

Enhanced secretion of azadirachtin by permeabilized margosa (*Azadirachta indica*) cells*

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Role of permeabilizing agents on secretion of *Azadirachtin* from margosa callus cultures was investigated under laboratory conditions. The results indicated that most of the synthesised *azadirachtin* remains inside the cells. Different permeabilizers showed variation in secretion pattern. The most effective was found to be Triton X-100, which secreted 10 mg/l at 150 ppm level followed by DMSO and chitosan. This is the first report of its nature on permeabilizing agent induced enhanced secretion of *azadirachtin* from neem callus cultures.

Azadirachta indica, *A. juss* (Meliaceae) commonly known as neem or margosa is well known for its utility as a source of bio-pesticide against a variety of insects. The active substance azadirachtin² has received increased attention as a bio-pesticide^{1,2}. The neem seed extract has been used as a source of active compound. Economics for its wide spread application is limited due to low productivity of azadirachtin and other environmental factors from the plant source, necessitating to search alternative methods for production of azadirachtin. In this context the development of callus culture of margosa plant received importance³⁻⁵. By culturing the cells under controlled conditions, the limitations of growing field plants can be overcome. The demonstrated success of producing shikonin from the cells of *Lithospermom erythrorhizon*⁶ in commercial scale has created an awareness for such attempts with other plants. Use of plant cell cultures for production of important secondary metabolites is receiving increasing attention in recent years. One of the limitation observed with plant cell cultures is poor productivity and lower secretion capabilities. Majority of favoured metabolites remain within the cell vacuoles and a small portion is secreted into the supported

medium. This intracellular accumulation of product restrains use of the grown cells repeatedly because the cells have to be macerated to obtain the product. This also limits the use of cell immobilization technology which was used only for the products secreted into the medium. There are reports on release of secondary metabolites from *Catharanthus roseus* and other plant cells by pre-treating with various detergents and organic solvents⁷. There are few reports on azadirachtin synthesis as its accumulation pattern. In the present study, we report the influence of permeabilizing agents such as dimethyl sulfoxide (DMSO), triton X-100 and chitosan on margosa cells for the secretion of azadirachtin.

Callus cultures—Callus culture of *Azadirachta indica* was obtained from leaf explants on MS basal media supplemented with indole butyric acid (4 mg/L), naphthalene acetic acid (4 mg/L), 6-benzyle aminopurine (2 mg/L) and 2% sucrose adjusted to pH 5.8 and solidified with agar (0.8%) before sterilization. Callus culture was maintained at 26°C under 16 hr light (40 W Fluorescent tubes) period in the above medium for routine work.

Suspension culture was also maintained in the same medium (without addition of agar) on a gyratory shaker (120 rpm) at 26°C with repeated sub-culturing.

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Preparation of permeabilizing agents—Different concentrations of Triton X-100 and DMSO solutions (v/v), were prepared in sterile distilled water, while chitosan was dissolved in glacial acetic acid (1%) and volume made upto 100 ml in sterile distilled water (w/v).

Permeabilization—Cell suspension culture was harvested after six week growth period from the individual flasks collected by filtration and resuspended the cells (~1 g) in 25 ml of medium containing permeabilizing agents, for half an hour on a gyratory shaker (120 rpm). Then the treated cells were collected and washed 3 times with sterilized double distilled water to remove remaining permeabilizing agents and resuspended in MS basal media. The suspension was kept on a gyratory shaker (120 rpm) for 48 hr in the dark and the supernatant was analysed for release of azadirachtin by using TLC and HPLC standard methods.

Extraction and identification of azadirachtin—The suspension culture was filtered using whatman filter paper and the filtrate was extracted twice with methanol:hexane (60:40), and the aqueous layer was further extracted twice with dichloromethane. Pooled fraction of dichloromethane was evaporated under vacuum at 40°C, redissolved

residue in methanol and analysed on TLC with toluene:methanol (80:20). Spots were developed by spraying vanillin reagent. HPLC was performed on Shimadzu model LC10AD pump, reverse phase shimpack CLC-ODS (M) column (4.6 mm×250 id) using mobile phase, methanol:water (50:50) at 241 nm. The presence of azadirachtin was confirmed by comparison with authentic standard.

For extraction of intracellular azadirachtin, control cells were separated from the medium by filtration (Whatman no.41) and was washed with distilled water. The freeze-dried callus was blended with methanol (100 ml) in a porcelain mortar and filtered. The callus residue was resuspended in methanol and the blending and filtration was repeated three times. The solvent was removed on a rotary vapour keeping the temperature below 40°C, redissolved residue in methanol:water (60:40) and further extracted as mentioned earlier.

Chemicals—All chemicals used in this investigation were analar grade and were obtained from Sigma where as HPLC grade solvents obtained from spectrochem.

Intra (4.22 mg/L) and extra (1.14 mg/L) cellular concentration of azadirachtin in untreated suspen-

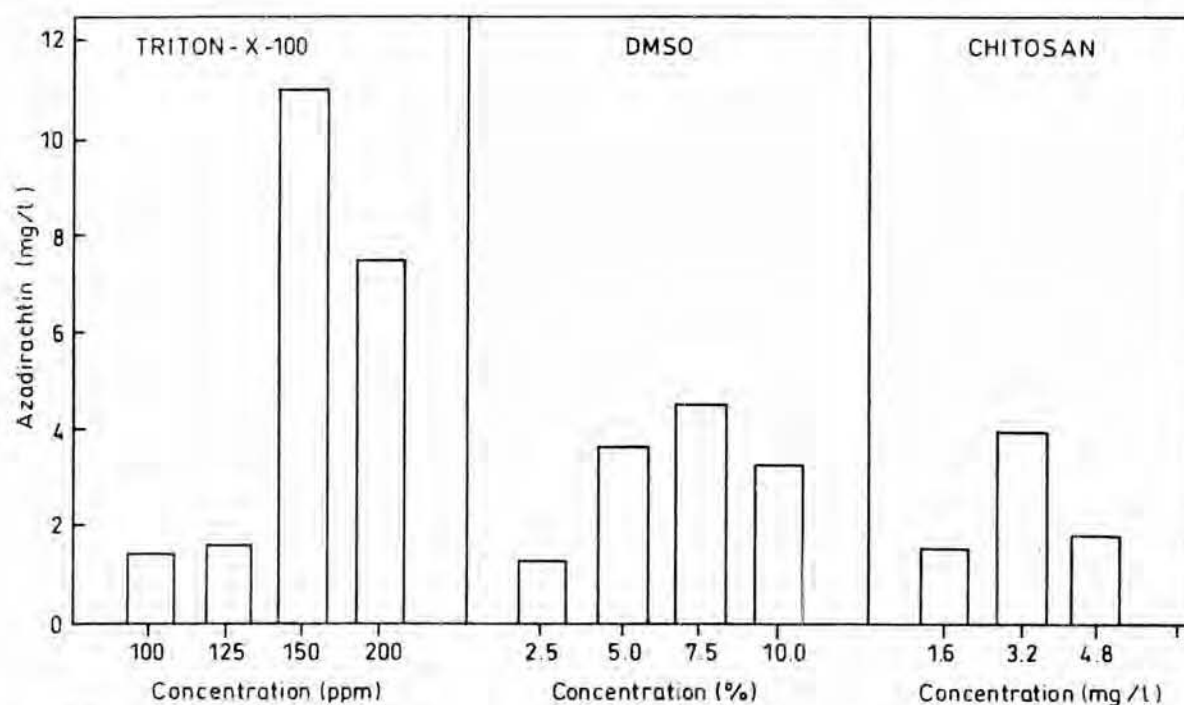


Fig. 1—Effect of DMSO, triton X-100 and chitosan on neem cell permeabilization. [Untreated sample contains 1.14 mg/l azadirachtin]

sion cultures of *A. indica* obtained indicates that only 20% of synthesized product released into the medium and majority of it still remains inside the vacuoles. This observation was the basis for our study on permeabilization. Pretreatment of the cells with permeabilizing agent was carried out to modify the cell wall characteristics and to facilitate improved secretion of the product. Permeabilizing agents, DMSO, tritonX-100 and chitosan, in various concentrations were tried for pretreatment and duration of pretreatment was kept constant (30 min.). DMSO at different concentrations ranging from 2.5 to 10% was used to permeabilize the cells (Fig. 1). The product secretion increased gradually by increasing concentration of permeabilizing agent (DMSO) up to 7.5 %. Of the four concentrations tried, maximum yield (4.2 mg/L) was obtained at 7.5% which was found to be four times higher than untreated cells. However, beyond 7.5%, treatment appears to be detrimental to the cells. TritonX-100 at lower concentration (100 to 125 ppm), extra cellular product concentration remained similar to that of untreated cells. Concentration of the product enhanced to 10-fold at higher concentration of tritonX-100(150 ppm). These results strongly indicated the effect of permeabilization on the cell wall.

The total concentration of the product (intra and extracellular levels) was 5 mg/L which increased to 10 mg/L in the culture medium of treated cells. However the higher concentration of permeabilizing agent above 150 ppm showed declined activity of the cells. In case of chitosan, positive influence of permeabilization was noticed. At 3.2 mg/L level, 4-fold increase in product secretion was ob-

served. At higher concentrations chitosan also showed adverse effect on the cell metabolism.

It has been observed that all the permeabilizing agents showed positive influence on secretion of the azadiractin. TritonX-100 (150ppm) exhibited considerable influence and the product secretion could be enhanced to 10 fold as compared to untreated cells. Pretreatment not only facilitated the release of the product but also accelerated the product synthesis process similar to 'in situ' removal of product by adsorbent resin which enhances the product in some cultures¹⁴.

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