantium*(9), *Musa paradiasca* (10),*Achras sapota* (11) etc. In the present study, it is demonstrated that fine charcoal powder obtained from *Citrus lemonii* can be a suitable alternative to activated carbon for treating coloured effluents.

Materials and Methods

Congo red was supplied by S.D.Fine Chemicals Ltd., Mumbai,Methylene blue was supplied by Qualigens Ltd., Mumbai. These Chemicals were used without further purification. The synthetic effluent solutions were made by dissolving the required amount of dye in double distilled water. Lemon fruit peels were collected and washed repeatedly with water to remove dust and soluble impurities and were allowed to dry at room temperature in a shade and then in an air oven at 333-343^0 K for a long time till the peels become completely dry and crisp. Then peels were burned in presence of incomplete oxygen to peel charcoal powder. The adsorption experiments were carried out in a batch process by using aqueous solutions of the dye in a fixed concentration range. The other variable parameters were adsorbent dose, contact time (1-5 hrs) and pH of the medium (2.0-10.0). In each experiment, an accurately weighed amount of peel charcoal powder was added to a fixed volume of the dye solution in a 100 ml conical

flask and the mixture was agitated on a thermostatic magnetic stirrer for a given length of time at a fixed temperature. The mixture was centrifuged rapidly and the dye remaining unadsorbed was determined spectrophoto metrically (Elico-SL 180). The concentrations were determined with the help of carefully prepared calibration curves with standard dye solutions and the amount adsorbed was found by mass balance procedure.

Results and Discussion

Adsorption at a surface largely the result of binding forces between the individual atoms,ions or molecules of an adsorptive and the surface, all of these forces having their origin in electromagnetic interactions. The net dispersion, electrostatic,chemisorptive and functional group interactions broadly define the affinity of an adsorbent for a specific adsorption. A number of parameters, specific to a given system,affect adsorption. With respect to the adsorptive, these factors are concentration, molecular weight, molecular size,molecular structure, molecular polarity, steric form or configuration and the nature of background or competitive adsorption. The important characteristics of the adsorbent that determine equilibrium capacity and rate, are the surface area, the physio-chemical nature of the surface, the availability of that surface to adsorptive molecules or ions, the physical size and form of the adsorbent particles. System parameters such as temperature and pH can also markedly influence adsorption to the extent that they effect changes in any one or more of the above mentioned parameters.

Effect of contact time

It was observed that *Citrus lemonii* peels can be used as low cost adsorbent effectively. The maximum time required for 25 to 42% adsorption of congo red is 1 hr.The contact time has little impact on adsorption of congo red (42 to 65%) on peel charcoal powder.The maximum time required for 65 to 77% adsorption of methylene blue is 1 hr. The contact time has positive impact on adsorption of methylene blue (77 to 99%) on peel charcoal powder (Tables. 1and 2). In both cases, the effect of contact time on adsorption continued even after 1 hr.

Effect of pH

The pH of the medium does not have much effect on adsorption of congo red on peel charcoal powder. Thus as the pH of the medium changes from 2.0 to 10.0, the adsorption of dye oscillates between 79 to 86%.This is contrary to what other workers have observed for adsorption of the basic dye on a variety of adsorbents where the pH was shown to have a positive influence (12). It is likely that the surfaces of peel charcoal powder particles are neither acidic nor basic and congo red seems to have equal preference for the adsorption sites at all pH values. Thus the adsorption can be carried out without a control of the pH. Almost similar observations have been recorded with the Methylene blue.

Adsorption equilibrium

The adsorption process was described by the following isotherms:

Freundlich adsorption isotherm: $q_c = k_f c^n e$

Langmuir isotherm: $C_e/q_e = (1/k_d)c_1 + (1/b)C_e$

C_e is the equilibrium adsorptive concentrations in aqueous phase, q_c is the amount of dye adsorbed on unit mass of peel charcoal powder, n and k_f are Freundlich constants,c_1 and k_d are Langmuir constants.

The linear Freundlich and Langmuir plots are obtained by plotting log q_c vs logc_e and c_e/q_e vs c_e, respectively,from which adsorption constants are evaluated.

A further analysis of the Langmuir equation was made on the basis of a dimensionless equilibrium parameter R_l.

$R_l = 1/(1+k_d C_{ref})$

C_{ref} is any equilibrium liquid phase concentration of the solute.The range $0<R_l<1$ reflects favourable adsorption.With both congo red and methylene blue,excellent fit with Freundlich isotherm and Langmuir isotherms are obtained. The values of the adsorption coefficients are given in (Table. 3).

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Table 1. Effect of contact time on adsorption of Congo red on peel charcoal powder at 300 K.(Dye Concentration 40 mg/dm^3).

Contact time(min)	Amount adsorbed (%) for peel charcoal powder dose of				
	2 gm dm^{-3}	4 gm dm^{-3}	6 gm dm^{-3}	8 gm dm^{-3}	10 gm dm^{-3}
60	25.6	30.1	34.9	37.7	41.2
90	29.4	32.9	36.7	40.9	44.6
120	32.3	35.5	39.8	43.7	47.1
150	35.5	39.7	43.3	46.5	49.6
180	38.2	41.9	44.1	47.9	51.3
210	41.1	44.2	47.7	51.9	54.7
240	44.5	47.9	50.2	53.9	57.9
270	47.1	51.4	54.7	57.9	60.7
300	50.2	53.4	56.9	59.1	63.2

Table 2. Effect of contact time on adsorption of Methylene blue on peel charcoal powder at 300 K. (Dye Concentration 40 mg/dm^3).

Contact time (min)	Amount adsorbed (%) for peel charcoal powder dose of				
	2 gm dm^{-3}	4 gm dm^{-3}	6 gm dm^{-3}	8 gm dm^{-3}	10 gm dm^{-3}
60	65.3	68.9	71.6	74.3	76.2
90	67.5	71.6	74.8	77.9	80.2
120	69.8	72.9	75.2	78.8	83.8
150	73.5	76.7	79.2	83.1	86.2
180	75.5	78.8	82.8	86.7	89.1
210	77.7	81.9	84.5	88.2	92.8
240	80.2	83.1	86.2	89.1	94.9
270	83.1	86.5	89.2	93.1	96.2
300	86.3	89.2	93.4	95.5	98.8

Table 3. Freundlich and Langmuir Adsorption co-efficients for the adsorption of congo red on peel charcoal powder (PCP) at 300 K.

PCP dose (gm dm^{-3})	Freundlich Constants		Langmuir Constants		
	n	K_f(dm^{3}g^{-1})	C_1(dm^{3}g^{-1})	K_d(mg g^{-1})	R_L
2	0.55	8.31	0.31	38.32	0.072
4	0.47	6.35	0.23	28.43	0.075
6	0.57	4.21	0.26	24.34	0.082
8	0.47	3.12	0.35	15.43	0.061
10	0.43	2.50	0.39	12.85	0.067
Mean	0.498	4.898	0.308	23.874	0.0714

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Biodegradation of Pentachlorophenol by white rot fungi isolated from forests of Western Ghats of Karnataka India

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Abstract

Pentachlorophenol (PCP) is a major organic compound used in the wood preservation. White rot fungal strains screened from local sources such as wood trunks and twigs of higher plants. Five selected isolates of White Rot Fungi (WRF) Viz: *Laetiporus cincinnatus, Trametes versicolor, Fomes fomentarius, Ganoderma aplanutum* and *Pleurotus ostreatus* isolated from Sambrani and Dandeli forests of Western Ghats of Karnataka. The isolates were evaluated for the in-vitro biodegradation of PCP. The presence of extracellular laccase shows that these isolates are capable of degrading variety of xenobiotics including biodegradation of PCP. The maximum laccase activity (0.14 ± 003U) of *F. fomentarius*, *P. ostreatus* followed by *T. versicolor* study has shown significant efficiency on biodegradation of PCP which was quantitatively estimated and analyzed by High Performance Liquid Chromatography (HPLC). The static culture studies have been worked on all the isolates. *F. fomentarius* (100%) and *P. ostreatus* (99.40±1.29%) which have degraded the PCP over 30 days of incubation.

Key words: Pentachlorophenol, White Rot Fungi, HPLC, Western Ghats.

Abbreviations: PCBs - Poly chlorinated biphenyls, **DDT** – Dichlorodiphenyl trichloroethane, **PCP** - Pentachlorophenol.

Introduction

Pentachlorophenol (PCP) has been used extensively as a wood preservative, pesticide and fungicide. The wood preserving industry is the primary source of producing PCP containing wastewater and ground waters. However, the process of PCP biodegradation has been observed to be very slow. Chlorophenols are the most dangerous classes of environmental pollutants have been produced in thousands of tons annually by the pulp, paper and agrochemical industries. Chlorophenols are known substrates for the oxidative enzyme laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) that can react with all the chlorophenols including the most recalcitrant compound pentachlorophenol (1). Many reports showed that white rot fungi can degrade a variety of persistent aromatic organopolluntants such as PCP, PCBs, DDT, and phenanthrene (2).

Pentachlorophenol synthesized for the first time in 1872 due to its broad spectrum and low cost. Its esters have also been used as biocides. This generalized use has led to the contamination of many ecosystems, with PCP currently considered to be a product of priority for decontamination studies according to the European Community and the American Environmental Protection Agency (3). The capacity of Basidiomycetes to degrade PCP as well the possibility of using these organisms in processes of reclamation of PCP contaminated

soils, have been the subject of much previous research.

White rot fungi are a group of filamentous wood-decay fungi, primarily Basidiomycetes with some Ascomycetes, which share the ability to simultaneously metabolize lignin and the polysaccharide components of wood. Research has demonstrated that these fungi are capable of degrading a variety of xenobiotics (recalcitrant chemicals) including PCP. White-rot fungi vary greatly in their ability to degrade wood and remove constituents. The combined ability to degrade wood and PCP makes those fungi attractive candidates for use in destroying PCP treated wood products (4).

The literature concerning biodegradation and bioremediation of organic chemical wastes (xenobiotics) dealt almost exclusively with bacteria. It is now becoming apparent that fungi also play an important role in degrading organic materials in the ecosystem, and that they have potential for remediating contaminated soils and waters. The higher Basidiomycetous fungi probably play the major role in recycling the carbon of lignocellulosics, which are the most abundant renewable organic materials on earth (5). Because of its environmental significance, biotreatment is of great interest. Both anaerobic and aerobic biodegradation pathways of PCP and other chlorophenols have been widely reported. Due to improper treatment of PCP, soil and groundwater has been widely contaminated and their toxicity seriously affected living organisms (6).

In 1990, PCP use was restricted to that of a wood preservative since it is a probable human carcinogen (7). Short term ingestion or inhalation results in neurological disorders, Leukemia, liver damage, and eye irritation (7). Long-term exposure damages the respiratory tract, blood, kidneys, liver, immune system, eyes, and skin (7). The wide spread use, accumulation in the environment, and negative impact on human health and environment has rendered PCP remediation to be of critical importance (7).

Laccase are glycosylated phenol oxidases that exist as monomers, homodimers or homotertamers. Fungal laccase have a very broad substrate range compared to other multi copper oxidase (8). Fungal reactivity with chlorophenols is due to the production of lignin degrading enzymes, particularly laccase and Mn-peroxidase that are secreted in increased quantities by the fungus during ligninolytic activity (1). The White rot fungi have been widely studied for their ability to degrade variety of environmental soil pollutants, including PCP and efficiently degrade lignin, a complex aromatic polymer in the wood, where the White rot fungi *Phanerochate chrysosporium* can be used for bioremediation of phenolic, xenobiotic compounds and decolonization and remediation of the effluent (9).

In the present experiment in-vitro study of laccase production and degradation of PCP were evaluated for the bioremediation potential of white rot fungi isolated from Sambrani and Dandeli forests of Western Ghats of Karnataka, India. The coastal areas of India have some of the best developed industrial areas, metros and ports. The Indian coastal area is under considerable stress from the effluents discharged by the industries and the municipalities. The industrialized centers release a horde of chemical pollutants through their effluents in the streams and nalas draining in to the nature. Most dreaded are the municipal wastes, sewage sludge and dredged spill dumping, oil spills and leakages. The wastes have a wide range of hazardous elements such as petroleum hydrocarbons, chlorinated hydrocarbons and heavy metals (10). The table-2 shows the compilation of different types of pollutant and annual quantum of pollution report by Tata Energy Research

Institute, New Delhi, Elrich de Sa, Director, National Institute of Oceanography (NIO).

Materials and Methods

Mushrooms

Fruiting bodies were collected on dead woods from different localities of Sambrani and Dandeli forests of Western Ghats of Karnataka. Isolates are identified as *Laetiporus cincinnatus* Morgan, *Trametes versicolor* Arora*, Fomes fomentarius* Kickx*, Ganoderma aplanutum* Smith & Weber and *Pleurotus ostreatus* Smith, A.H. Identification was done based on their morphological, physiological and biochemical characteristics. Taxonomic features are verified with monographs and descriptions of the Manual (11), taxonomic research articles (12) and also through the electronic data on identification keys of mushrooms (13). Herbariums of the isolates were deposited at Mycology laboratory, Department of Botany, Karnatak University, Dharwad, India. These isolates of white rot fungi were selected based on their known high lignin degrading capacity for evaluating the PCP degradation. Isolates were raised by aseptically transferring a piece of fruiting bodies into cultures on 5 % Malt Extract Agar (MEA) at pH – 4.5 and incubated statically for fungal colonization at ambient temperature in complete darkness. Isolates were sub cultured and transferred to the fresh slant for every fortnight. Each agar plugs (6mm diameter) from the outer circumference of a fungal colony growing on MEA plates (7 days) used as the inoculum. Young cultures (mycelial state) were used to determine efficacy of laccase activity and PCP degradation. All the experiments were performed in triplicate.

Chemicals

Pentachlorophenol Obtained from Aldrich Chemical Co. (Milwaukee WI, USA) Acetonitrile, Glacial acetic acid, ABTS (2, 2'-azino–bis 3-ethylbenzothiazoline-6-sulphonic acid), Sodium azide, and other chemicals are of analytical grade from Merck Bombay.

Laccase assay

For laccase enzyme assay all the isolates were grown on nutrient deficient (PCP solution 50 ppm/L) liquid medium consisted of 5g glucose, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.2g NH_4NO_3, 0.1g yeast extract, 0.01g $CaCl_2$, 1mg $CuSO_4.5H_2O$, 1 mg $FeSO_4.7H_2O$ and 1mg $MnSO_4$ per liter of water (14) Finally 50 ppm/L of PCP was added into 250ml erlenmeyer flasks containing 50mL of liquid medium with PCP as carbon source and incubated at room temperature on shaker at 150 rpm and final adjusted to pH - 4.5. Laccase activity was measured by using the method described by Godliving and Rose (15). The oxidation rate of (2, 2-azino–bis 3-ethylbenzothiazoline 6 sulphonic acid) ABTS was determined by Spectrophotometric method. The reaction mixture contained 600 ìL sodium acetate buffer (0.1 M, pH 5.0 at 27^{o}C), 300 ìL ABTS (5 mM), 300 ìL mycelial liquid fraction and 1.4ml distilled water. The mixture was then incubated for 2 min at 30^{o}C. The absorbance was measured at 420 nm at room temperature. One Unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 m mole of ABTS per minute. Laccase activity was defined as 1ìmole of ABTS oxidized product formed per minute. The experiment was performed in triplicate. Control was done without substrates.

PCP concentration analysis

Mycelial plugs inoculated in 50ml of liquid broth with PCP as carbon source (50 ppm/L) were harvested after the 10th, 20th and 30th day of incubation. One ìmole of sodium azide was added to the cultures to inhibit enzyme activity. The harvested cultures were stored at -20°C. The cultures were homogenized and flasks were rinsed with absolute ethanol (5ml), followed by water (5 ml), and the rinses were pooled with the homogenates and stored at -20°C. PCP was extracted from 0.5- to 5-ml samples of culture homogenates with hexane (0.5 to 2 ml) following

the addition of 1 ml of H_20 saturated with NaCl. The sample was filtered through membrane filter of 0.4ìm and used for quantification of PCP by HPLC. The amount of PCP remaining was determined by HPLC (16). A standard curve was determined daily by plotting the peak area obtained from the integrator versus known standard amounts of PCP injected. PCP was analyzed using (Model. HPLC WATERS 2487 with EMPOWER software) with a Symmetry C-18 reverse-phase column (4.6 by 250 mm, 5 µm). 20 µl of each sample was eluted from the column with acetonitrile-H_20-glacial acetic acid (75:25: 0.125) at a flow rate of 1 mL/min. Elution was monitored at 238 nm. The remaining PCP concentration of each sample was calculated with known concentration and peak area of standard PCP.

All the test organisms were studied for adsorption of PCP to mycelial mass. Cultures used in this study were grown only up to inoculum level and incubation period maintained as described above for degradation experiments so as to facilitate sufficient adsorption of PCP. Heating at 55°C for 24hr killed all the cultures.

Statistical analysis

All the data was subjected to one-way analysis of variance (ANOVA) and the significance of the difference between the means was determined by the Duncan's multiple range tests significant difference ($p < 0.05$) by using SPSS 9 Student version software.

Results and Discussion

In vitro studies were performed with five isolates for their ability to produce extra cellular laccase and PCP degradation. These two criteria are important in assessing the bioremediation potential of white rot isolates. During the experiment all the sets of 10th, 20th and 30th day samples were assessed for laccase activity and biodegradation of PCP. The table-1 shows percentage of degradation, remaining Pentachlorophenol and laccase production at different interval of incubation where increasing rates of PCP removal recorded from these isolates. Initial rate of PCP degradation (10th day) is very slow when compared with final incubation period (30th day). This indicates initially culture had not become fully acclimatized to this concentration of PCP. Biodegradation of PCP in static culture was evaluated based on disappearance of PCP. When compared to the control all the five isolates of white rot fungi have shown the significant results were PCP degradation ranged (91.94±1.34% to 100±00%) and laccase production ranged (0.10±003U to 0.14±003U) over the 30th day of incubation. The static culture experiments were done in triplicate.

Laccase activity

All the isolates were produced laccase at some stages over the 10th, 20th and 30th day of incubation period. The fig. 1 shows laccase activity was highest was found (0.14±003U) in *F.fomentarius, T.versicolor* and *P.ostreatus* over the 30th day of incubation. The lowest laccase activity was found (0.10±003U) in *L. cincinnatus.* Laccase activity was varying slowly on initial 10th day when compared to 30th day of analysis. The initial concentration 50 ppm of PCP supported Laccase activity by enhancing production of laccase two fold from 0.03 ± 003U to 0.14±003U in active sample. The results obtained clearly showed that the laccase activity assayed by the ABTS substrate was significantly higher and the experiment support the research work done in previous literatures. According to workers (1) In the free cell cultures the initial rise to 8 units when the first addition of 200 ppm PCP was induced the production of laccase accompanied by a 10-fold increase in laccase activity When 1000 ppm of PCP was added to the culture, both laccase and MnP activities increased from 0.3 to 0.5 units and from 0.001 to 0.18 units respectively. The catalytic efficiency of laccase from fungi tested was much lower for the substrates of o-

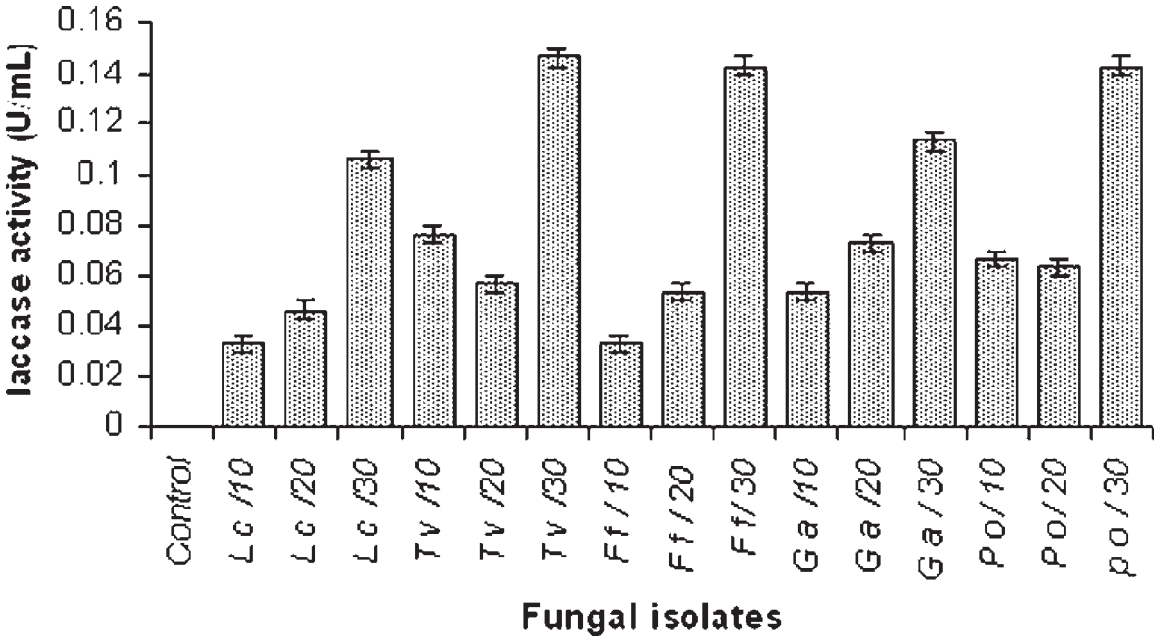

Fig. 1. Laccase activity by fungal isolates at 10th, 20th and 30th day of incubation period against respective species Viz: L c - *Laetiporus cincinnatus*, T v - *Trametes versicolor,* F f - *Fomes fomentarius*, G a -*Ganoderma aplanutun,* P o - *Pleurotus ostreatus.*

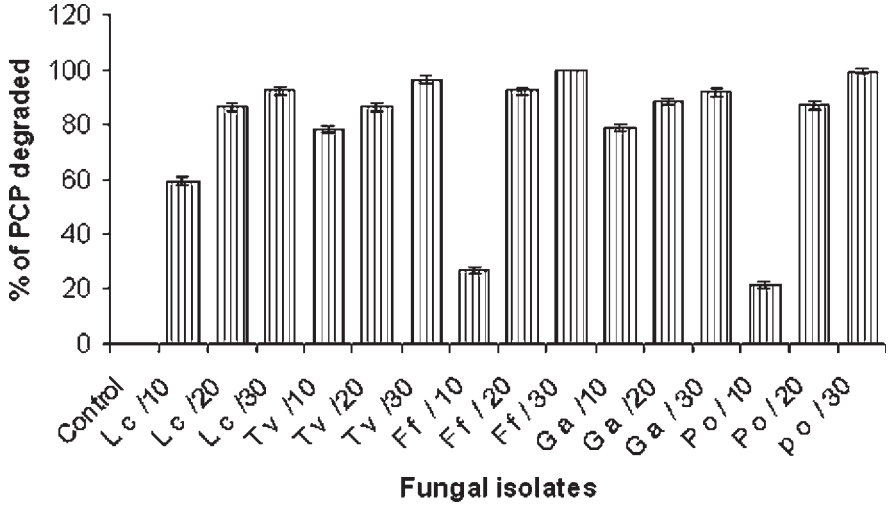

Fig. 2. PCP degradation by fungal isolates at 10th, 20th and 30th day of incubation period against respective species Viz: L c - *Laetiporus cincinnatus*, T v - *Trametes versicolor* F f - *Fomes fomentarius*, G a -*Ganoderma aplanutun,* P o - *Pleurotus ostreatus..*

dianisidine and guaiacol In case of *Trametes versicolor Fomes fomentarius* and *Pleurotus ostreatus* the laccase activity decrease at 20th day analysis and immediate raise in laccase production at 30th day of analysis. Such kind of variation indicates initiation and utilization of PCP as carbon sources (17).

Laccase synthesis was induced by phenolic compounds present in medium, leading to increasing of laccase production. This induction mechanism may help fungus to degrade lignin or aromatic compounds in media to supply further nutrients especially carbon and nitrogen. *Trametes versicolor* produced the highest xylanase and cellulase from solid state culture at 15th day, While those of laccase and peroxidase were at 60th days, However, addition of rice straw in mineral salt broth (MSB) increased laccase production by white rot fungus *Daedalea flavida* MTCC 145 from 0.06 U/mL to 9.04 U/mL (14).

PCP degradation

All the 5 isolates significantly reduced PCP supplied in the liquid medium over the 30 days incubation period when compared to PCP control. The level of PCP removal varied between isolates. And fig. 2 shows the initial concentration of PCP was (50 ppm/L) degraded 100% by the *F. fomentarius,* 99.40±1.29% by *Pleurotus ostreatus* followed by average degradation i.e. 96.14 ± 1.37 %, 92.16 ± 1.41%, 91.94 ± 1.34% by *T. versicolor L. cincinnatus* and G. *aplanutum* respectively at 30th day when compared to initial 10th and 20th day of incubation period. Since many previous studies have shown *Trametes versicolor* was able to mineralize PCP (21% in 17 days) while secreting Laccase (but not peroxidases) in a ligninolytic culture medium. (18). For the removal of PCP, reaction with laccase as a predominant enzyme in many white-rot species such as *C. versicolor* and *P ostreatus* has been described here, in this reaction, PCP is a substrate for laccase. With 100 units of laccase, 100% of 25 µg/ml PCP and 60% of 200µg/ml PCP were removed respectively over 72 hours (19). Inoculation with *P. chrysosporium,* the concentration of PCP was decreased 98% compared to that in control cultures (20) Aerobic PCP transformation initially produced small amounts of pentachloroanisole; however more than 75% of both chemicals disappeared in 30 days from the test soil (21).

Organisms like *Hyphoderma praetemissum,* (11.6%) *Irpex lacteus* (11.8%) and *Phanerochaete chrysosporium* (12.5%) were able to mineralize significant amounts of PCP. The mycelium of the brown-tot fungi *Gloeophyllum trabeum* (13.5%) and *Posita placenta* (12.9%) possessed the capacity for mineralizing PCP (22). According to Workers (23) two white-rot fungi *Bjerkandera adusta* and *Anthracophyllum discolor* Both fungi were grown on Kirk medium and shown the highest PCP degradation was attained by *A. discolor* using DDGS (95% after 28 days).

PCP was added into reactor R_1 and observed in the effluent at the 4th day. 2,3,5,6 TeCP was detected at 10th day and maintained low level. At 23rd day a sharp peak of 2, 3, 5-TCP was observed after introduction of PCP in the influent, a higher peak of 3, 5 DCP was observed at 34th day and a highest peak of 3-CP was observed at 60th days after PCP introduction (6). HPLC analysis shows there is an immediate increase in percentage of degradation with increasing rates of removal recorded from these isolates. Initial rate of PCP degradation (i.e. 10th day) is very slow when compared with 30 days of incubation period. In **Fig. 3** Peaks with retention time shown here is one of the active isolate (*P.ostreatus*) The HPLC peaks showed the clear evidence of the PCP degradation at 10th, 20th, and 30th days of incubation.

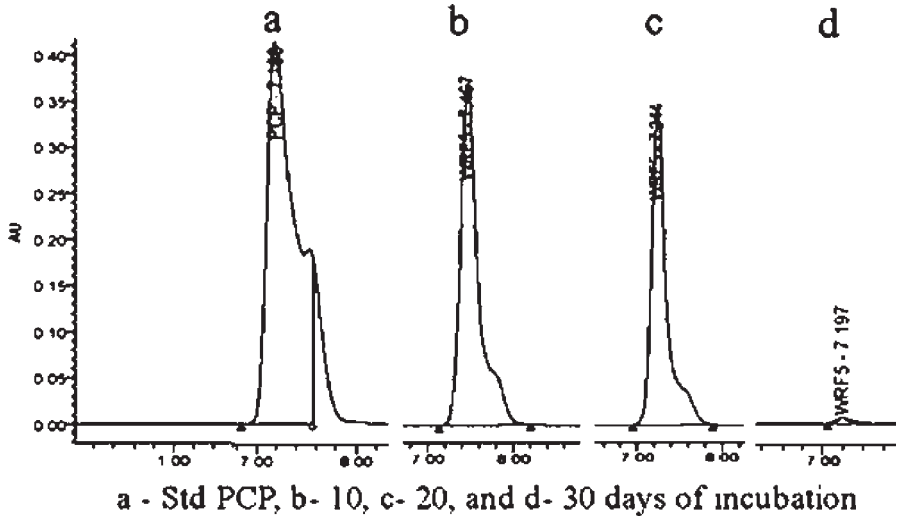

Fig. 3. HPLC peaks of Std PCP and PCP degraded by active sample with retention time at different incubation period *i.e. Pleurotus ostreatus* A - 10th days, 20th days and 30th days.

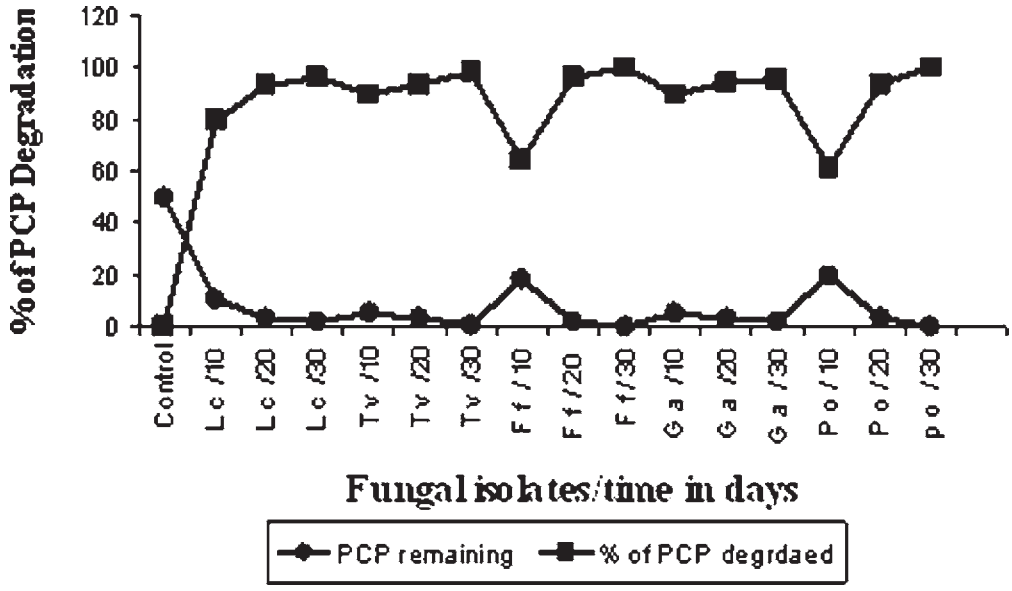

Fig. 4. Comparison of PCP Remaining and Degraded by fungal isolates at 10th, 20th and 30th day of incubation period against respective species. Viz: L c - *Laetiporus cincinnatus*, T v - *Trametes versicolor,* F f - *Fomes fomentarius*, G a -*Ganoderma aplanutun,* P o - *Pleurotus ostreatus.*

The PCP biodegradation rate in the 30 days aged controls was found in traces on a dry weight basis. Recovery of PCP from heat killed control cultures appeared to be unaffected. There is rate is similar to a killed control biodegradation rate reported in another study (24) with a similar PCP–soil mix. Therefore, this result was no different from a zero PCP biodegradation rate. It is reasonable to assume that mineralization would follow dehalogenation since aromatic compounds such as phenol have been shown to serve as sole carbon sources for growth of *P. chrysosporium.*

Comparison of these results presumed that the initially culture had not become fully acclimatized to this concentration (50 ppm/L) of PCP. But 30th day of analysis indicates the ability of White rot isolates to use the PCP as sole carbon source. Fig. 4 shows the PCP remaining and biodegradation rate calculated in the 30 days of the time course in the culture medium at different time of analysis. PCP remaining at different incubation periods it was found in trace quantity with *F. fomentarius* (0.001ppm), and maximum with *Ganoderma aplanutum* (2.106ppm), In the

Table 1. Concentration of PCP remaining and PCP Degraded by isolates at 10th, 20th and 30th day of incubation period (Mean ± Standard deviation; n=3)

Isolates/ time in days	PCP Degraded in ppm	Laccases activity in Units	PCP Remaining in ppm
Control	00±00	00±00	50.000
L c /10	59.53 ± 1.34	0.03 ± 003	10.216
L c /20	86.14 ± 1.17	0.04 ± 003	3.466
L c /30	92.16 ± 1.41	0.10 ± 003	1.871
T v /10	78.23 ± 1.26	0.07 ± 003	5.310
T v /20	86.27 ± 1.23	0.05 ± 003	3.174
T v /30	96.14 ± 1.37	0.14 ± 003	0.901
F f / 10	26.94 ± 1.41	0.03 ± 003	18.195
F f / 20	92.10 ± 1.21	0.05 ± 003	1.967
F f / 30	100 ± 00	0.14 ± 003	0.001
G a /10	78.72 ± 1.24	0.05 ± 003	5.273
G a /20	88.36 ± 1.23	0.07 ± 003	2.909
G a / 30	91.94 ± 1.34	0.11 ± 003	2.106
P o / 10	21.79 ± 1.32	0.06 ± 003	19.512
P o / 20	87.13 ± 1.35	0.06 ± 003	3.341
p o / 30	99.40 ± 1.29	0.14 ± 003	0.052

L c - *Laetiporus cincinnatus*, T v - *Trametes versicolor,* F f - *Fomes fomentarius*, G a - *Ganoderma aplanutun,* P o - *Pleurotus ostreatus.*

Table 2. Type of pollutants and annual quantum of pollution

Sl No	Input /pollutant	Quantum- Annual
1.	Sediments	1600 million tonnes
2.	Industrial Effluents	50 x 10^6 m^3
3	Sewage - Largely Untreated	0.41 x 10^9 m^3
4	Garbage and Other Solids	34 x 10^6 tonnes
5.	Fertilizer – Residue	5 x 10^6
6.	Synthetic Detergents – Residue	1,30,000 tonnes
7.	Pesticides – Residue	65,000 tonnes
8.	PetroleumHydrocarbons (Tar balls residue)	3,500 tonnes
9.	Mining Rejects, Dredged Spoils and Sand Extractions	0.2 x 10^6 tonnes

(Data by Tata Energy Research Institute, New Delhi)

present study PCP remaining and PCP degraded did not correlate. PCP concentrations decreased through the incubation with the 41 mg kg^{-1} being the most persistent in the soil and detected until day 35. In the other two treatments, PCP remained was detected only until 7th day (25).

Conclusion

Fungi producing large quantities of laccase may offer preferable enzyme system for the removal of PCP. Relation between laccase producing and PCP degradation shows the potentiality of white rot fungi in bioremediation of aqueous effluents, where polymerized products could be readily removed. 30 days of incubation with white rot isolates like *F. fomentarius, P. ostreatus* and *T. versicolor* Laccase production (0.14±003U) and PCP degradation (91.94±1.34% to 100±00%) found quite interesting and satisfactory when compare to the remaining isolates.

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Vitamin E reduces reactive oxygen species mediated damage to bio-molecules in leprosy during multi-drug therapy

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Abstract

Chronic infectious disease process of leprosy caused by the intracellular parasitic pathogen *Mycobacterium leprae* involves accumulation of highly reactive oxygen species (ROS). The present treatment method available for leprosy is MDT (Multi Drug Therapy) involves combination of Rifampicin, Dapsone and Clofazimine, although MDT disinfect leprosy patients, it has limited impact on the increased ROS production and decreased antioxidant status in affected individuals. In the present study we have investigated the causative bacillary load, lipid peroxidation, DNA damage in single cells using single cell gel electrophoresis (alkaline comet assay), oxidative stress indices in multi bacillary leprosy patients. The mean values plasma lipid peroxidation, erythrocyte oxidative stress indices in untreated leprosy cases were higher than the controls, this further increases significantly ($p<0.05$) with MDT. Upon supplementation with 400 IU antioxidant vitamin E we noticed a significant decrease was noticed in plasma lipid peroxidation, oxidative stress indices, and DNA damage. Hence we conclude that MDT along with vitamin E supplementation reduces the oxidative stress mediated insult to cells and bio-molecules during the chronic course of treatment in leprosy.

Keywords: *Mycobacterium leprae,* Multi Drug Therapy, Alkaline Comet Assay, Peroxidation, Oxidative stress.

Abbreviations

MDT : Multi Drug Therapy, ROS : Reactive oxygen species, LPO: Lipid Peroxidation , TBARS: Thio Barbutric Acid Reactive Substances, DNA: Deoxy Ribo nuclic Acid, MDA: Malondialdehyde, DMSO: Dimethyl Sulphoxide, WBC: White Blood Cell, SOD: Superoxide Dismutase, NBT: Nitroblue tetrazolium, EDTA: Ethelene Diamine Tetra Acetic acid.

Introduction

Hansen's disease "*historically known as leprosy*" is a mutilating, debilitating, devastating and deforming disease of mankind. The causative intra cellular germ *Mycobacterium leprae* mediate strong inflammatory response and cause gross destruction of tissues during the chronic course of infection in affected individuals. Patients with leprosy are treated with Multi Drug Therapy (MDT) consisting of Rifampicin, Dapsone and Clofazimine. Despite of large-scale implementation of MDT by the world health organization, the incidence of the disease is still cause of concerned in several hyper-endemic countries (1). MDT although disinfect leprosy

patients, it has limited impact on the increased ROS production and decreased antioxidant status in affected individuals (2,3,4). In our earlier studies, we reported apparent involvement of oxidative stress together with a significant decrease in the antioxidant status in leprosy cases (5)

In leprosy affected persons, the exact mechanism by which intracellular *M leprae* resist destructions of bactericidal activity of the host cell still remains poorly understood. Possible mechanism of bactericidal activity is believed to include the effects of toxic oxygen derivatives or ROS (Reactive Oxygen Species) such as superoxide anion (O_2), hydroxyl radical (OH) or hydrogen peroxide (H_2O_2) derived from the phagocytic cell respiratory burst (6). Other mechanism that mediate ROS production is the drugs used in MDT in releasing ROS during the mode of action (4,7,8). Recent evidences reveal the possible association of ROS in causing injury to cell and damaging effect on DNA such as oxidation of purine and pyramidine bases, break in double strand and single strands (9). Apart from the severity caused by the infectious agent, malnutrition often seen in leprosy patients aggravates infection, while infection with intracellular *Mycobacterium leprae* adversely affect the nutritional status(10). Earlier studies shown that in the malnourished, the severity of the disease and susceptibility disease is relatively higher (11). Malnutrition usually co-exists with the depletion of micronutrients and antioxidant vitamins (12). Under normal condition the production of ROS is kept in control by the antioxidant defense system but in leprosy cases this antioxidant defense system is affected and leprosy patients are exposed for oxidative stress which affect the cell structures and bio-molecules (13,14).

Several intervention studies support the long-term supplementation of antioxidant vitamin E results in declining in the oxidative damage to DNA during the chronic pathology caused by *Mycobacterium* (15). Earlier studies reveal reduction of clastogenic effect was noticed upon treatment with vitamin A and vitamin C in murine models infected with *M.leprae* (16). Apparent renal toxicity owning to anti leprosy drug administration is also reported in recent years (17). Low levels of antioxidants as seen in patients with leprosy could expose the leprosy patients to oxidative stress mediated insult through ROS.

Hence exogenous supplementation of micronutrients and intervention of antioxidants is an attractive approach in the treatment of leprosy. In the present study we have investigated the causative bacillary load, lipid peroxidation, DNA damage in single cells using single cell gel electrophoresis (alkaline comet assay), oxidative stress indices in multi bacillary leprosy patients and we have supplemented 400 IU of antioxidant vitamin E to evaluate the protective role against the oxidative stress mediated changes to cells and bio-molecules during the chronic course of infection and anti leprosy chemotherapy (MDT).

Materials and Methods

Laboratory diagnosis of leprosy is made on the basis of the skin smear examination and the causative bacillary load is recorded as (BI) Bacteriological index (18). A total of 80 untreated multi bacillary (severe) type of leprosy patients attending the OPD of Central Leprosy Teaching and Research Institute in the age group of 25 to 40 years of both the gender were enrolled in this study. Of the 80 diagnosed leprosy cases only 50 cases fully cooperated for the study and they are enrolled as Group II (remaining 30 cases did not fully co-operate for this study). Enrolled 50 cases in the Group II are further divided in to Group II a and Group II b. Group II a (n=25) leprosy cases are treated with MDT and Group II b (n =25) consisted of leprosy patients treated with MDT + antioxidant vitamin E 400 IU/ L

supplementation. Group I (n=25) consist of control subjects and they are age matched healthy without any signs and symptoms of the disease. Informed consent from the participants and clearance of ethical bodies of the institution was obtained. Persons with systemic ailments having the habit of smoking, consuming alcohol, taking treatment for any other ailments were excluded from the study. No study subject consumed vitamin E in the last 6 months.

All diagnosed leprosy patients were treated with anti leprosy chemotherapy (MDT) and patients in group II b is treated with vitamin E 400 IU along with MDT. Following diagnosis, anti coagulated blood samples were collected from the control and experimental subjects (group II at the time of diagnosis) and group II a and group II b (after the treatment period) were processed for the separation of plasma, WBC (Using Ficoll Hypaque) and red blood cells and haemolysate. Lipid peroxidation assay was estimated using plasma samples. Detection of DNA damage in single cells (WBC) were estimated using the separated WBC fraction, erythrocyte and haemolysate was used for the estimation of oxidative stress indices. Haemolysate was prepared according to standard bio-chemical method (19). Skin smear grading is used to understand the grading of bacterial load in a leprosy patient (20). Mean standard deviation values are tabulated and when found significant ANOVA tests is performed for inter group comparison at $P<0.05$ significance level.

Oxidative stress indices

Superoxide radical assay

Superoxide radical is a very labile radical and analysis was made in all samples processed identically from the collection site. The blood samples were cooled during the transport from the collection site during the transport and the nitro blue tetrazolium (NBT) reduction was measured exactly at the end of one hour after blood collection to obtain uniformity. For each sample of blood superoxide was measured as NBT reduction in two sets of tubes of which SOD was inhibited in one set. The difference gave the superoxide radicals present at the time of assay. A stock solution containing 100 mM of NBT was prepared in water and diluted to make a working standard of 10mM/ml of NBT served as standard for the assay of superoxide radicals. Superoxide levels in erythrocytes were expressed in terms of milli moles of NBT reduced/ 10^{12} cells/ 10 minutes (21).

Hydroxyl radical assay

Hydroxyl radicals in the haemolysate were estimated by the method of Gutteridge by their reaction (hydrogen abstraction) from 2-deoxyribose, resulting in the formation of thio barbutric acid and reactive substances. 2.0 ml of 0.5 % hemolysate, 2.0ml of deoxyribose was added and incubated at 37-C for one hour. Then 0.5 ml of TBA (thiobarbituric acid solution) and 0.5 ml of trichloroacetic acid solution were added and heated in a boiling water bath for 15minutes. The mixture was cooled and the reading was taken at absorbance at 530 nm. Blank were included without 2-deoxy ribose to assess the basal thiobabuturic acid reactive substance in the hemolysate for each blood samples analyzed. Standards in the range of 5 to 30 nanomoles were also developed. Amount of hydroxyl radicals present in the erythrocytes were expressed in terms of nanomoles of MDA/10^{12} cells/ hour (22).

Hydrogen peroxide assay

Hydrogen peroxide in the erythrocytes were estimated by the method of Wolf (23). Hydrogen peroxide oxidizes ferrous iron to ferric iron selectively and the resultant Fe3+ can be determined by sensitive dye xylenol orange (O-cresol sulfonapthalene, 3'3'-bis methylamino diacetic acid which is highly sensitive to Fe3+ to form a blue-purple complex measured at 560 nm.

Fresh blood samples containing heparin and 1mM sodium azide a catalase inhibitor was used in this assay. Solution containing 1-5µM of hydrogen peroxide were treated in a similar way is used a standard. Erythrocyte hydrogen peroxide levels were expressed as µ moles/ 10^{12} cells.

Lipid peroxidation

Lipid peroxidation in the plasma was estimated using TBA (Thiobarbutric acid) reaction. Standard MDA 50 mM solution of malondialdehyde was prepared in distilled water using 1,1,3,3 tetrahydroxypropane. This was stored in 4 °C and diluted just before use such that working standard contains 50nM/ml. Plasma TBARS values were expressed as n moles of MDA/ L (24).

Detection of DNA damage in single cells (Comet assay)

DNA damage in the separated portion of white blood cells (WBC's) was carried out by the method of detection of DNA damage in single cells (25). Comet assay: single cell gel electrophoresis is a technique which detects DNA damage and repair in individual cells like WBC. 100 µl of normal melting agarose in phosphate buffer was dropped on a frosted slide, immediately covered with a cover slip and kept for 10 minutes in a refrigerator to solidify. Then, cover slips were removed and 100 µl low melting agarose containing 75 µl of WBC in PBS and 100 µl low melting point agarose) were added to the slides. Again the cover slips were replaced and slides were kept in refrigerator for another 10 minutes. After this, the cover slips were removed and the top layer of 100 µl low melting agarose was added and cooled again for another 10 minutes. After this step, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris-Hydrochloric acid, pH adjusted to 10.0 with NaOH; 1% Triton X – 100 and 10% DMSO were added freshly) and slides were kept in dark at 4°C for at least 1 hr to prevent additional DNA damage the following procedures were carried out under dim light. The slides were removed from lysing solution and placed on a horizontal electrophoresis tank. The electrophoresis unit was filled freshly made electrophoresis buffer (300 mM NaOH and 1mM EDTA, pH 13.0) to a level of 0.25 cm above the slides. The cells were exposed to alkali for 20 minutes to allow unwinding of DNA. An electric current of 25 volt and 300mA (high voltage electrophoresis) was applied for 20 minutes. After electrophoresis, slides were placed horizontally and neutralized with Tris-HCl. Finally, 50µl of ethidium bromide was added to each slide and covered with cover slip again and analyzed under the high power magnification of fluorescent microscope with a calibrated ocular. Images of 50 randomly selected cells were analyzed from each sample. For each cell, the length of the image (diameter of the nucleus plus migrated DNA) was measured. The intact DNA appear like a spot while the damage in DNA appear as comet with tail. The tail length is directly proportional to the DNA damage in single cell (WBC). The damage is represented by an increase of DNA fragments that have migrated out of the cell nucleus in the form of a characteristic streak similar to the tail of a comet.

Results

Table 1: depicts the levels of toxic metabolites of oxygen derived free radicals like superoxide radicals, hydroxide radicals, and hydrogen peroxide in controls and experimental subjects, Superoxide and hydroxyl levels were estimated in the haemolysates prepared from erythrocytes and the levels of hydrogen peroxide were estimated in the erythrocytes of control and experimental subjects. Nearly two fold increase in the levels of oxygen-derived free radicals noticed in group II. The levels of oxygen derived free radicals increase further upon treatment with

Table 1. Levels of Superoxide, Hydroxyl and Hydrogen peroxide in the control and experimental subjects

Oxidative Stress Indices	Group I	Group II	Group II a	Group II b
Superoxide radical in haemolysate (mmoles NBTreduced / 10^{12} cells/ 10 min)	44 ± 5	110 ± 10 [a]	135 ± 11 [b]	73 ± 8 [c]
Hydroxyl radical in haemolysate (nmoles of MDA/ 10^{12} cells / hr) / 10 min)	10 ± 1	26 ± 2 [a]	36 ± 3 [b]	20 ± 2 [c]
Erythrocyte Hydrogen peroxide (mmoles / 10^{12} cells)	62 ± 5	135 ± 13 [a]	172 ± 16[b]	90 ± 10 [c]

Values expressed as mean ± S.D.
a- Group II compared with group I
b- Group II a compared with group II
c- Group II b compared with group II a
Values are significant at $p<0.05$

MDT (compare group II verses group II a). Upon co-supplementation of vitamin E oxidative stress decreases

Fig. 1 represents the bacterial load scored after examining 100 oil-immersion fields by number ranging from 0 to 4. 5 + each representing, on the average of 10 times as many bacilli as the smaller number is 1 +. Untreated MB leprosy cases had a high bacillary index of 4. 5 + on Ridley's logarithmic scale. Upon treatment with MDT the BI fall from 4.5 + to 2 +. Group II (untreated leprosy) patients showed a profound increase in the BI which may be due to the lack of immunity against the invading intra cellular *M.leprae*. Treatment with MDT showed a progressive reduction in the bacterial index from 4 + to 2 + (compare group II vs group II a). There is no significant change in the bacterial index in group II b (vitamin E supplemented subjects) which means vitamin E supplementation did not either induce or reduce the bacterial load in leprosy patients.

Fig. 2 depicts the levels of plasma lipid peroxidation in control and experimental subjects. Lipid peroxidation is an organic expression of free radical mediated changes to lipid and the end product of lipid peroxidation (LPO) is malondialdehyde which can be measured in plasma samples by their reaction with thiobarbituric acid reactive substances. We noticed a sharp increase in the LPO in plasma was observed in leprosy patients when compared to control (compare group I vs group II), LPO levels further increased in leprosy subjects undergoing MDT (compare group II a vs group II). Upon supplementation with antioxidant vitamin E (group II b) the LPO levels decreased (compare group II a Vs group II b).

Fig. 3 depicts the extent of DNA damage in single cells (WBC) in control and leprosy affected persons. The tail length of the comet is directly proportional to the extent of DNA damage in single cells. Single cells of multi bacillary leprosy cases shows a significantly

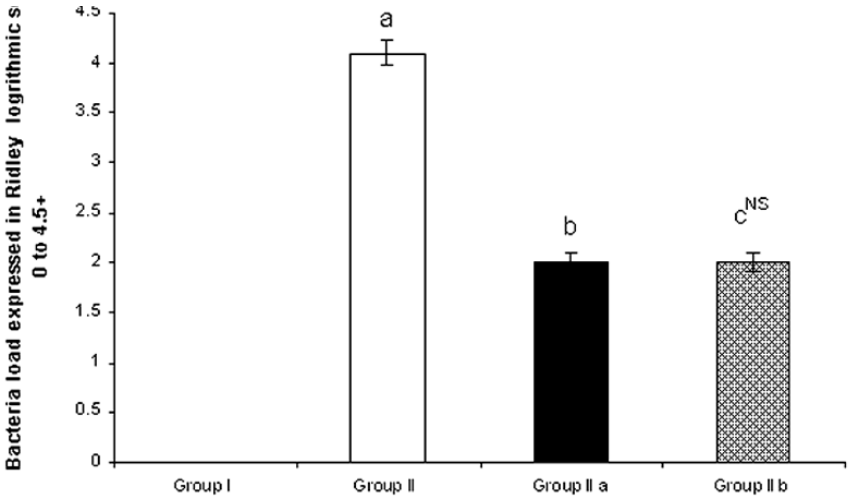

Fig. 1. Bacteriological index of experimental subjects

Values expressed as mean ± S.D
Group I (not applicable)
a -group II compared with group I
b- group II a compared with group II
a,b statistically significant at $p<0.05$
c^{NS} - not significant at $p<0.05$ (group II b compared with group II a)

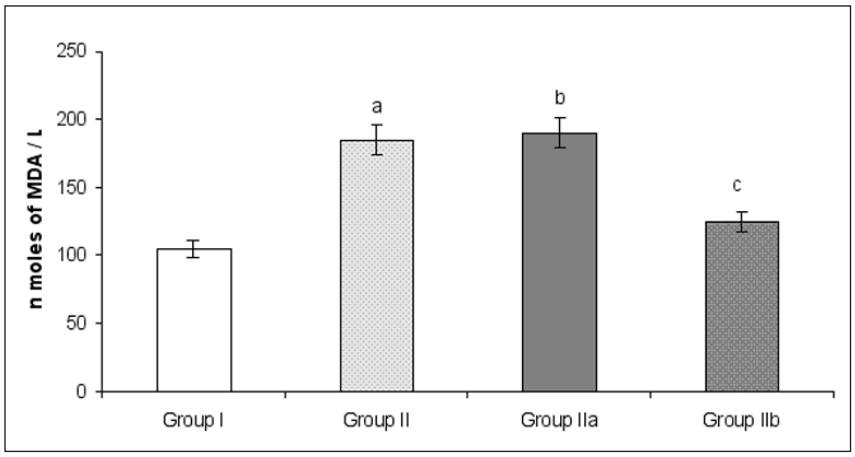

Fig. 2. Lipid peroxidation products (TBARS) in the plasma of control and experimental subjects

Values expressed as mean ± S.D
a -group II compared with group I
b- group II a compared with group II
c- group II b compared with group II a
a,b and c statistically significant at $p<0.05$

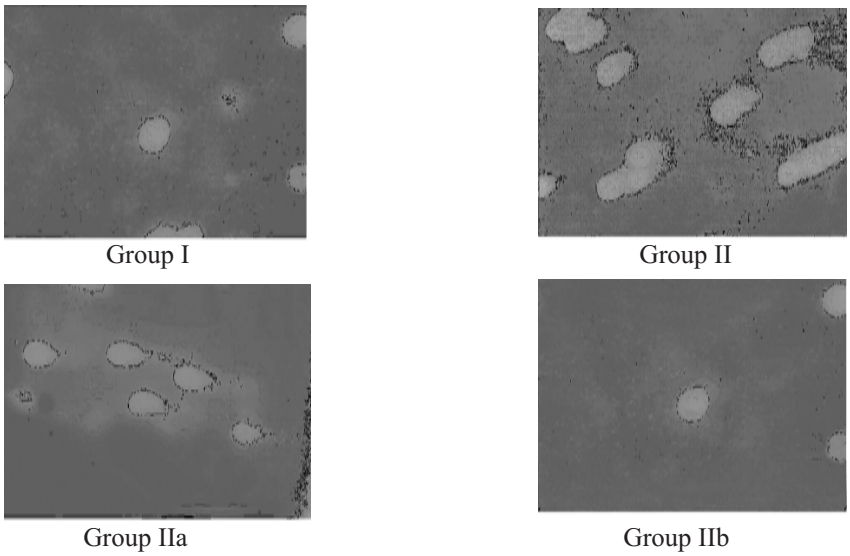

Fig. 3. Demonstration of DNA damage in single cells (White Blood Cells) by the alkaline comet assay stained with Ethidium bromide fluorescent staining technique in control group I , untreated group II, MDT treated (group II a) and MDT + vitamin E co-supplementation (group II b). Magnification X 400 times.-

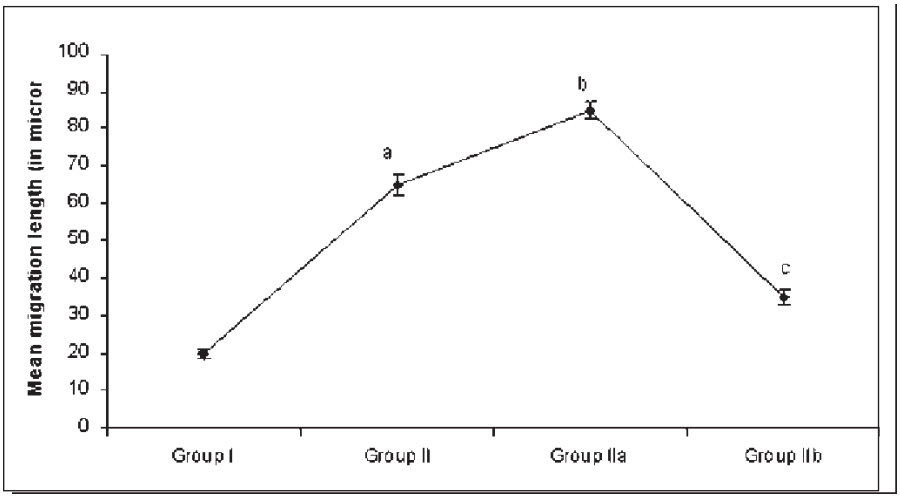

Fig. 4. DNA migration (comet tail length) control and experiental subjects.

Values expressed as mean ± S.D
a -group II compared with group I
b- group II a compared with group II
c- group II b compared with group II a
a,b and c statistically significant at $p<0.05$

increased tail length ($P<0.05$) indicates the increased DNA damage in response to the infection, Fig. 4. There was a significant protection against the DNA damage in single cells upon treatment with antioxidant vitamin E.

Discussion

The greatest paradox of aerobic respiration is oxygen, which is essential for energy production may also be detrimental, because it leads to the production of reactive oxygen species (26). Oxidative stress is a condition in which the elevated levels of ROS damage cells, tissues and internal organs (27,28). Oxygen derived free radicals are produced as a result of metabolism of oxygen biradical during reduction reactions (29). Superoxide radicals are unique in that it can lead to the formation of many other reactive oxygen species including hydroxyl radical (HO_2). Superoxide radicals also reacts with Hydrogen peroxide to generate the singlet oxygen molecule (30). The hydroxyl radicals are most potent oxidant encountered in the biological system, because they readily react with almost all biological substrates (31). Hydrogen peroxide is not a radical by definition but it remains most extensively as they are formed as a secondary product of one-electron transfer oxidation of oxygen biradical during reduction reaction (32). Oxidative DNA damage refers to the functional or structural alterations of DNA resulting from the insults of ROS (33). DNA base modification and DNA strand breaks are two of the major forms of oxidative DNA damage caused by ROS. DNA also has the ability to repair itself against the toxic substances and deleterious effects of reactive oxygen species (34,35). When the oxidative stress override the antioxidant status, a physiological imbalance is produced in multi bacillary leprosy patients, such imbalance expose the DNA to persistent attack of ROS.

At molecular level, damage to DNA caused by ROS, oxidize the purine and pyrimidine bases, there by result in gross changes in DNA such as single strand breaks, sister chromatid exchanges and the formation of micronuclei during the chronic infectious disease process and treatment with drugs used in MDT. They are produced continuously in the cells either as accidental by-products of metabolism or deliberately during the pathogenesis of chronic infectious, inflammatory diseases. It is difficult to block the oxidative stress-induced injury to cells or tissues because ROS are continuously produced by cellular aerobic metabolism (36). Oxidative stress may be limited by using chain-breaking antioxidants such as vitamin E which neutralizes hydroxyl, superoxide, and hydrogen peroxide radicals and prevents oxidative stress (37). In addition, it also helps in membrane stability and recycling of vitamin C. In our earlier studies we have reported free radical mediated oxidation of lipids and proteins in untreated and MDT treated leprosy patients (38,39).

Dietary antioxidants form an essential part of the human antioxidant defense system. Fruits and vegetables as well as daily dietary supplements constitute the potential sources of various antioxidants (38). The daily requirement of vitamin E varies from 50 to 800 mg. The *'antioxidant hypothesis'* proposes that antioxidants like vitamin C, vitamin E, carotenoids present in fruits and fresh leafy vegetables afford protection against the oxidative damage to cells and bio-molecules (39). But leprosy patients do not get antioxidant rich diet like fruits and fresh leafy vegetables everyday and moreover, the deranged liver function affects the homeostasis of *in vivo* antioxidant status in affected individuals (40). Agnihotri et al, reported higher plasma LPO with decreased activity of renal brush border membrane in *M.leprae* infected mouse models (41). The elevation of LPO in the plasma is considered as a cause for degeneration of organs and tissues in leprosy patients. Lipid peroxides formed at the primary

site could be transferred through blood circulation to other organs and tissues provoke damage by lipid peroxidation (42).

Mycobacterium leprae does not affect all the individuals who are exposed to it, nor does it produce the same degree of illness in those who become infected by it. A variety of risk factors have been invoked to explain this variation in disease susceptibility and morbidity of which nutrition plays a key role in determining the type of disease. Leprosy afflicted patients are devoid of balanced food for various reasons like: lack of job opportunity, loss of social status, deformity, disfigurement, social stigma and further the deranged liver function as seen in leprosy patients could affect the homeostasis of micronutrients. Further, the drugs used in MDT (multi drug therapy) for treatment of leprosy is very effective in controlling the proliferation of *M.leprae* but, during the mode of action produce copious amount of oxygen-derived free radicals this process could affect the functional integrity of the cells. In the present study we have noticed a decline in the oxidative stress indices upon supplementation of antioxidant vitamin E which compel us to examine more and more on oxidative stress mediated changes to cells and bio-molecules in affected individuals. Advances in understanding of the oxidative stress related changes to biomolecules like lipid and DNA may lead to tools for use of specific drugs used in control programmes in leprosy and will provide new insights on *Mycobacterium leprae* virulence, pathogenecity and treatment of leprosy. This study offers an opportunity for correlating levels of MDT therapy-induced DNA damage with administered dose and modulating effect of exogenous supplementation of antioxidant vitamin E in genotoxicity during the chronic course of the disease and anti-leprosy chemotherapy.

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Formulation and Evaluation of Controlled release of Losartan potassium matrix tablets using poly (Ethyleneoxides)

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Abstract

In the present investigation an attempt has been made to increase therapeutic efficacy, reduced frequency of administration and improved patient compliance by developing controlled release matrix tablets of Losartan Potassium. Losartan Potassium was formulated as oral controlled release matrix tablets by using poly(ethylene oxides) { Polyox WSR 303 }.The aim of this study was to investigate the influence of polymer level and type if fillers namely lactose [soluble filler], microcrystalline cellulose and anhydrous dibasic calcium phosphate [insoluble fillers] on the release rate and mechanism of Losartan Potassium from matrix tablets prepared by direct compression process. Higher polymeric content in the matrix decreased the release rate of drug because of increased tortuosity and decreased porosity. At lower polymeric level the rate and extent of drug release was elevated. On the other hand, replacement of lactose with anhydrous dibasic calcium phosphate and microcrystalline cellulose have significantly retarded the release rate of Losartan Potassium.

Key words: Losartan Potassium, Matrix tablets, Poly (ethylene oxides).

Introduction

Oral route has been one of the most popular routes of drug delivery due to its ease of administration, patient compliance, least sterility constraints and flexible design of dosage forms. Hydrophilic polymeric matrices have attracted many researchers due to their wide applications in controlled drug delivery. When the release medium (i.e. water) is thermodynamically compatible with a polymer, the polymer may undergo relaxation process so that the polymer chains become more flexible and the matrix swells. This could allow the encapsulated drug to diffuse more rapidly out of the matrix. On the other hand, it would take more time for drug to diffuse out of the matrix since the diffusion path is lengthened by matrix swelling. Moreover, it has been widely known that swelling and diffusion are not the only factors that determine the rate of drug release (1). For dissolvable polymer matrix, polymer dissolution is another important mechanism that can modulate the drug delivery rate. While either swelling or dissolution can be the predominant factor for a specific type of polymer (2), in most cases drug release kinetics is a result of a combination of these two mechanisms (3,4). Among the variety of

hydrophilic polymers, poly(ethylene oxide) (PEO) is one of the most important material used in the pharmaceutical industries mainly because of its non-toxicity, high water-solubility and swellability, insensitivity to the pH of the biological medium and ease of production. Recently the swelling and dissolution behavior of PEO tablets (5–8) and hydrogels [9] as well as their influences on drug release characteristics have been studied. It is found that, compared with low molecular weight PEO, the high molecular weight PEO tablet swells to a greater extent and the swelling of the polymer rather than the dissolution of the polymer is the governing factor for drug release. The compression force applied during the manufacturing process, pH of the release medium and the stirring rate do not affect the drug release behavior significantly. One method of fabricating controlled-release dosage forms is the incorporation of the drug in a matrix containing a hydrophilic, rate-controlling polymer. Matrix systems are popular in controlled-release formulation systems in terms of economic, process development and scale-up procedures (10–13). Various cellulose derivatives are widely used in the pharmaceutical field as excipients for the preparation of matrix systems used in sustained release formulations (14). Several factors, such as the polymer type and concentration, the drug particle size and the presence of additives and excipients in the final formulation can modify the drug release from the matrices (15–17). The present study on controlled release PEO matrix tablets containing Losartan Potassium with different types of diluents such as Lactose, DCP & Microcrystalline cellulose, and their influence on matrix erosion and drug diffusion to achieve steady state drug release was performed.

Materials and Methods

Losartan Potassium was kindly supplied from Dr.Reddy's Laboratories Ltd (Hyderabad), PEO's { Polyox WSR 303}, Microcrystalline cellulose (MCC)(Avicel PH 102) were obtained from Dow chemicals Asia Pvt., Ltd., Mumbai. Dibasic calcium phosphate(DCP) and lactose and Magnesium stearate is of analytical grade and procured commercially. All other chemicals were of analytical grade and were used as received.

Preparation of matrix tablets

The PEO matrix tablets containing Losartan Potassium was prepared by a direct compression method. Poly (ethylene oxides) (POLYOX WSR 303) was used as a swellable hydrophilic polymer that controls drug release rates. The Controlled release tablet formulations consisted of a drug & polymer were in the ratio of 1: 0.5, 1:0.75 and 1: 1 for Polyox WSR 303. The diluents such as Lactose, DCP & Microcrystalline cellulose were added at different proportions to the matrix tablets to achieve uniform weight of all the matrix tablet formulations. The drug, polymer and diluent were screened through #45 sieve and blended in a lab scale double cone blender. The lubricant such as magnesium stearate in the concentration of 0.5 % was added and the blend was mixed again prior to compression. The drug blends were directly compressed by using cadmach rotary compression machine using 9 mm flat punches. The different forms of tablets compressed together with their compositions are given in the table 1.

Evaluation of Tablets

The prepared tablets were tested as per standard procedure for weight variation (n = 20), hardness (n = 6), drug content, thickness (n = 20) and friability(n = 20) characteristics. Matrix tablet hardness was determined by using a Monsanto tablet hardness tester (Campbell Electronics,Mumbai, India). Friability test (n = 20) was conducted using Roche friabilator. Thickness of the tablets was measured by digital Vernier caliper . Drug content of Losartan Potassium was analyzed by measuring the

absorbance of standard and samples at λ = 205 nm using UV/Visible spectrophotometer (Elico model SL-159).

***In Vitro* Drug Release Characteristics**

Drug release was assessed by dissolution test under the following conditions: n = 6 (in triplicate), USP type II dissolution apparatus at 75 rpm using 900 mL of 0.1 N HCl (2 h) and phosphate buffered solution, pH 6.8 (PBS) (24 h), as the dissolution media. Dissolution studies were carried out in triplicate, maintaining the sink conditions for all the formulations. A 5 ml aliquot of samples were withdrawn at regular time intervals, filtered and assayed spectrophotometrically at 205 nm. Some diffusion models

Table 1. Compositions of Various Matrix Tablet Formulations of Losartan Potassium

	Formulations								
Ingredients [mg/tablet]	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Losartan Potassium	100	100	100	100	100	100	100	100	10050
Polyox-WSR 303	50	75	100	50	75	100	50	75	100
Microcrystalline cellulose	148.5	123.5	98.5	—	—	—	—	—	—
Dicalcium phosphate	—	—	—	148.5	123.5	98.5	—	—	—
Lactose	—	—	—	—	—	—	148.5	123.5	98.5
Magnesium stearate	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Total wt of tablet (mg)	300	300	300	300	300	300	300	300	300

Table 2. Pharmacokinetic Parameters of Various Matrix Tablet Formulations of Losartan Potassium

Formulations	First order constants (hr^{-1})	Correlation coefficient (r)	Dissolution rate constants ($mg/hr^{1\backslash 2}$)	Correlation coefficient (r)	Peppas constant (n)	Correlation coefficient (r)
F01	0.268	0.9976	34.65	0.9971	0.77	0.9974
F02	0.152	0.9957	26.62	0.9980	0.59	0.9917
F03	0.116	0.9952	23.62	0.9979	0.62	0.9922
F04	0.199	0.9970	33.17	0.9995	0.65	0.9931
F05	0.110	0.9967	24.99	0.9983	0.63	0.9911
F06	0.087	0.9895	22.32	0.9988	0.64	0.9915
F07	0.338	0.9803	43.07	0.9963	0.89	0.9958
F08	0.151	0.9819	32.69	0.9916	0.85	0.9983
F09	0.138	0.9856	26.51	0.9934	0.80	0.9940

(Korsmeyer-Peppas) are expected to be valid up to ~60% cumulative drug released, therefore the data for analysis were restricted to that range. To analyze the mechanism of drug release from the matrix tablets, data obtained from the drug release studies were analyzed according to Equations 1, 2, and 3 of the First-order model, Higuchi model, and the Korsmeyer-Peppas model respectively.

$$InQ=k.t \quad \text{——————1}$$
$$Q = k.t^{\circ} \quad \text{——————2}$$
$$M_t/M_\infty = Kt^n \quad \text{——————3}$$

where Q in the equation (1) is Cumulative percent drug remained, while Q in the equation (2) is Cumulative percent drug released, where $Mt/M\infty$ is the fraction of drug released, t is the release time and k is the constant incorporating the structural and geometrical characteristics of the release device. The values of n were obtained by linear regression analysis. A value of n=0.45 indicates Case I (Fickian) diffusion or square root of time kinetics, $0.45<n<0.89$ indicates anomalous (non-Fickian, drug diffusion in the hydrated matrix and the polymer relaxation,) diffusion, n=0.89 indicates Case II transport and $n>0.89$ indicates Super Case II transport (18). Linear regression analysis was performed for all these equations and regression coefficients (r) were determined and the results were given in the table 2.

Stability studies

Stability studies on the optimized matrix tablets were carried out as per ICH guidelines at $25^0C \pm 2^0C/60\% \pm 5\%$ RH and $40^0C \pm 2^0C/75\% \pm 5\%$ RH. Physical attributes of the tablets, % drug content and in vitro drug release profiles were studied over a period of 6 months and the results were given in the table 3.

Results and Discussion

All batches of matrix tablets were produced under similar conditions to avoid processing variables. The compressed tablets were evaluated for various physical parameters such as weight uniformity , hardness, drug content, friability and thickness. Drug content of the formulations were assayed spectrophotometrically at 205 nm. The assayed content of drug in various formulations varied between 98% and 100% (average 99 %). Tablet weights varied between 300and 303mg (average 301.5 mg), hardness between 3.0 to 5.0 kg/cm^2 (average 4.0 kg/cm^2), thickness between 3.00 and 3.10 mm and friability loss was in the range of 0.32% to 0.47% (average 0.40%). Thus all the physical parameters of the compressed matrix tablets were found to be practically within the official limits. The *in vitr*o drug release studies were conducted for all the matrix tablet formulations. The tablets extended the drug release from 8hrs to 24hrs. Among the matrix

Table 3. Physical Parameters of Various Matrix Tablet Formulations of Losartan Potassium before and after stability studies.

Formulation	Weight uniformity(mg)		Friability loss (%)		Hardness (kg|cm^2)		Drug Content(%)	
	Before stability studies	After stability studies	Before stability studies	After stability studies	Before stability studies	After stability studies	Before stability studies	After stability studies
F3	300	300	0.33	0.32	4.0	4.0	99.68	99.24
F6	300	300	0.32	0.32	4.0	4.0	99.75	99.25

tablets, formulations F03 & F06 showed linear drug release over the period of 24hrs. The drug release from the matrix tablets containing lactose as diluent was rapid when compared to the other diluents such as MCC and DCP. The order of drug release from various matrix tablets along with diluents is Lactose>M.C.C. >D.C.P. This may be due to higher hydrophilicity of the organic diluent than the inorganic counterpart, resulting in faster movement of solvent front i.e. easier penetration of dissolution medium into the tablet matrix, lead to the faster matrix erosion. It was also observed that the matrix delivery system has potential to function in relatively pH – independent manner and it was able to control the release of Losartan Potassium in a linear manner over a prolonged period of time. Drug released from the tablets were with in 98-100 % and found to be best explained by first order plots and Korsmeyer-Peppas model (the critical value of n = 0.593–0.850 suggesting non-Fickian diffusion i.e, the drug release is by diffusion from the hydrated matrix and by polymer relaxation).It is also indicated that the higher proportion of PEO in

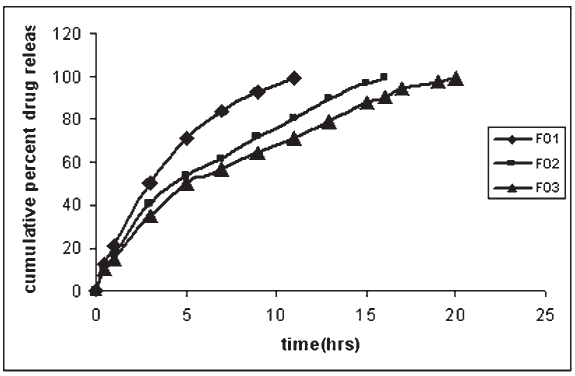

Fig. 1.1. Drug release profiles of controlled release matrix tablet formulations of Losartan Potassium. (MCC as diluent)

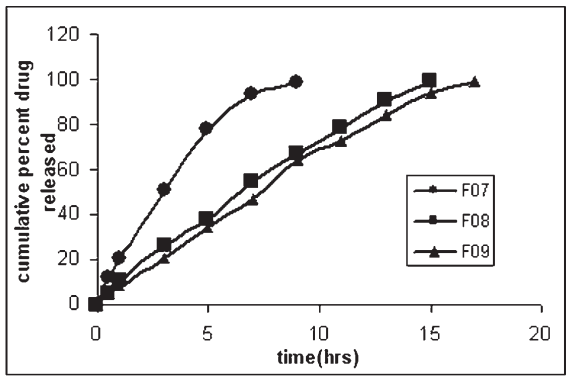

Fig. 1.3. Drug release profiles of controlled release matrix tablet formulations of Losartan Potassium (Lactose as diluent).

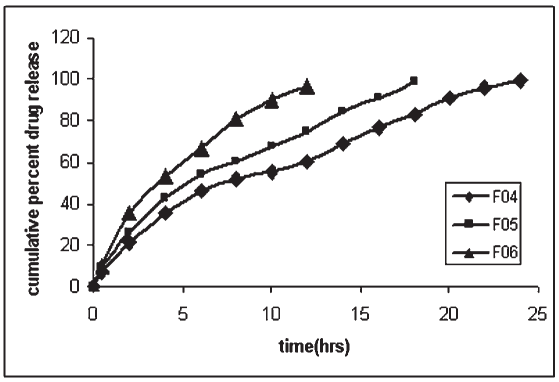

Fig. 1.2. Drug release profiles of controlled release matrix tablet formulations of Losartan Potassium. (DCP as diluent)

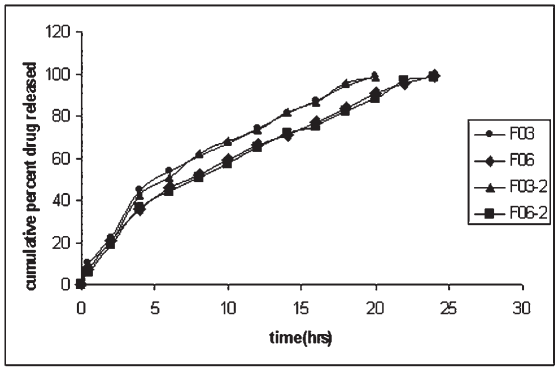

Fig. 1.4. Drug release profiles of selected matrix tablet formulations of Losartan Potassium before and after stability studies.

the matrix results in the delay of drug release over an extended period of time. Formulations F3 and F6 are prepared with highest proportion of PEO than other formulations. The stability studies on selected formulations were carried out for 6 months as per ICH guidelines. Under the specific storage conditions, no significant changes in Losartan Potassium profiles from the prepared tablets was observed. The result of stability tests indicated that Losartan Potassium release properties from the prepared tablets were stable under the accelerated stability storage conditions.

Conclusions

This work has provided a novel and simple approach to formulate an oral, swellable monolithic controlled release delivery system designed for delivery of Losartan Potassium over an extended period of time. An important feature of this system is the potential for generating constant drug release. The formulations F3 & F6 are considered to be best formulations and can further evaluated for *Invivo* pharmacokinetic studies.

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In vitro rooting from callus cultures derived from seedling explants of *Erythrina variegata* L.

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Abstract

Shoot differentiation from seedling explants of *Erythrina variegata*, an important medicinal plant, was tried on Murashige and Skoog's (MS) medium containing different plant growth regulators, but without any success. Thus, *Erythrina variegata* appeared to be a highly recalcitrant leguminous plant. However, rhizogenesis was frequent and achieved directly from different seedling explants (stem, leaf and roots) as well as from explant derived calli. Roots were produced along with callus formation. NAA is the most effective auxin for induction of roots. Natural auxins IAA and IBA were also used for rooting. The media used were (1) MS + 2 mg/l 2,4-D + 1 mg/l NAA and (2) MS + 2 mg/l 2,4-D + 1 mg/l BAP + 1 mg/l IBA for *in vitro* induction of roots from root, stem and leaf calli respectively. Thus, like in other legumes, in the present study also, *Erythrina* displayed a strong ability for *in vitro* rhizogenesis than caulogenesis.

Key words: Rhizogenesis, Auxins, Adventitious roots.

Abbreviations: BAP- 6-benzylaminopurine, **NAA-** a-naphthaleneacetic acid, **IBA-** indole-3-butyric acid, **IAA-** indole-3-acetic acid, **2,4-D-** 2,4-dichlorophenoxyacetic acid.

Introduction

Most seed legumes have a higher propensity for root formation than for shoot formation. For most species, the frequency of root initiation is quite high despite the concentrations of cytokinins in media. Only root initiation was observed in earlier efforts to obtain shoot regeneration for *Psophocarpus tetragonolobus* (1), *Glycine max* (2) and *Phaseolus vulgaris* (3). Root formation occurs prior to shoot regeneration in *Stylosanthes hamata* (4). Forage legumes will form roots but at a lower frequency than seed legumes. Though root differentiation in *in vitro* is frequent, difficulty in root formation has also been reported in both *Trifolium pratense* (5) and *Lathyrus sativus* (6). Numerous studies have indicated that, among the common auxins, NAA is the most effective auxin for induction of root regeneration (7, 8). Usually natural auxin IAA and synthetic auxin NAA and IBA are used for rooting. Janes *et al* (9) used filter sterilized IBA in their experiments. Auxins alone or with cytokinins, GA_3, ABA and phenolics exert their effect mainly during the root induction and initiation phase. In many plant species it was shown that optimal root formation occurred in the presence of auxins and cytokinins (10). The aim of the present study was to induce somatic embryogenesis or whole plants from *Erythrina*.

However, all our efforts failed in the induction of shoots or somatic embryos.

Materials and Methods

The seeds of *Erythrina variegata* were collected from a locally available variety. The seeds were germinated in pots. Young healthy leaf, stem, cotyledon and root explants were isolated, washed with 1% laboline and kept under running tap water for 30 minutes and subsequently surface sterilized with 0.1% mercuric chloride for 2 minutes. The explants were washed with sterile distilled water 3-4 times. The medium was supplemented with 0.5 mg/l to 2 mg/l auxins (NAA, IBA, IAA and 2,4-D) and 2 mg/l to 4 mg/l cytokinins (Kn and BAP). All media were autoclaved at a temperature of 121 ^{0}C (15 psi) for 20 minutes. The percentage of rhizogenesis was recorded from each explant directly or via explant derived callus. Root and shoot (only on one occasion) lengths in mm were

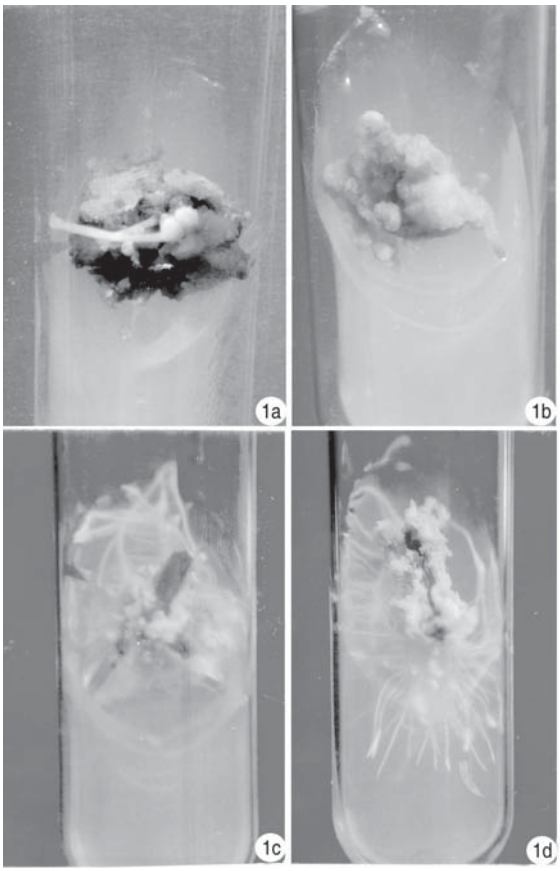

Fig. 1 Rhizogenesis from stem cultures of *Erythrina variegata L.* a) Induction of roots from stem derived callus on MS + 2.0 mg/l 2,4-D + 1.0mg/l BAP + 1.0 mg/l IBA. b) Induction of single root from cotyledon derived callus on MS +2.0 mg/l 2,4-D+1.0 mg/l BAP + 2.0 mg/l NAA. c) The direct rooting from stem explant before callus is induced on MS +2.0 mg/l 2,4-D+1.0 mg/l BAP + 2.0 mg/l IBA. d) Roots with root caps formed from stem explant cultures on MS +2.0 mg/l 2,4-D+1.0 mg/l Kn + 1.5 mg/l IBA.

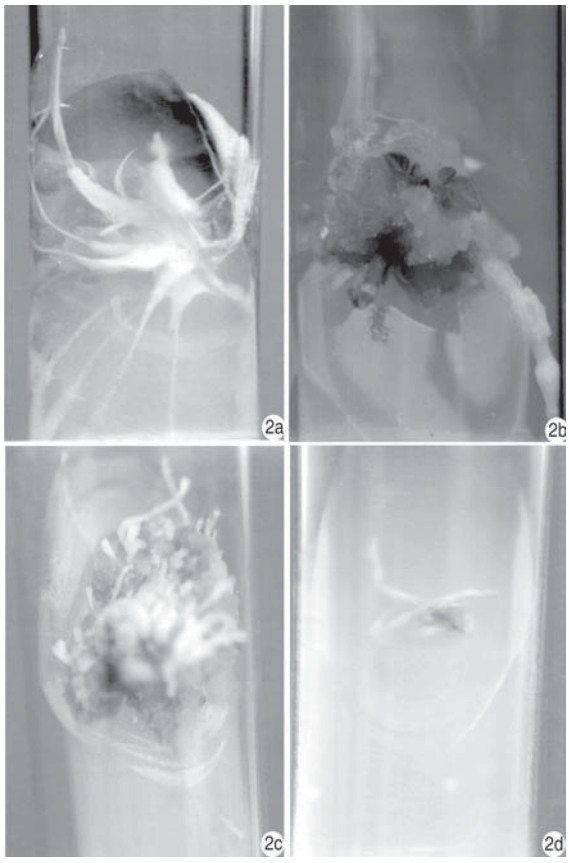

Fig. 2 Rhizogenesis from different explant cultures of *Erythrina variegata L.* a) Direct multiple root production from leaf cultures on MS + 1.0mg/l BAP + 2.0 mg/ NAA. b) Single root production from stem explant culture on the MS + 2.0 mg/l 2,4-D + 2.0 mg/l BAP + 2.0 mg/l NAA. c) Induction of small adventitious roots from stem explant on MS + 2.0 mg/l 2,4-D+1.0 mg/l NAA + 1.0 mg/l IAA. d) Rooting from root cultures on MS + 2.0 mg/l 2,4-D + 1.0 mg/l NAA.

measured in stem explant cultures and are presented in tables 1 and 2.

Results and Discussion

During the present studies, large number of combinations and permutations with Cytokinins were tried for shoot differentiation directly from explants as well as callus cultures (data not shown). But, no shoots were regenerated from explants of *Erythrina*. However, frequent rhizogenesis was noticed from the callus cultures (Figs. 1a & 1b). When stem derived callus was

Table 1. Effect of tissue sources and composition of culture media on callusing and rhizogenesis from responses of explant after 6 week of culture.

Medium	Tissue Source	Callusing response	Caulogenesis (%)	Rhizogenesis (%)
1	Stem	+	6.5	39.5
	Root	--	--	20.0
	Cotyledon	+	10.7	0.0
	Leaf	+	7.0	0.0
2	Stem	+	8.2	12.0
	Root	--	--	0.0
	Cotyledon	+	8.4	0.0
	Leaf	++	26.2	0.0
3	Stem	+	7.5	13.5
	Root	--	--	6.0
	Cotyledon	+	21.5	0.0
	Leaf	+++	35.4	0.0
4	Stem	++	24.1	0.0
	Root	+	8.0	13.0
	Cotyledon	++	17.2	0.0
	Leaf	++	27.5	0.0
5	Stem	++	16.4	15.0
	Root	--	--	20.0
	Cotyledon	++	30.7	16.5
	Leaf	+++	38.2	10.6
6	Stem	++	10.5	52.5
	Root	--	--	0.0
	Cotyledon	+	7.5	0.0
	Leaf	++	21.8	0.0

1) MS + 2.0 mg/l kn + 2.0 mg/l NAA
2) MS + 3.0 mg/l kn + 1.0 mg/l L-glutamic acid
3) MS + 4.0 mg/l Kn + 1.0 mg/l L-glutamic acid
4) MS + 2.0 mg/l 2,4-D+1.0 mg/l NAA
5) MS + 2.0 mg/l 2,4-D + 1.0 BAP + 2.0 mg/l NAA
6) MS + 2.0 mg/l 2,4-D + 1.0 mg/l kn + 1.5 mg/l NAA

subcultured on MS medium fortified with 2 mg/l 2,4-D, 1 mg/l BAP and 1 mg/l IBA, two roots were produced (Fig. 1a). These roots elongated after two weeks of culture. When cotyledon derived callus was subcultured on MS medium containing 2 mg/l of 2,4-D, BAP and NAA, the cultures produced a single root (Fig. 1b). Roots were also produced directly from the stem (Fig. 1c & 1d) and leaf explants (Fig. 2a) after the formation of callus around the explants. On one occasion, roots were differentiated from the regenerated shoots (Fig. 2b). Adventitious root formation occurred from the leaf and stem explants after the callus formation (Fig. 2a; Fig. 1c, Tables 1 & 2). In the present studies, growth regulators were used in the following combinations for root induction: 2,4-D, IBA, BAP or kinetin. Root explants were cultured on MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l NAA (Figs. 2c & 2d). Lower concentration of these two auxins facilitated better rooting without any callus formation (Table 1). These roots were subcultured on MS medium supplemented with auxins and cytokinins. After six weeks of culture, friable callus developed which ultimately turned brown. The brown callus with roots was subcultured again on MS medium containing auxins, two cytokinins (BAP or TDZ and 15% coconut milk) and L-glutamic acid for shoot differentiation, but no shoots were observed.

It was observed that all explants do not have the equal potential to regenerate roots. Stem explants displayed higher percentage of rhizogenesis than other explants. Various growth regulators influenced the induction of roots as well as their elongation (11, 12). Roots are mostly induced in the presence of an auxin. IAA, IBA and NAA promoted high percentage of

Table 2. Effect of IBA, NAA, IAA and 2,4-D (auxin) in various concentrations on rhizogenesis from stem explants in *Erythrina variegata L.*

Auxin type	Auxin concentration (mg/l)							
	0.5	1.0	1.5	20	0.5	1.0	1.5	2.0
	Percentage rooting				No of roots per shoot			
IBA	25.0	35.0	40.0	82.0	1.5	1.9	2.0	2.3
NAA	35.0	42.0	55.0	90.0	2.5	2.8	3.8	5.2
IAA	15.0	24.0	30.0	45.0	1.0	1.3	1.8	2.1
2,4-D	5.0	5.0	6.0	8.0	0.1	0.3	1.0	1.0
	Root length (mm)				Shoot length (mm)			
IBA	8.2	10.5	11.3	15.0	17.6	18.2	19.4	20.1
NAA	12.8	13.5	13.8	17.3	20.4	22.1	24.0	26.7
IAA	7.8	8.2	8.4	9.8	8.2	8.8	9.1	10.3
2,4-D	5.6	5.8	5.9	6.2	2.1	3.4	5.6	5.8

After six weeks of culturing the regenerants before transfer to soil

rhizogenesis in general. However, in the present study, IBA showed no promotive effect on lateral root induction. On the other hand, NAA showed the most positive effect on induction and elongation of lateral roots (Table 2). Such an effect of NAA was also observed by Taylor *et al* (13) in tomato. The capacity of phenolic compounds to act as auxin synergists in the rooting process is well known, although the mode of action remains obscure. The difficulty in inducing *in vitro* rooting in woody species (14) led to explore the phenolics and they obtained satisfactory results with phloroglucinol. Subsequently, the *in vitro* root inducing capacity of phenolics was demonstrated in apple rootstocks (15), *Fragaria* (16), *Prunus insititia* (14). According to Mosella Chancel *et al* (17) phenolics specifically act on the middle phase, the "initiation phase" of rhizogenesis. James *et al* (18) reported that the presence of phloroglucinol during the shoot proliferation stage significantly promoted root formation when measured as rooting percentage or number of roots per shoot.

The concentration of agar in rooting should vary from 0.6-0.8%. Anderson (19) considers that its concentration should be as low as possible for rooting. Physiologically, agar is not a completely inert material, and is a source of various types of substances which may affect growth. Agar, thus, may result in poor root growth in certain sensitive species. Lane (20) reported rooting inhibition in *Sequea* and *Prunus* when 0.6% agar was used. Kitto *et al* (7) observed an inverse relationship between the rooting ability and agar concentration. In addition to the presence of possible growth inhibiting substances, the reason for poor rooting in agar medium may also be due to poor aeration and a slow rate of diffusion of the toxic metabolic wastes released by growing tissue. Two methods are commonly used to circumvent the inhibitory effect of agar medium: (a) supplementing fine powder of activated charcoal to the agar medium; and (b) using a liquid and filter-paper-bridge system in place of agar. Activated charcoal may absorb toxic substances in the medium, thereby improving root regeneration and development (21, 22). It may also absorb residual cytokinin from medium. Takayama *et al* (22) reported an inhibition of root formation of *Lilium* by BA. Such an inhibition was completely reversed by the addition of charcoal. Media, in general, having a low concentration of salts and sugar proved satisfactory for rooting of shoots.

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