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Volume 11 (4) **CONTENTS** **October 2017**

Current Trends in Biotechnology and Pharmacy
Vol. 11 (4) October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

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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, (4th edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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Current Trends in Biotechnology and Pharmacy
Vol. 11 (4) 334-344 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

Plant Regeneration of a Spring Vegetable -*Asparagus officinalis* L. Through Somatic Embryogenesis

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Abstract

A plant regeneration protocol was established from hypocotyl explants of *in vitro* grown seedlings of *Asparagus officinalis* and *in vitro*-proliferated shoots, respectively through either somatic embryogenesis or embryogenic callus. Types of auxins and cytokinins play an important role for producing somatic embryos. Friable calli with somatic embryos developed well in MS medium supplemented with 2.0-4.0 µM 6-benzylaminopurine (BAP) and 1.0-4.0 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA). The highest 96.3% embryogenic calli and maximum number of 60.0 somatic embryos formation were obtained from each explant when the MS medium was fortified with 4.0 µM BAP and 2.0 µM 2,4-D. The best embryo germination occurred in 1.0 µM BAP supplemented ½ strength MS medium. The highest 95.2% of shoot proliferation was observed in embryogenic calli in MS medium containing 2.0 µM BAP and 1.0 µM IBA or NAA. *In vitro*-grown shoots were rooted in ½ strength MS medium with 0.5-4.0 µM IBA. Regenerants were transferred to vermicompost and successfully established under an *ex vitro* environment in garden soil with 75.0% survival rate.

Key words: embryos, growth regulators, hypocotyl, embryogenic callus, coconut water.

Introduction

A. officinalis L. known as spring vegetable is a dioecious perennial herb belonging to the family Asparagaceae (formerly Liliaceae). It is native to Europe, Asia and northwestern Africa. It is grown worldwide as a crop, particularly in North and South America, China and Europe. The male plant of the species produces well developed and succulent spring stalks, popularly known as spears / strains. It is the best known and economically important garden species cultivated as a green tender vegetable (17, 32).

Asparagus is low in calories and is very low in sodium. It is a good source of vitamin B6, calcium, magnesium, and zinc, and a very good source of dietary fiber, protein, beta-carotene, vitamin C, vitamin E, vitamin K, thiamin, riboflavin, niacin, folic acid, iron, phosphorus, potassium, copper, manganese, and selenium, as well as chromium, a trace mineral that enhances the ability of insulin to transport glucose from the bloodstream into cells. The amino acid asparagine gets its name from asparagus, as the asparagus plant is relatively rich in this compound (19, 23). Asparagus is useful in the treatment of rheumatic conditions, is a mild laxative and sedative, and is considered useful in the treatment of a range of maladies from arthrosis to tuberculosis. It is also used for the treatment of urinary and kidney problems, jaundice and sciatica (12). Asparagus is a rich source of glutathione, a powerful antioxidant that is known to boost the immune system, reduce inflammation and maintain the health of the liver (11, 13, 14, 27).

There are several practical advantages to the use of somatic embryogenesis as a means to manufacture or develop plant clones. Moreover,

in vitro somatic embryogenesis is an important prerequisite for the use of many biotechnological tools for genetic improvement as well as mass propagation. Somatic embryogenesis produces a virtually unlimited number of embryos as long as the culture and the embryos thus produced can be maintained in a healthy manner in the laboratory. Somatic embryogenesis may prove to be a widely applicable process for crop improvement in agriculture and forestry (2).

A. officinalis is normally propagated through seeds and rhizomes; but this is a slow process that results in smaller number of new propagules (28). *In vitro* propagation technique can be of immense value in offsetting the pressure on natural populations, thereby conserving the species. The present study was aimed at developing an efficient propagation protocol for the production of clonally uniform plants through somatic embryogenesis and organogenesis method (25). We reported earlier the development of somatic embryos from cell suspension culture of *Aralia elata* (21). The aim of the present investigation reported here was successful plant regeneration through somatic embryogenesis or organogenesis from the embryogenic callus of *A. officinalis*.

Materials and Methods

Seeds were collected from Japan and were stirred in a Savlon (cetrimide and chlorhexidine gluconate) (ICI, Bangladesh) solution (1 ml Savlon mixed with 1 liter tap water) for 15 min and then thoroughly rinsed with running tap water for 20 min. These seeds were then surface-sterilized with 70.0% EtOH for 3 minutes, moved to a laminar-air-flow cabinet, and transferred to a sterilized conical flask. Subsequent surface disinfection was conducted with a 0.2% mercuric chloride ($HgCl_2$) solution (Uni-Chem, China) for 15 minutes. The seeds were then washed with at least three changes of sterilized distilled water for removing any trace of the sterilant. Sterilized seeds were germinated on hormone-free MS medium (26). The cultured seeds germinated within 3 weeks and gave rise to shoots that developed two to three nodes five to six weeks later. Shoots were then propagated by subculturing single-node cuttings at 4-week intervals in MS medium with 2.0-4.0 µM BAP. Prepared hypocotyl explants were collected from 6-week-old aseptically grown seedlings. These explants were cultured in MS medium containing different concentrations (2.0-4.0 µM) of BAP in combination with either 1.0-4.0 µM 2,4-D, NAA or IBA for the induction of embryogenic calli and somatic embryos. Hypocotyl explants were also cultured in different culture media, such as B_5 (16) and ½MS (half strength of MS basal salts), media, with the addition of 4.0 µM BAP plus 2.0 µM 2,4-D, NAA or IBA to determine the effects of media on somatic embryogenesis. For the germination of somatic embryos (SEs), the individual embryos were cultured in ½ strength MS basal medium with 2.0% sucrose and 0.5-2.0 µM cytokinin, 10-30% coconut water (CW) or without the addition of any plant-growth regulators. Fifty regenerated plants were transplanted to small plastic pots containing vermicompost and kept in a culture room for 8-weeks for hardening under diffuse light (16-h photoperiod; temperature 25 ± 1 °C; relative humidity 65.0%). The plants were irrigated once in 3-days and fertilized with MS (¼ strength) basal-salt solutions devoid of sucrose and *myo*-inositol at weekly intervals. Then, the plants were transferred to pots containing garden soil and reared in the polythene-tent. Irrigation and fertilizer treatment were continued. The embryogenic calli (approx. 0.5-1.0 g) were also transferred to MS medium supplemented with various concentrations and combinations of BAP (2.0-4.0 µM) and NAA or IBA (0.5-2.0 µM) for producing adventitious shoots. The following data were recorded after eight-weeks of culture initiation: percentage of shoot formation, number of total shoots per culture, and average length of shoots per culture. Embryogenic calli-derived shoots were rooted in ½MS medium supplemented with different concentrations (0.5-4.0 µM) of IBA, NAA, or indole-3-acetic acid (IAA). All culture media were adjusted to pH 5.7 ± 0.1, fortified with 3.0% sucrose (w/v) (except embryo germination medium), and gelled with 8.0% agar. The cultures were grown at 25 ±

Vol. 11 (4) 334-344 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

1°C under the illumination of cool-white fluorescence tubular lamps with a light intensity of 50 µmol.m^{-2}.s^{-1} for a 16-h photoperiod.

Twenty replicates were used for all the experiments conducted. Experiments were repeated four times. The effects of different treatments were quantified, and the data were analyzed using the analysis of variance (ANOVA). Tukey's multiple comparison was used to distinguish differences between treatments.

Results and Discussion

Plant growth regulators and culture media play an important role for induction of somatic embryogenesis (3, 15). Irrespective of the kinds of growth regulators used in this experiment callusing started at different points on the surfaces of the explants after 3-4 weeks of culture. The calli successfully produced globular- to early cotyledonary-stage embryos accompanied with callus proliferation (Fig. 1A-B). A somatic embryo

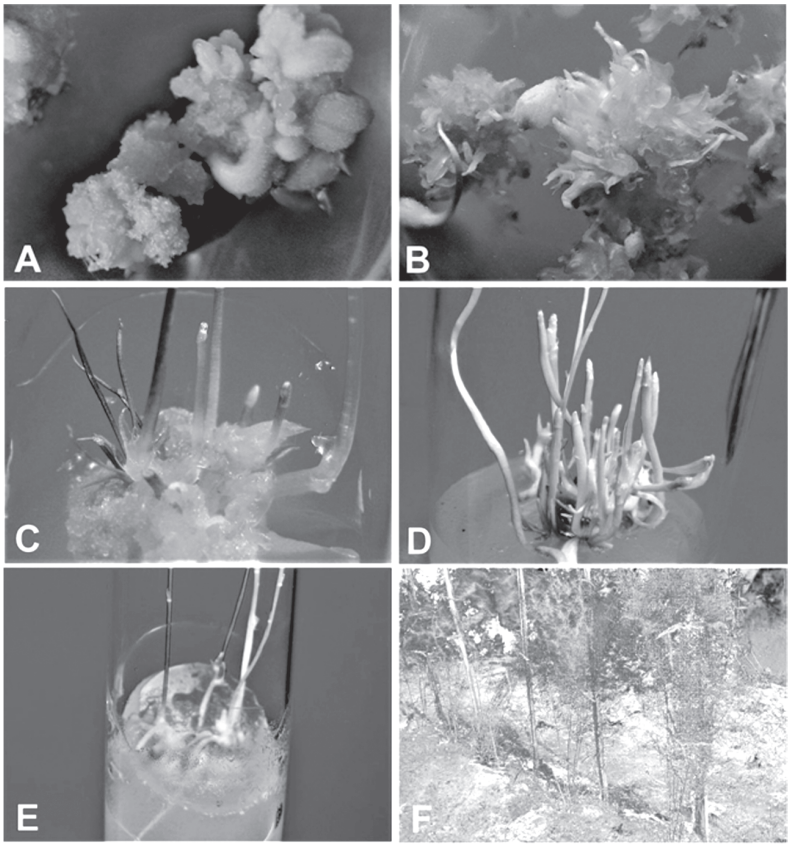

Fig.1: Plant regeneration through somatic embryo and embryogenic calli.

A. Proliferation of somatic embryos on MS medium containing 4.0 µM BAP plus 2.0 µM 2,4-D after 8 weeks of culture.
B. Somatic embryo germination on MS medium containing 1.0 µM BAP after 6 weeks of culture.
C-D. Development of adventitious shoots from embryogenic calli on MS medium containing 2.0 µM BAP plus 1.0 µM IBA after 7 weeks (C) and 10 weeks (D) of culture.
E. Rooting of *in vitro* proliferated shoots on ½ strength MS medium containing 2.0 µM IBA after 6 weeks of culture.
F. Acclimatized plants growing under field conditions after 3 months of transfer to soil.

Table 1. Effects of different concentrations and combinations of cytokinins and auxins on embryogenic callus induction and embryo formation from hypocotyl explants.

Plant growth regulators (µM)	Embryogenic callus formation (%)	Ave. no. of embryos / culture
BAP + 2,4-D		
2.0 + 1.0	60.1 ± 1.1 d	36.1 ± 1.4 c
+ 2.0	73.2 ± 1.7 c	47.2 ± 1.0 b
+ 4.0	81.3 ± 1.2 b	42.0 ± 1.2 b
4.0 + 1.0	86.2 ± 1.0 b	44.2 ± 1.1 b
+ 2.0	94.9 ± 0.8 a	51.7 ± 1.5 a
+ 4.0	88.5 ± 1.3 b	46.3 ± 1.2 b
BAP + NAA		
2.0 + 1.0	64.7 ± 1.3 d	13.5 ± 1.0 f
+ 2.0	72.1 ± 1.1 c	35.5 ± 1.1 c
+ 4.0	74.1 ± 1.0 c	30.7 ± 1.5 d
4.0 + 1.0	70.1 ± 1.5 c	29.5 ± 1.3 d
+ 2.0	85.1 ± 1.4 b	38.2 ± 1.3 c
+ 4.0	77.1 ± 1.3 c	36.6 ± 1.5 c
BAP + IBA		
2.0 + 1.0	42.7 ± 1.1 f	9.5 ± 1.3 g
+ 2.0	52.4 ± 1.1 e	21.5 ± 1.1 e
+ 4.0	55.8 ± 1.4 e	18.7 ± 1.2 e
4.0 + 1.0	52.8 ± 1.1 e	18.1 ± 1.5 e
+ 2.0	60.5 ± 0.8 d	34.2 ± 1.2 c
+ 4.0	64.8 ± 1.4 d	26.6 ± 1.1d

Values represent means ± standard error of 20 explants per treatment in four repeated experiments. Means followed by the same letters are not significantly different by Tukey's multiple comparison test at 0.05 probability level.

is a bipolar structure raised from vegetative cells that have no vascular connection with the maternal tissues. *In vitro* somatic embryogenesis occurred from different types of explants and can be induced by the addition of auxin and cytokinin.

In this experiment, BAP with 2,4-D or NAA was the most efficient cytokinin-auxin combination for producing embryogenic calli and somatic embryos from hypocotyl explants for *A. officinalis*. Among the different concentrations and combinations of growth regulators in MS medium, 4.0 µM BAP plus 2.0 µM 2,4-D or NAA gave better performance of embryogenic callus formation and somatic embryo proliferation from the hypocotyl explants, the frequencies was 94.9% and 85.1%, respectively (Table 1). The highest average numbers of somatic embryos per culture was 52.7 and 35.2 were recorded from hypocotyl explants. The proliferation of embryogenic calli was faster in the media with BAP plus 2,4-D than in those with BAP plus NAA or BAP plus IBA which required a short period to produce an appreciable number of calli. In this study, the initial primary calli were white-greenish, nodular, and friable in the case of the hypocotyl. Tawfik and Noga (31) reported that 2,4-D and Kn was the most suitable PGR combination for producing embryogenic callus in *Cuminum cyminum*. Unfortunately this combination failed to induce any somatic embryo. When these calli were subcultured in hormone

free MS medium, they produced somatic embryos. In our experiment we showed that BAP with 2,4-D combination produced embryogenic calli simultaneously along with somatic embryos. No embryo formation was observed in hormone free medium.

The culture media and types of plant growth regulators influenced the frequency of embryogenic callus formation and number of embryos produced. The results are shown in Fig. 2, as well as the formation of SEs at different frequencies, and the differences among them were statistically significant. Among the different treatments, MS medium containing 4.0 μM BAP plus 2.0 μM 2, 4-D induced embryo differentiation at high rates, and the frequency was 94.9 from hypocotyl explants. The lowest percentage (25.3) of embryo development was obtained in cultures in WPM medium supplemented with 4.0 μM BAP and 2.0 μM IBA. Considerable embryogenic callus and SE formation were achieved from hypocotyl explant of *A. officinalis* in ½MS and B5 medium having 4.0 μM BAP plus 2.0 μM 2,4-D.

Lee *et al.* (22) have attempted to find out the optimization of the conditions for efficient induction of embryogenic calli and regeneration of plants from mature seeds of *Japonica* rice cultivars. The number, color, size, shape, and appearance time of the induced embryogenic calli varied among the rice cultivars depending on the type of basal medium (LS, MS, N6). Lee *et al.* (22) reported that N6 medium was the most efficient for induction of embryogenic calli, where the overall rates ranged from 30 to 56%. In that medium, Kn was more effective for shoot regeneration compared with BA, while the highest shoot regeneration frequencies were 67-77% when either cytokinin was combined with high concentration (10.74 μM) of NAA. In the present study, we also used three types of basal media (MS, ½MS and B5) and plant growth regulators respectively. Among these media and plant growth regulators, MS medium and 4.0 μM BAP plus 2.0 μM 2,4-D were the most efficient basal medium and plant growth regulator, respectively for induction of embryogenic callus and somatic embryos.

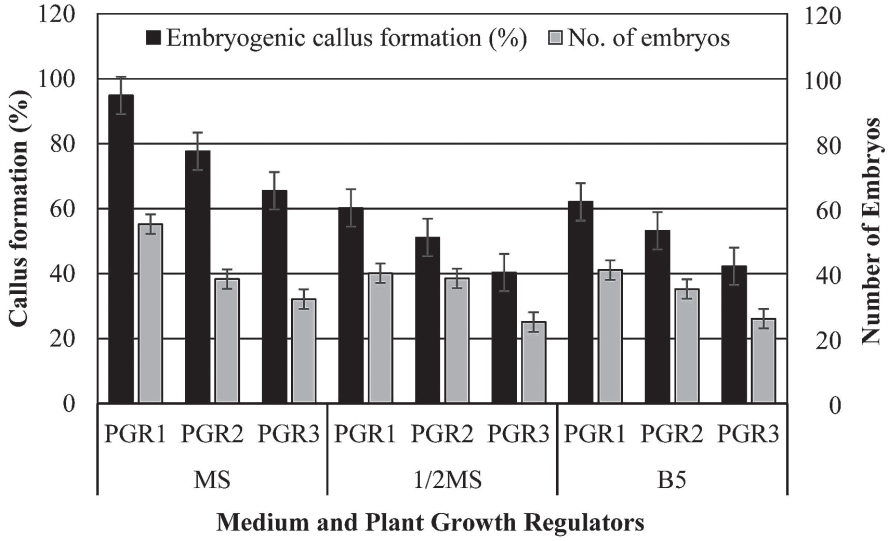

Fig. 2: Figure showing the effects of the culture medium and plant growth regulators (PGRs) on somatic embryogenesis from hypocotyl explants. PGR1 = 4.0 μM BAP + 2.0 μM 2,4-D; PGR2 = 4.0 μM BAP + 2.0 μM NAA; PGR3 = 4.0 μM BAP + 2.0 μM IBA

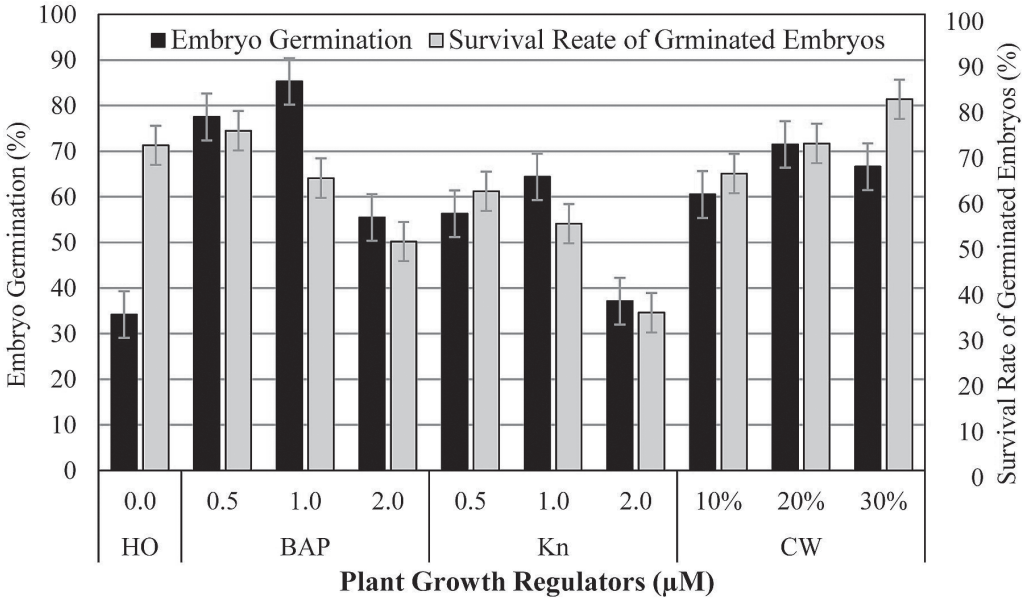

Fig. 3: Figure showing the effects of cytokinins on somatic embryogenesis from hypocotyl explants.

In this experiment some embryos showed a tendency to undergo dedifferentiation, the developed calli underwent embryogenesis. About eighty percent of embryos showed maturation, but these embryos did not regenerate in the media. These embryos or embryogenic calli developed plantlets only when transferred to other media.

Embryos can be distinguished from adventitious shoots because they are bipolar, having both a shoot pole and a root pole, and do not have any vascular connections with the underlying parental tissues (18). Individual embryos were easily separated from the maternal tissues, as they were loosely attached at the radicle end to the mother explant (Fig. 1B). Such embryos were easily converted into plantlets in ½ strength MS medium containing 2.0% sucrose without the addition of any PGRs. In this medium, the highest embryo germination rate of 85.3% was observed. On the other hand, addition of 20% CW in MS medium showed a considerable germination rate followed by 1.0 µM Kn (Fig. 3). Moreover, satisfactory embryo germination was observed on MS medium with a low (0.5 µM) concentration of NAA (data not shown in Fig. 3). When SEs of *A. officinalis* were maintained in an embryo-induction medium (MS + 4.0 µM BAP + 2.0 µM 2,4-D, NAA or IBA) without transfer for approximately 3 months, about 20.0-45.0% (data not shown) of the embryos germinated and developed into normal plantlets, probably due to desiccation caused by the increased osmoticum in the solid medium. Junaid *et al*. (20) reported that somatic embryo maturation and germination in *Catharanthus roseus* occurred in MS medium supplemented with 3.0% sucrose and 2.22 µM BAP. In our study, SEs that were induced in a 2,4-D-containing medium developed only shoots but failed to produce any roots. During the maturation stage, somatic embryos undergo various morphological and biochemical changes. Seventy percent of somatic embryos germinated into plantlets within 6 weeks. Out of 50 plants transferred, 40 plants survived after one month of transplantation to the polythene-tent. The surviving plantlets did not show any detectable variations

Vol. 11 (4) 334-344 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

Table 2. Effects of different concentrations and combinations of cytokinins and auxins on adventitious shoot regeneration from embryogenic calli.

Plant growth regulators (µM)	Shoot formation (%)	Total shoots / culture	Ave. length of shoots / culture (cm)
BAP + IBA			
2.0 + 0.5	92.5 ± 1.1 a	42.3 ± 1.0 b	5.4 ± 1.3 b
+ 1.0	95.2 ± 2.0 a	47.1 ± 1.2 a	7.1 ± 1.2 a
+ 2.0	58.6 ± 1.2 e	12.2 ± 1.0 f	4.5 ± 1.7 c
4.0 + 0.5	82.1 ± 1.1 b	32.4 ± 1.1 c	5.3 ± 1.8 b
+ 1.0	88.8 ± 1.4 a	39.3 ± 1.2 b	5.6 ± 0.6 b
+ 2.0	76.5 ± 1.7 c	22.6 ± 1.1 e	4.1 ± 1.1 c
BAP + NAA			
2.0 + 0.5	75.3 ± 0.5 c	38.4 ± 1.1 b	5.7 ± 1.2 b
+ 1.0	83.1 ± 1.1 b	45.3 ± 1.3 a	6.4 ± 1.0 b
+ 2.0	50.3 ±1.3 e	7.2 ± 1.5 f	2.2 ± 0.3 e
4.0 + 0.5	76.2 ±1.7 c	29.3 ± 1.1 d	4.1 ± 1.1 c
+ 1.0	81.0 ± 1.5 b	35.7 ± 1.7 b	5.2 ± 0.9 b
+ 2.0	75.3 ± 1.0 c	17.4 ± 1.2 f	3.9 ± 1.1 c
Kn + IBA			
2.0 + 0.5	59.2 ± 1.1 e	13.4 ± 1.4 f	3.4 ± 1.7 d
+ 1.0	58.1 ± 1.4 e	15.2 ± 1.5 f	4.9 ± 1.5 c
+ 2.0	23.4 ± 1.2 h	5.2 ± 1.0 f	2.5 ± 1.2 e
4.0 + 0.5	53.4 ± 1.0 e	9.2 ± 1.2 f	3.1 ± 1.6 d
+ 1.0	63.3 ± 1.6 d	17.4 ± 1.2 f	3.4 ± 1.9 d
+ 2.0	54.2 ± 1.0 e	6.4 ± 1.1 f	2.9 ± 1.0 d
Kn + NAA			
2.0 + 0.5	43.3 ± 0.8 f	9.5 ± 1.4 f	3.3 ± 1.2 d
+ 1.0	54.7 ± 1.2 e	14.3 ± 1.7 f	4.7 ± 1.5 c
+ 2.0	14.4 ± 1.3 i	4.4 ± 1.2 f	1.6 ± 1.3 f
4.0 + 0.5	30.3 ± 1.7 g	7.3 ± 1.2 f	3.7 ± 1.1 c
+ 1.0	55.1 ± 1.1 e	13.5 ± 0.9 f	4.3 ± 0.6 c
+ 2.0	31.3 ± 1.0 g	3.9 ± 1.2 f	2.1 ± 1.5 e

Values represent means ± standard error of 20 explants per treatment in four repeated experiments. Means followed by the same letters are not significantly different by Tukey's multiple comparison test at 0.05 probability level.

Table 3. Effects of auxins on adventitious root formation from microcuttings in MS medium.

Type of auxin	Con. of auxin (µM)	Rooted cuttings(%)	No. of roots / rooted cutting	Ave. length of roots (cm)
IBA	0.5	67.4 ± 1.5 d	2.1 ± 1.1 c	2.2 ± 1.1 e
	1.0	87.4 ± 1.3 b	4.2 ± 1.3 b	3.1 ± 1.2 c
	2.0	95.3 ± 2.1 a	5.4 ± 1.7 a	4.5 ± 1.8 a
	4.0	37.5 ± 2.2 e	2.5 ± 1.8 c	1.3 ± 1.3 e
NAA	0.5	64.3 ± 1.8 d	1.4 ± 1.1 d	3.5 ± 1.5 b
	1.0	79.4 ± 2.0 c	2.3 ± 1.2 c	3.1 ± 1.9 c
	2.0	60.1 ± 1.1 d	1.2 ± 1.4 d	2.6 ± 1.7 d
	4.0	-	-	-
IAA	0.5	32.2 ± 1.6 e	1.0 ± 1.4 e	1.5 ± 1.6 e
	1.0	35.2 ± 1.7 e	1.1 ± 1.2 e	1.8 ± 1.6 e
	2.0	39.5 ± 2.1 e	2.0 ± 1.6 d	2.1 ± 1.6 e
	4.0	42.6 ± 1.4 e	2.4 ± 1.5 c	2.7 ± 1.5 d

'-' indicates no response. Values represent means ± standard error of 20 explants per treatment in four repeated experiments. Means followed by the same letters are not significantly different by Tukey's multiple comparison test at 0.05 probability level.

in morphological or growth characteristics from the donor plants (data not shown).

Hypocotyl-derived embryogenic calli produced adventitious shoots in MS media with either 2.0 µM BAP plus 1.0 µM IBA or NAA; and 4.0 µM Kn plus 1.0 µM IBA or NAA, where the shoot formation rates were 95.2, 83.1, 65.3 and 55.1%, respectively (Table 2). The combination of 2.0 µM BAP with 1.0 µM IBA gave the highest frequency (95.2%) of shoot proliferation, 47.1 ± 2.0 shoots per culture, and the highest average length of 7.1 ± 1.2 cm (Fig. 1C,D). On the other hand, considerable shoot proliferation (83.1%) was found in the medium containing 2.0 µM BAP plus 1.0 µM NAA. The lowest performance was shown in Kn plus IBA or NAA formulations. Similar results were found in leaf and node explants of *Lilium (*9), callus derived protoplast cultures in *Phellodendron amurense* (4), cotyledon explants in *Acacia mangium* (29), epicotyl and internode explants in *Cajanus cajan* (5). Our present study also revealed that BAP-IBA and BAP-NAA formulation was better than Kn-IBA or Kn-NAA combinations. The present study noticed that the stimulation of shoot regeneration by the PGR combinations is significantly higher than the 2:1 cytokinin/auxin ratio and the hypocotyl-derived embryogenic calli showed better response for shoot regeneration than internode-derived embryogenic calli. Štajner *et al.* (30) reported that crown formation, essential for further subculturing, was positively correlated with the number of shoots. Ancymidol in combination with different growth regulators played an important role by promoting the initiation of shoots (except in combination with kinetin and NAA). Our study revealed that BAP and 2,4-D combination was more suitable for producing embryogenic callus and somatic embryos.

In vitro-proliferated shoots were prepared for micro-cuttings (1.5-2.0 cm length) and were transferred for rooting to an agar gelled ½ strength MS medium supplemented with 0.5, 1.0, 2.0, and 4.0 µM IBA, NAA, or IAA. Among the different

auxins used, IBA was found to be the best for root induction. The percentage of root induction and the number of roots differentiated per shoot were also highly influenced by the concentration and type of auxin (Table 3). The maximum rooting of cultures was 95.3% with 5.4 ± 1.7 number of roots per shoot, and 4.5 ± 1.8 cm average length of roots when the shoots were cultured in a medium containing 2.0 µM IBA (Fig. 1E). Although the media containing NAA and IAA also resulted in root formation, the rooting response was not as good as that in the media containing IBA. The maximum rooting frequency was 79.4% in the medium supplemented with 1.0 µM NAA and 42.6% in the medium supplemented with 4.0 µM IAA. No rooting was found in 4.0 µM NAA-supplemented medium. In this case, only calli were formed at the cut margin of the shoots. Being stable, IBA is the preferred auxin for adventitious root initiation in many species (1, 8, 33). In addition, Litz and Jaiswal (24) found IBA to be a superior auxin to IAA or NAA for the *in vitro* rooting of apple shoots. In the case of *A. officinalis*, IBA is also considered to be the best auxin for rooting from microcuttings. In this experiment, the minimum number of days required for rooting was 7, and the maximum was 25 for culture initiation. The rooting frequency increased gradually with the increase in the culture incubation period. It is a common practice to transfer shoots from a high strength media to less concentrated solutions to induce rooting (10). In many species such as *Adhatoda vaisca* (6), *Phellodendron amurense* (7, 8, 34), and *Curcuma caesia* (29*)* rooting frequency was higher when shoots were rooted on low strength MS medium. The rationale behind the favorable effect of reduced macronutrient concentration is that the concentration of nitrogen ions needed for root formation is much lower than for shoot formation and growth. The regenerated plantlets were gradually acclimatized and successfully established in the soil under natural conditions (Fig. 1F), with a survival rate of 75.0%. The acclimatization procedures for *in vitro*-regenerated plantlets of *P. amurense* were reported in our previous paper (8).

In conclusion, this is the first report on *A. officinalis* demonstrating a significant finding of somatic embryos from hypocotyl explants. Our studies may allow SEs to be used for clonal propagation of elite *A. officinalis* clones and other applications that require the production of a large number of plants from limited source materials. The present study provides a useful model system for further studies on *in vitro* morphogenesis, and in particular, on the comparison of plants regenerated either via organogenesis or somatic embryogenesis for their genetic homogeneity or variability.

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An updated review on Phytochemical and Pharmacological propeties of *Piper sarmentosum*

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Abstract

Piper sarmentosum is a well-known medicinal plant of Southeast Asia consist of many medicinal properties such as Anti-microbial, antifungal, antimalarial, antituberculosis, anti-mycobacterial, anticarcinogenic, antioxidant, anti-nociceptive, anti-inflammatory, antiamoebic, neuromuscular blocking activity, anti-obesity, antiangiogenesis, wound healing, cardiovascular activity, anti-hypertensive, antiulcer and Anticoccidial activity. It consists of adifferent class of compounds such as amide, phenylpropanoid, alkaloid, steroid, C- benzylateddihydroxyflavone, alkaloid amide, Phenylpropanoyl amides, and lignin. Therefore, current article reviews its phytochemical and pharmacological activities that will help for the future scientist.

Keywords:*Piper sarmentosum*; Phytochemistry; Pharmacology; alkaloid amide; Southeast Asia

Introduction:

Natural source is the vital part of basic human requirement especially in the field of health care (1). Plants are the essential foundation of medicine (2). It is used in different forms in different civilizations such as Ayurveda (3), traditional Chinese medicine (4), Unani herbalism, traditional Malaysian health care system (5), African herbal medicine (6) and many more. Piper *sarmentosum* Roxb belongs to family Piperaceae and is famous in the tropical and subtropical region (7). It is used in treatment of different kinds of ailments such as anti-microbial (8), anti-oxidant or oxidative stress (9-13), wound healing (14), anti-nociceptive, anti-inflammatory, anti-pyretic (15-16), osteoporosis (17), atherosclerotic (18), antimalarial (19), cardiovascular problems (20), antiangiogenesis (21-22), anti-hypertensive (23), antituberculosis (24), It is distributed in adifferent parts of Southeast Asia such as Malaysia, Indonesia, Thailand, Cambodia, Vietnam (25). Leaves and fruit of *Piper sarmentosum*can be seen in fig. 1.

Traditional uses : It has been known by different names in different parts of theworld. Leaves of *Piper sarmentosum* has been used traditionally as natural anti-oxidant. It is usually boiled in water and used to relieve fever in malaria and treating cough, flue, and rheumatism. It has been used as antifungal against foot's dermatitis. It has been known as food flavoring agents in Thailand (14). List of traditional uses along with common names is represented in table 1.

Bioactive compounds isolated from *Piper sarmentosum*: Bioactive compounds differ in different parts of the plant. Fruits of *Piper sarmentosum* possess adifferent class of compounds such as amides, lignan, piperic acid, steroidal class of compounds and phenyl alkane.

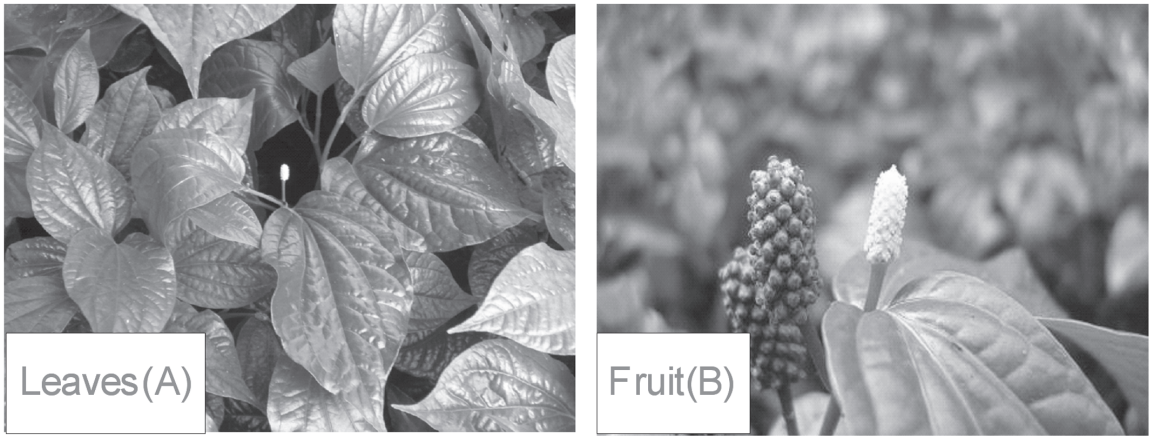

Fig. 1. (A) Leaves and (B) fruit of *Piper sarmentosum*(26)(27)

Table 1: Traditional medicinal uses

Plants parts	Common names	Traditional Medicinal uses	Reference
Leaves	Kadok, PokokKadok (Malaysia)	Natural antioxidant.In Malaysia, they are also eaten raw as ulam and the leaves are boiled in water and taken to relieve fever in malaria and treat coughs, flu, and rheumatism	(28)(29)
Roots, Leaves	Kudak (Malaysia) Sirihduduk, Akarbugu or Mengkadak (Indonesia)	The root is a remedy for tooth-ache and may be made into a wash for fungoid dermatitis on the feet. A decoction of the boiled leaves may be utilized to treat coughs, influenza, tooth-aches, and rheumatism	(19)
Leaves	Cha-plu, (Thailand)	Food flavoring agents and traditional medicines	(14)

Phenyl propanoids is the most common class of compounds found in leaves followed by alkaloid amide and piperamide. The aerial part of possessing C- benzylateddihydroflavones such as Sarmentosumins. List of bioactive compounds of *Piper sarmentosum* is represented in table 2. They are traditionally known as an antifungal agent. It is due to the prescene of alkaloid amides such as sarmentosine, sarmentine and brachyamide B (30)(34). Some of the benzene class of compounds inside *Piper sarmentosum* showed anti-microbial activity (31). The relationship among different pharmacological activities and their bioactive compounds have mentioned in table 3.

Pharmacological activities: *Piper sarmentosum* possess different pharmacological activities. They were tested in vivo and in vitro test models. They contain different pharmacological activities in different parts of plants under different conditions. List of pharmacological activities was mentioned table 4.

Hypoglycemic activity: *Piper sarmentosum* possess hypoglycemic activity in animal models. The whole plant of was extracted with water macerated at 70°C and fractionated with methanol to give a soluble and insoluble portion. Male Wistar rats of 120-140 g were used. Diabetes was induced using streptozotocin.The hypoglycemic effect of the methanol soluble fraction of the water extract was found to be more active than water extract (39).

Anti-oxidant activity: Ethanolic extract of leaves and fruit of *Piper sarmentosum* possess in-vivo antioxidant activity. Oxidative stress was induced by CCl_4 in rats. It was administered orally for fourteen days. The level of hepatic function markers was observed and found effective. Fruits and leaves possess anti-oxidant activity (11). The methanolic leave extracts of *Piper sarmentosum* at 250 ug/ml were tested using Xanthine/Xanthine Oxidase (X/XOD) superoxide scavenging assay. It possesses high anti-oxidant activity (88%) as compared to superoxide dismutase standard (28).*Piper sarmentosum* is capable of reducing the oxidative stress in lungs by decreasing lipid peroxidation and maintaining the glutathione peroxidase activity towards the normal level. Leaves of *Piper sarmentosum* were macerated with absolute methanol and were tested thein-vivo model using male Wistar rats (10).

Anti-inflammatory activity: Methanolic extract of leaves of *Piper sarmentosum* leaves possesses anti-inflammatory activity while lacking antipyretic activity. It was extracted using cold extraction by macerating in 20 L of methanol for 7 days at room temperature (15). Leaves of *Piper sarmentosum* was extracted by soaking in distilled water and express anti-nociceptive activity, anti-inflammatory activity in a dose dependentmanner. Anti-inflammatory activity was performed by carrageenan-induced paw edema test whereas anti-nociceptive activity was performed by abdominal constriction and hot plate test (16).

Cardiovascular activity: Aqueous extract of *Piper sarmentosum* decreases atherosclerotic lesions in high cholesterolemic experimental rabbits. After treatment animals were sacrificed and aortic tissue was examined histologically (18). Furthermore, it was also effective to heal the integrity of diabetic cardiovascular tissues (left ventricular cardiac tissues and proximal aorta) examined under transmission electron microscope (20). It reduces oxidative stress damage, increases NO production and able to reduce blood pressure and cholesterol level (23).

Anti-microbial activity: *Piper sarmentosum* possess anti-fungal properties due to the presence of bioactive amide alkaloids. The crude extract was also active against methicilin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Vibrio cholera* and *Streptococcus pneumonia*. There are a number of bioactive compounds isolated from *Piper sarmentosum* that possesses anti-fungal activities as mentioned in table 3.

Miscellaneous activities: The leaves of aqueous plant extract of *Piper sarmentosum* was used for the healing of oral wounds (14). Fresh leaves of *Piper sarmentosum* was also effective to improve fracture healing which was assessed by the callus volume and callus scores (44).The methanol extracts of roots of appeared to be effective against caecalamoebiasis in female swiss albino mice (40). It induces anticarcinogenic activity through an intrinsic apoptosis pathway in HepG2 cells in vitro. Fresh plants were used in extraction, macerated under ethanolic extract (41). Leaves of methanolic extract of *Piper sarmentosum* possess a similar protective effect against stress-induced gastric lesions as omeprazole (46).

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Table 2: List of Bioactive compounds

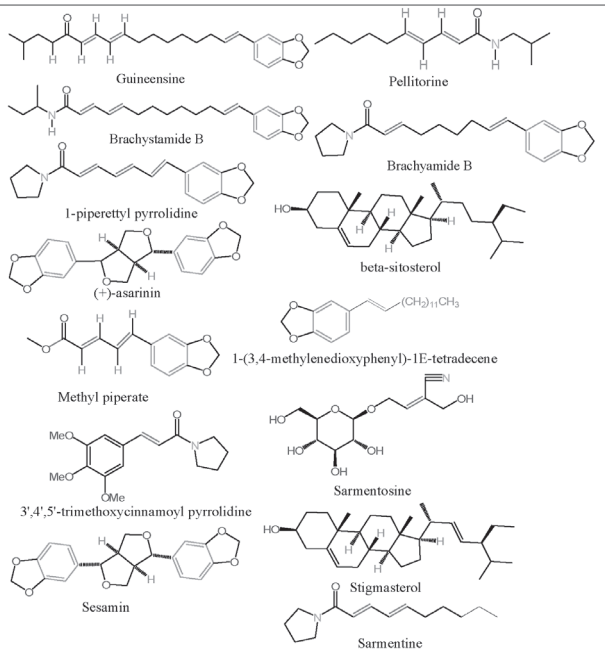
Name	Plant part	Class of compound	Structure	Reference
Pellitorine, guineensine, brachystamide B, brachyamide B, 1-piperettylpyrrolidine, 3',4',5'-trimethoxycinnamoylpyrrolidine	Fruits	Amide		(30)
(+)-asarinin, sesamin		Lignan		
β-sitosterol, stigmasterol		Steroidal class		
Methyl piperate		Piperic acid		
1-(3,4-methylenedioxyphenyl)-1E-tetradecene		Phenyl alkane		
Sarmentine, Sarmentosine		Amide		

Table 2: (Continue)

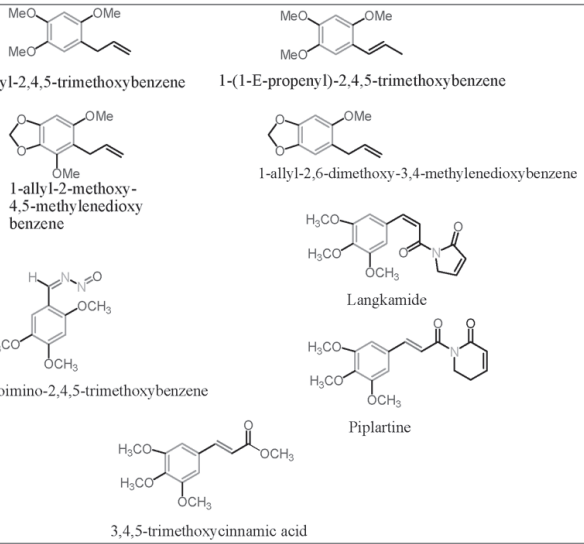
Name	Plant part	Class of compound	Structure	Reference
1-allyl-2,6-dimethoxy-3,4-methylenedioxybenzene; 1-allyl-2,4,5-trimethoxybenzene; 1-allyl-2-methoxy-4,5-methylenedioxybenzene; 1-(1-E-propenyl)-2,4,5-trimethoxybenzene	Leaves	Phenyl propanoid		(31)
1-nitrosoimino-2,4,5-trimethoxybenzene	Roots			(32)
Langkamide	Roots and stems	Alkaloid		(33)
Piplartine				
3,4,5-trimethoxycinnamic acid				

Table 2: (Continue)

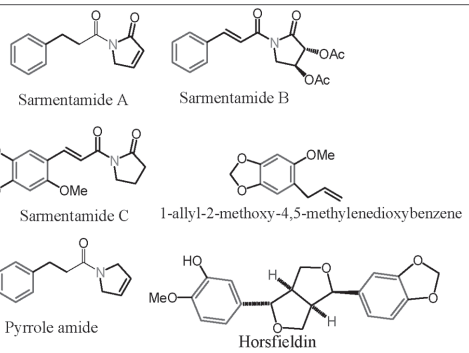
Name	Plant part	Class of compound	Structure	Reference`
Aromatic alkene	Roots			(34)
Sarmentamide A, Sarmentamide B, Sarmentamide C,		Amide		
Horsfieldin				
1-allyl-2-methoxy-4,5-methylenedioxybenzene				
pyrrole amide		Amide		

Table 2: (Continue)

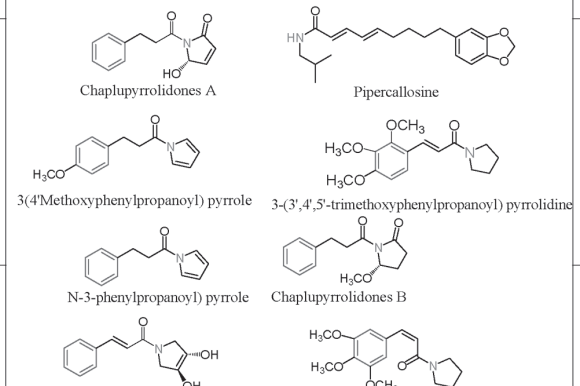
Name	Plant part	Class of compound	Structure	Reference
Chaplupyrrolidones A ChaplupyrrolidonesB Deacetylsarmentamide B	Leaves	Phenylpropanoyl amides		(35)
3-(3',4',5'-trimethoxyphenylpropanoyl) pyrrolidine N-3-phenylpropanoyl) pyrrole 3(4'Methoxyphenylpropanoyl) pyrrole	Aerial parts			(36)
Sarmentosumins A Sarmentosumins B Sarmentosumins C Sarmentosumins D	Aerial parts	C-benzylateddihydroflavones		(37)
Pipercallosine		Piperamide		
N-(3',4',5',-trimethoxy-cis-cinnamoyl)pyrrolidine	Leaves	Alkaloid amide		(38)

Table 3: Compound reported with respect to pharmacological activities

Compound identified	Pharmacological activity	Reference
Sarmentine;sarmentosine	Antituberculosis and antiplasmodial activities	(30)(34)
Pellitorine;guineensine;brachyamide B,sarmentosine,1-(3,4-methylenedioxyphenyl)-1E-tetradecene	Antituberculosis activity	
1-allyl-2,6-dimethoxy-3,4-methylenedioxybenzene;1-allyl-2,4,5-trimethoxybenzene;1-allyl-2-methoxy-4,5-methylenedioxybenzene;1-(1-E-propenyl)-2,4,5-trimethoxybenzene	Anti-microbial activity	(31)
Sarmentosine,brachyamide B	Anti-fungal activity	(34)
N-[9-(3,4-methylenedioxyphenyl)-2E,4E,8E-nonatrienoyl]-Pyrrolidine;aromatic alkene	Anti-mycobacterial activity	
Langkamide; piplartine; 3,4,5-trimethoxycinnamic acid	Hypoxia Inducible Factor-2	(33)
Chaplupyrrolidones-B	α-glucosidase inhibitory activities	(35)
3-(3',4',5'-trimethoxyphenylpropanoyl) pyrrolidine; N-3-phenylpropanoyl) pyrrole; β-sitosterol	Anti-bacterial (gram-positive bacteria)	(36)

Table 4: Pharmacological activities reported

Pharmacological activity	Part used	Extract/Fraction/Isolate	Testing method; Experimental model	Animals/ Cell line culture	Dose	Results	Reference
Hypoglycemic effect	Whole plant	Macerated with Water at 70°C and methanol soluble and insoluble fraction	Diabetic induced in vivo via STZ-diabetes induction	Male Wistar rats	125mg/kg; 250mg/kg	The methanol soluble fraction of the water extract was found to be more potent than that of the water extract.	(39)
Anti-amoebic activity	Root	Macerated in absolute methanol	In-vivo model	Female Swiss albino mice	125, 250,500, 1000 mg/kg/day	The methanol extracts appeared to be effective against caecalamoebiasis in mice	(40)
Anticarcinogenic activity	Fresh plant material	Macerated in ethanolic extract	In-vitro	human hepatoma cell line (HepG2)	12.5 µg mL^{-1}	It inducesanticarcinogenic activity through an intrinsic apoptosis pathway in HepG2 cells in vitro	(41)
Anti-nociceptive and anti-inflammatory activities	Leaves	soaking (1:8; w/v) in distilled water for 72 h.	In-vivo model	Male Balb/c mice and Sprague–Dawley rats	30,100 and 300 mg/kg	It possessesanti-nociceptive and anti-inflammatory activities in a dose dependent manner	(16)
Anti-oxidant activity	Fruit and leaves	Ethanol extract	In-vivo model	Female Sprague–Dawley rats	250 and 500 mg/kg	Fruits and leaves possess anti-oxidant activity	(11)

Table 4: (Continue)

Pharmacological activity	Part use	Extract/Fraction /Isolate	Testing method; Experimental model	Animals/ Cell line culture	Dose	Results	Reference
Antioxidant activity	Leaves	Methanolic leave extracts	Xanthine/xanthine oxidase (X/XOD) superoxide scavenging assay	--	250µg/ml	Fractions of *Piper sarmentosum* consist of naringenin which is natural anti-oxidant	(28)
Neuromuscular blocking activity	Leaves	Macerated with methanol	In-vivo	Adult Wistar rat	3.2, 4.0, 4.8 and 6.4 mg/ml	*Piper sarmentosum* likely to inhibit neurotransmitter (acetylcholine) release at the presynaptic terminal	(42)
Atherosclerosis activity	Leaves	Aqueous extraction	In-vivo	Rabbit	62.5-500 mg/kg	*Piper sarmentosum* produce protective effect against atherosclerosis	(18)
Antimalarial activity	Leaves	Chloroform and methanol	In-vitro	--	2.5 mg/ml	*Andrographispaniculata* also demonstrated higher antimalarial effect than *Piper sarmentosum*	(19)
Anti-obesity	Leaves	Decoction	In-vivo	Female Sprague–Dawley rats	1.25mg/kg	*Piper sarmentosum*water extract reduce the activity on 11-β-Hydroxysteroid dehydrogenase Type-1 Bioactivity	(43)

Table 4: (Continue)

Pharmacological activity	Part use	Extract/Fraction/Isolate	Testing method; Experimental model	Animals/ Cell line culture	Dose	Results	Reference
Antioxidant and antiatherosclerotic activities	Leaves	100 g of the powdered leaves in 900 ml of purified water and incubated in a high-speed mixer at 80°C for 3 hours.	In-vitro	--	150 µg/mL	The expressional suppression of ICAM-1 and Nox4 and induction of antioxidant enzymes might be an an important component of the vascular protective effect of *Piper sarmentosum*	(13)
Anti-tuberculosis activity	Leaves	Methanolic extract. Chloroform, ethyl acetate fractions of methanol. Combination of Isoniazid with different fractions	MIT in-vitro Assay	--	100µg/ml from the stock solution	Aqueous, ethanolic and Methanolic extract possess anti-TB activity with MIC 12.5 µg/ml, whereas chloroform and ethyl acetate possess 3.12 µg/ml. Concentration of Isoniazid (INH) was reduced to 75% when used in combination	(7)
	Root, stem, leaves and fruit	Aqueous and ethanolic extract					

Table 4: (Continue)

Pharmacological activity	Part use	Extract/Fraction/Isolate	Testing method; Experimental model	Animals/ Cell line culture	Dose	Results	Reference
Fracture healing	Fresh Leaves	Aqueous	In-vivo	Female Sprague-Dawleyrats	125 mg/kg	Improved fracture healing, as assessed by the reduced callus volumes and reduced callus scores.	(44)
Anti-inflammatory and antipyretic activities	Leaves	Extracted using cold extraction by macerating in 20 L of methanol for 7 days at room temperature.	In-vivo	Male Wistar rats	50, 100 and 200 mg/kg p.o	Methanolic extract of *P. sarmentosum* leaves possesses anti-inflammatory activity while lacking antipyretic activity	(15)
Antiangiogenesis Activity	Leaves	Petroleum ether, chloroform, methanol, n-hexane, chloroform, ethyl acetate fractions of methanol	In-vivo	Rat aorta model using adult Sprague-Dawley rats	100µg/ml	Chloroform extract and fraction have promising antiangiogenesis activity	(21)
Anti-microbial activity	Leaves	Extracted by percolation in 70% methanol	In-vitro	Disc Diffusion Test	100 mg/ml	*P. sarmentosum* has shown to have some antimicrobial properties against methicilin-resistant Staphylococcus aureus (MRSA).	(45)

Pharmacological activity	Part use	Extract/Fraction/Isolate	Testing method; Experimental model	Animals/ Cell line culture	Dose	Results	Reference
Oxidative stress	Leaves	Macerated in absolute methanol	In-vivo	Male Wistar rats	125 mg/kg body weight	*P. sarmentosum* is capable of reducing the oxidative stress in lungs by decreasing lipid peroxidation and maintaining the glutathione peroxidase activity towards the normal level.	(10)
Cardiovascular activity	Leaves	Mixed with 1L of water and boiled for 1 hr	In-vivo	Sprague-Dawley Rats	0.125 g/kg	The results indicate that *P. sarmentosum* restores ultrastructural integrity in the diabetic cardiovascular tissues.	(20)
Wound Closure Activity	Leaves	Aqueous Plant extract	In-vitrocell proliferation assay	--	1 to 100 µg/mL	*P. sarmentosum*used for the healing of oral wounds	(14)
Antihypertensive agent	Leaves	An amount of 100 grams of dried leaves were added to 900 ml of distilled water and boiled at 80°C for three hours.	In-vivo	Wistar rats	Not more than 2 ml/ 100 g body weight	*P. sarmentosum* possess antioxidant activity thatreduces oxidative stress damage, increase NO production and able to reduce blood pressure and cholesterol level	(23)

Table 4: (Continue)

Pharmacological activity	Part use	Extract/Fraction/Isolate	Testing method; Experimental model	Animals/ Cell line culture	Dose	Results	Reference
Anti-Ulcer	Leaves	250 g leaves were mixed with 2.5 L of methanol	In-vivo	Male Wistar rats	500 mg/kg	*Piper sarmentosum* possesses a similar protective effect against stress-induced gastric lesions as omeprazole	(46)
Anticoccidial effect	stem leaves	Extracted using supercritical carbon dioxide	In-vivo	Wenchang broiler chicks	100-300 mg/kg of feed	It possessesanticoccidialproperties and beneficial effect on intestinal mucosa damage in broiler chickens that had been challenged by coccidiosis	(47)

Conclusion:

Although a comprehensive literature available on *Piper sarmentosum* about its pharmacological properties and phytochemical potential but aclinical trial on identified compounds were not available yet. Furthermore, there is no identified compound of some pharmacological activities such as anti-cancer, anti-obesity, anti-ulcer, cardiovascular and wound healing activities. These pharmacological activities were may be due to thesynergistic effect of multiple bioactive compounds. There is still need of bioassay-guided fractionation of *Piper sarmentosum* on these pharmacological activities. Research on green technologies such as green solvents, new green extraction methods, and green processing technologies is yet to be evaluated.The use of silver nanoparticles as anantimicrobial agent is growing rapidly. *Piper sarmentosum* possesses anti-microbial activity. There is a significant scope of research in green synthesis of *Piper sarmentosum* silver nanomaterial.

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Formulation and Optimization of Porous Osmotic Pump Tablets based Controlled Release System of Stavudine for the Treatment of HIV Infection

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Abstract

The current research was designed to develop controlled release osmotic pump tablets of stavudine a nucleoside reverse transcriptase inhibitor for the treatment of HIV infection. Wet granulation method was adopted for the development of tablets containing dose 80mg of stavudine. The coating membrane of core tablets were prepared by using cellulose acetate as wall forming agent, poly ethylene glycol as flux regulating agent, and sorbitol as pore forming agent. The formulated tablets were characterized by FTIR, DSC, pre compression parameters, post compression parameters, *in vitro* drug release study and scanning electron microscopy study. Among developed formulations SM5 Batch showed 96.06% of drug release in controlled manner at the end of 18hrs.The *in vitro* release kinetics data were fitted for different batches in various pharmacokinetic models such as zero order, first order, Higuchi, Korsmeyer Peppas and Hixon Crowell model. The optimized formulation was carried out for effect of pH in dissolution media, agitation intensity and osmotic pressure effect on dissolution media. Short term stability study (at 40±2ºC/75±5% RH for three months) on the best formulation confirmed that there was no significant changes in thickness, weight variation, %friability, drug content and *in vitro* drug release.

Keywords: HIV infection, DSC wet granulation, in vitro drug release, stability study**.**

Inroduction

Most conventional oral drug products are developed to release the active drug immediately after oral administration to gain rapid and complete systemic drug absorption, but it is unable to control the release of drug and effective concentration of drug at the target site. The bioavailability (1) of drug for conventional dosage forms depends on physiochemical properties of drug, presence of excipients, physiological factors such as presence or absence of food, pH of GI tract, GI motility etc.Hence to avoid the limitations of conventional dosage forms various modified release drug products have been developed to control the release rate of drug and the time of drug release. Among several types of modified release drug products extended release drug product in the form of controlled release cover a wide range of prolonged action which provide continuous release of their active ingredients at predetermined rate and predetermined time. Out of various controlled drug delivery system oral osmotic controlled drug delivery system have advantages like independent of pH and hydrodynamic condition of the body, agitation intensity, presence or absence of food etc.Osmotic controlled drug delivery system works on principle

Vol. 11 (4) 357-369 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

of osmotic pressure for controlled delivery of drugs in the form of zero order.

The present study is to develop controlled porosity osmotic pump (CPOP) tablets of stavudine.CPOP tablets comprise of a core including the drug, an osmotic agent, other additives and semi permeable membrane (SPM) with porogen.Water leachable substances (2) are present in SPM which get dissolved when it comes in contact with release media, creating in situ micropore formation generating osmotic pressure within CPOP tablet to release drug in controlled manner.

HIV infection is a serious and a pandemic disease which is transmitted (3) primarily via unprotected sexual intercourse, contaminated blood transfusions through hypodermic needles and from mother to child during pregnancy, delivery or breastfeeding. Person infected with HIV has a CD4+ count of less than 200cells/μL in blood. The management of HIV infection can be controlled by antiretroviral therapy, male circumcision, needle exchange program, use of diaphragms, topical protection, use of condoms and alternative medicine (4). Stavudine is a nucleoside reverse transcriptase inhibitor (NRTI) with activity against (5) Human Immunodeficiency Virus Type 1 (HIV-1) which is chemically 2',3'-didehydro-3'-deoxythymidine.The active metabolite stavudine 5' triphosphate is an inhibitor of the HIV reverse transcriptase and acts as a chain terminator during DNA synthesis.Stavudine is absorbed rapidly orally producing peak plasma concentration within 1hour with 86% bioavailability and elimination half life of 1 to 1.5 hour following single or multiple doses (6). The conventional dose of stavudine 40mg twice daily. Converting twice daily regimen of stavudine into once daily formulation of controlled release dose enhances the effectiveness of antiretroviral therapy.

Materials and Methods

***Materials*:** Stavudine was obtained from Hetero Drugs Pvt. Ltd. India. Mannitol (Qualigens Fine Chemicals, India) and Cellulose acetate (CA) was obtained from Eastman Chemical Inc, Kingsport, TN. Sorbitol,HPMC E5LV,polyethylene glycol (PEG) 400,600,1500,4000,6000, Magnesium stearate and talc were purchased from S.D. Fine Chemicals Ltd, Mumbai, India. Microcrystaline cellulose(MCC),PVPK30 were purchased from Signet Pharma,Mumbai,India.All other solvents and reagents used were of analytical grade.

Compatibility Studies

Fourier Transform Infrared Spectroscopy (FTIR): In this method individual samples (7) as well as the mixture of drug and excipients were ground mixed thoroughly with potassium bromide (1:100) for 3-5 minutes in a mortar and compressed into disc by applying pressure of 10kg/cm to form a transparent pellet in hydraulic press. The pellet was kept in the sample holder and scanned from 4000 to 400 cm^{-1} in FTIR spectrophotometer (Bruker, Germany).

Differential Scanning Calorimetry (DSC): Physical mixtures of drug and individual excipients in the ratio of 1:1 were taken and examined in DSC (Shimadzu DSC-50,Japan).Individual samples as well as physical mixture of drug and excipients were weighed to about 5mg in DSC pan. The sample pan was crimped for effective heat conduction and scanned (8) in the temperature range of 50-300^{0}C.Heating rate of 20^{0}C $min^{-1}$was used and the thermogram obtained was reviewed for evidence of any interactions.

Methods

***Preparation of osmotic pump tablets*:** Wet granulation technique was used to develop CPOP core tablets. Accurately weighed quantities of ingredients mentioned in Table 1 were sifted through sieve No. 30. Lubricant (magnesium stearate) and glidant (talc) were sifted through sieve No. 80.The ingredients were manually blended homogenously in a mortar by way of geometric dilution except lubricant and glidant. The mixture was moistened with aqueous solution and granulated through sieve No.30 and dried in a hot air oven at 60ºC for sufficient time (3-4 hrs). The dried granules were passed through sieve No.30 and blended with talc and magnesium stearate.

Vol. 11 (4) 357-369 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

The homogenous blend was then compressed into round tablets with standard concave punches using 10 station rotary compression machine (Mini press,Karnavati, India).

***Coating of Core Tablets*:** The coating solution was prepared taking required ingredients from table 2 and acetone was added quantity sufficient maintaining proper viscosity of solution. The coatings of tablets were performed by spray pan coating in a perforated pan (GAC-205, Gansons Ltd, Mumbai, India). Hot air is supplied to tablet bed by rotating lower speed 5-8 rpm initially. The coating of tablets was carried out with the rotation speed of 10-12 rpm.The spray rate and atomizing air pressure were 4-6 ml/min and 1.75 kg/cm^2 respectively. Inlet and outlet air temperature were 50ºC and 40ºC respectively. Coated tablets were dried at 50ºC for 12 hrs.

***Evaluation of granules*:** The prepared granules were evaluated for pre compression parameters (9) such as angle of repose, bulk density, tapped density and compressibility index (Carr's index).Fixed funnel method was used to determine angle of repose. The bulk density and tapped density were determined by bulk density apparatus (Sisco, India).
The Carr's index (10) can be calculated by the following formula.

$$\% \text{ Carr's index} = \frac{et - eb}{et} \times 100 \quad (1)$$

Where e_t is the tapped density of granules and e_b is bulk density of granules.

The Hausner's ratio can be calculated by the taking the ratio of tapped density to the ratio of bulk density.

Evaluation of tablets (11)

Thickness: The thickness of individual tablets is measured by using vernier caliper (Absolute digimatic, Mitutoyo Corp. Japan).The limit of the thickness deviation of each tablet is ±5%.

***Measurement of coat thickness*:** Film was isolated from the tablets after 18hrs of dissolution and dried at 40^{0}C for 1hr.Thickness was measured by using electronic digital calipers (Absolute digimatic, Mitutoyo Corp. Japan)

Hardness: The hardness of tablets can be determined by using Monsanto hardness tester (Sisco,India).

Friability: Friability (12) of tablets was performed in a Roche friabilator (SISCO, India).Twenty tablets of known weight ($W_{initial}$) were de-dusted in plastic chamber of friabilator for a fixed time of 25 rpm for 4 minutes and weighed again of weight (W_{final}).The percentage of friability was calculated using the following equation.

Table 1. Composition of Osmotic Pump Stavudine tablets

Ingredients (mg)	SM1	SM2	SM3	SM4	SM5
SD(mg)	80	80	80	80	80
MCC(mg)	175	155	135	115	95
PVPK30(mg)	20	20	20	20	20
HPMC E5LV(mg)	100	100	100	100	100
Mannitol(mg)	20	40	60	80	100
Magnesium stearate(mg)	2	2	2	2	2
Talc(mg)	3	3	3	3	3
Total weight(mg)	400	400	400	400	400

Table 2. Coating Composition for Controlled porosity Osmotic Pump Tablets

Formulation code	CA (g)	PEG 400 (g)	PEG 600 (g)	PEG 1500(g)	PEG 4000 (g)	PEG 6000 (g)	Sorbitol (g)	Acetone (ml)
SM1	6	2	0	0	0	0	0.4	300
SM2	6	0	2	0	0	0	0.8	300
SM3	6	0	0	2	0	0	1.2	300
SM4	6	0	0	0	2	0	1.6	300
SM5	6	2	0	0	0	2	2	300

%Friability= $F = \left(1 - \frac{W\,final}{W\,initial}\right) \times 100$ (2)

Where, $W_{initial}$ and W_{final} are the weight of the tablets before and after the test respectively.

Weight variation test (13) : The weight variation test is performed by weighing 20 tablets individually calculating the average weight and comparing the individual tablet weights to the average. The percentage weight deviation was calculated and then compared with USP specifications.

***Uniformity of drug content test*:** Powder is made after triturating 10 CPOP tablets from each batch with mortar and pestle.The powder weight equivalent to one tablet was dissolved in a 100ml volumetric flask filled with 0.1N HCl using magnetic stirrer for 24hr.Solution was filtered through Whatman filter paper No.1 diluted suitably and analyzed spectro photometrically

***Diameter of tablet*:** The diameter of individual tablets is measured by using vernier caliper (Absolute digimatic, Mitutoyo Corp. Japan).

***In vitro dissolution studies*:** The in vitro dissolution studies were carried out using USP apparatus type II (Lab India 8000) at 75 rpm. For the first 2 hr the dissolution medium was 0.1N HCl (pH1.2) and phosphate buffer pH 6.8 for remaining hours (900 ml), maintained at 37±0.5^{0}C. At each time point 5 ml of sample was withdrawn and it was replaced with 5 ml of fresh medium. The drug release at different time interval was measured by UV-visible spectrophotometer (UV-1800, Shimadzu, Japan)

***In vitro* drug release kinetic studies (14, 15):** In order to investigate the mode of release from tablets, the release data of formulation was analyzed zero order kinetics, first order kinetics, Higuchi model and Korsmeyer and Peppas equations.

***Effect of osmogen concentration*:** Keeping all the parameters for a tablet constant different osmogen (16) concentrations were used to prepare tablets. The drug release was compared with the different osmogen concentration of formulated batches by using USP-II dissolution apparatus.

***Effect of pore former concentration*:** SPM for various batches were prepared by taking different concentrations of pore former (17). The effect of pore former on in vitro release profile is compared as well as number of formation of micropores were observed.

***Effect of membrane thickness*:** Tablets with varying coating thicknesses were developed to demonstrate the effect of coating thickness (18) on drug release. The drug release rate was measured using 0.1N HCl and phosphate buffer pH6.8 as a dissolution medium.

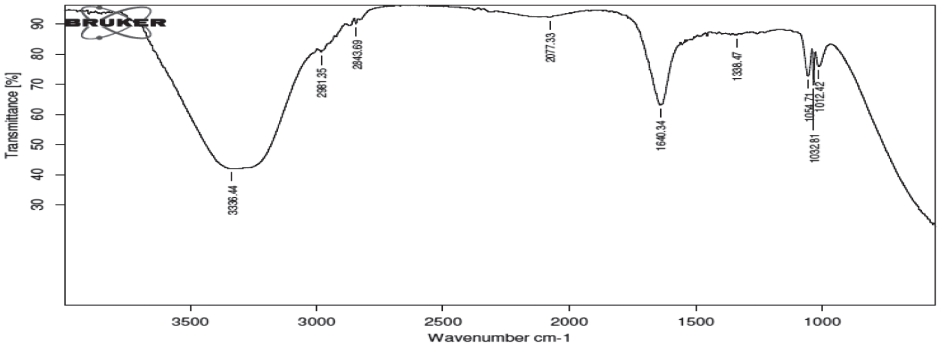
Effect of osmotic pressure (19) : The effect of osmotic pressure was demonstrated by adding different amount of mannitol of an osmotic agent to produce 30 atm,60 atm and 90 atm respectively in dissolution media 0.1N HCl for 2hrs and phosphate buffer pH 6.8. The drug release rate

was carried out in USP type II (Paddle) apparatus at 75 rpm maintained at 37±0.5^{0}C and compared for various dosage forms.

Effect of pH (20) : The effect of pH for developed formulations were observed by performing the release studies of optimized formulation in different media 0.1 N HCl(pH 1.2), pH 6.8 phosphate buffer and pH 7.4 phosphate buffer in USP type II dissolution apparatus at 75rpm. The temperature was maintained at 37±0.5°C. The release was studied at predetermined time intervals.

Effect of agitation intensity (21) : The effect of agitation intensity were observed by performing the release studies of optimized formulation in USP Type II(Paddle) dissolution apparatus containing 0.1NHCl for first 2hrs and phosphate buffer pH 6.8 for rest hours at different rotational speeds of 50,100 and 150rpm with maintaining temperature at 37±0.5°C.The samples were withdrawn at predetermined intervals and analyzed by UV spectrophotometer.

Scanning Electron Microscopy (SEM) : Coating membranes (22) of formulation were collected before and after complete dissolution of core contents and examined for their porous morphology (23) as well as mechanism of drug release by scanning electron microscope (Leica, Bensheim, Switzerland). Scans were taken at an



1(a)

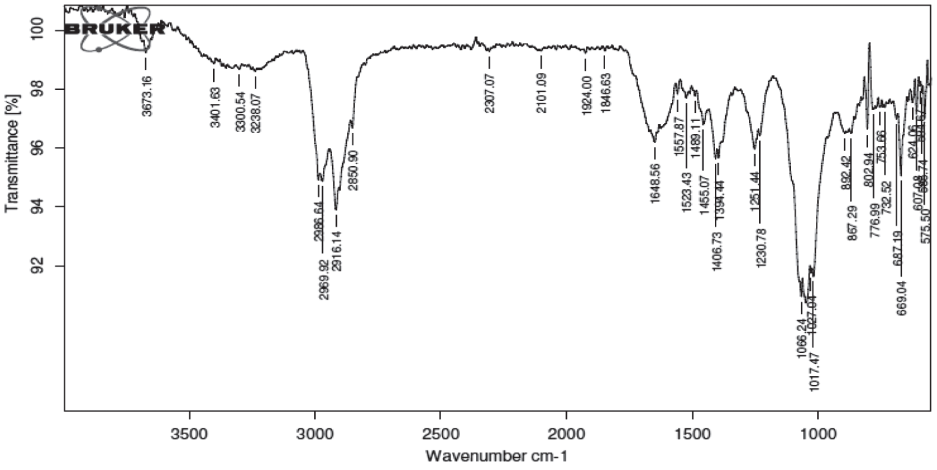
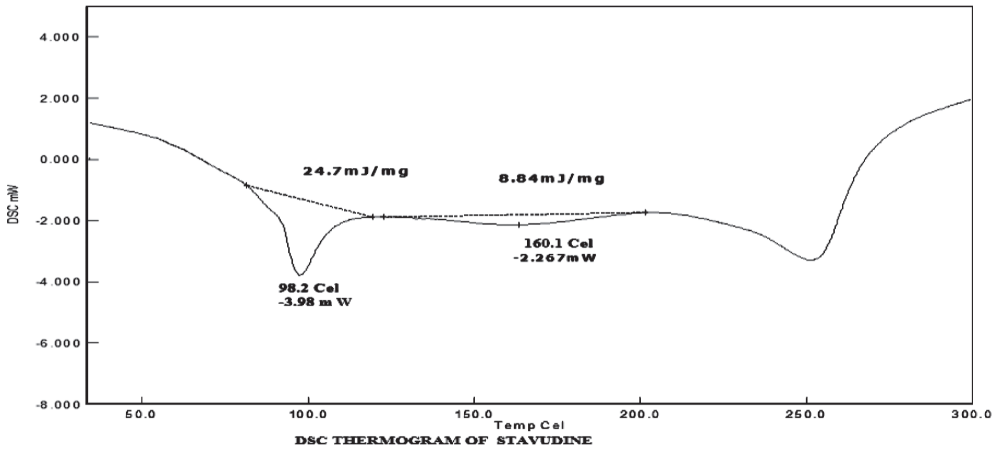


Fig. 1. FTIR spectroscopy study of (a) Stavudine (b) SM5 CPOP tablets



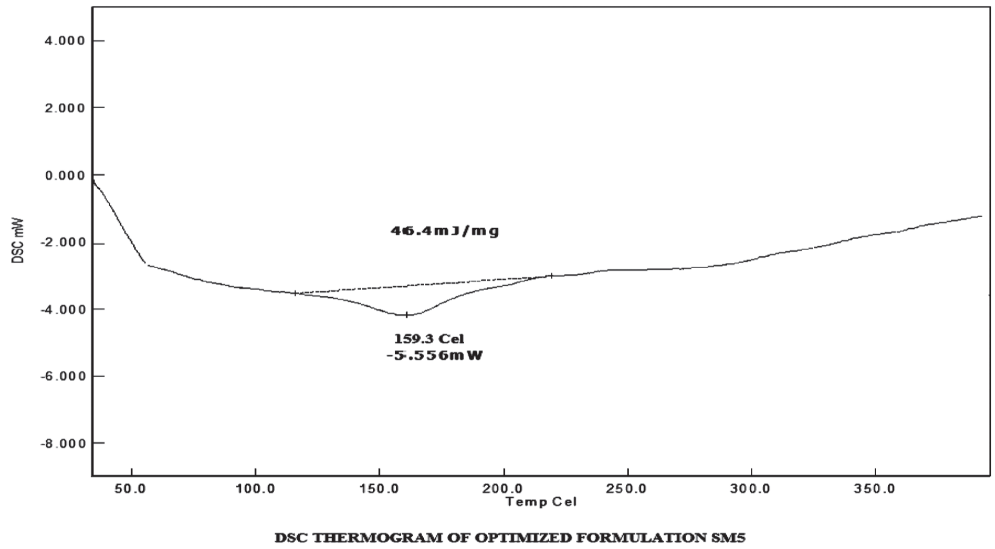


Fig. 2. DSC thermograms of (a) Stavudine (b) SM5 CPOP tablets

excitation voltage in SEM fitted with ion sputtering device.

Accelerated stability studies (24) : The packed tablets in air tight container were placed in stability chambers(Thermo lab Scientific equipment Pvt.Ltd.,Mumbai,India) maintained at 40±2^{0}C/ 75±5% RH conditions for accelerated testing) for 3 months. Tablets were periodically removed and evaluated for physical characteristics, drug content, in vitro drug release etc..

Results and Discussion

***FTIR studies*:** In the optimized formulation SM5 peak at 3673.16, 1455.07,1251.44, and776.99 cm^{-1} were due to presence of the polymer HPMCE5LV.In the formulation the peaks present due to mannitol were 2916.14,1017.47 and 669.04 cm^{-1}.Peaks at 2986.64, 1648.56 and 1066.24 cm^{-1} were due to presence of the drug stavudine in the optimized formulation. So from the study it can be concluded that the major peaks of drug

2986.64, 1648.56 and 1066.24 cm^{-1} remain intact and no interaction was found between the drug, polymer and osmogen. Hence drug-excipient mixture reveals that here is no incompatibility was observed between stavudine. It was found that there was no major shift in peaks of optimized formulation as a result of which drug-excipients were found to be compatible. It is shown in figure 1(a,b).

***DSC study*:** Fig. 2a indicates that the endothermic peak of stavudine is at 160.1^{0}C(Figure 2a).The endothermic peak of SM5 formulation (Figure 2 b) is observed at159.3^{0}C.There was no significant changes in the endotherm peak between drug and formulation. Hence drug and excipients in SM5 were compatible.

***Pre compression parameters*:** All the compressible excipients for various batches were evaluated for angle of repose, bulk density, tapped density, Carr's index and Hausner's ratio. The angle of repose was found in the ranges from 25.11± 0.11 to 29.73± 0.11 degrees, bulk density of pre-compression blends was found to be in the range of 0.467± 0.12 to 0.474 ± 0.06 gm/ml, tapped density in the range of 0.504 ± 0.13 to 0.519 ± 0.06 gm/ml, the Carr's index values were in the range of 7.34± 0.08 to 9.44± 0.07 %, and the Hausner's ratio was in the range between 1.07± 0.06 to 1.10± 0.05. All the values were found to be acceptable limits of pharmacopoeial specifications.It is given in Table 3.

Table 3. Pre compression parameters of granules

Formulation code	Angle of repose (degree)[a]± S.D	Bulk density (gm/ml)[a]± S.D	Tapped density (gm/ml)[a]± S.D	Carr's Index (%)[a]± S.D	Hausner's Ratio[a]± S.D
SM1	29.73 ± 0.11	0.472 ± 0.08	0.516 ± 0.06	8.52 ± 0.07	1.09 ± 0.08
SM2	28.92 ± 0.08	0.474 ± 0.06	0.518 ± 0.04	8.49 ± 0.05	1.09 ± 0.04
SM3	27.46 ± 0.12	0.470 ± 0.04	0.519 ± 0.06	9.44 ± 0.07	1.10 ± 0.05
SM4	28.12 ± 0.13	0.468 ± 0.11	0.508 ± 0.14	7.87 ± 0.12	1.08 ± 0.12
SM5	25.11 ± 0.11	0.467 ± 0.12	0.504 ± 0.13	7.34 ± 0.08	1.07 ± 0.06

N.B. All values are expressed as mean S.D, [a] n = 3

***Post compression evaluation tests*:** The thickness of the tablet formulations was found to be in the range of 2.9± 0.02 to 3.15± 0.06 mm, the hardness values were in the range of 6.5± 0.13 to 7.9± 0.09 kg/cm^2,the friability values were in range of 0.13± 0.12 to 0.37± 0.06, average weight of tablet was in the range of 400.6± 1.06 to 403.2± 1.13 mg, drug content of tablet was in the range of 98.45± 1.06 to 101.23± 1.05 and diameter of tablets values were ranges of 7.9± 0.03 to 8.2± 0.06 mm. All values were found to be in acceptable ranges for uncoated tablets.It is mentioned in Table 4a.Similarly for coated tablets it is shown in Table 4b.

***In vitro drug dissolution study*:** The in vitro drug release characteristics were studied in 900ml of 0.1N HCl (pH1.2) for a period of first 2hrs and remaining hrs in phosphate buffer pH 6.8 using USP type II dissolution apparatus (Paddle type).The cumulative percentage drug release for SM1, SM2, SM3, SM4 and SM5 core CPOP tablets were 77.56, 80.34, 83.01, 87.11 and 95.99 respectively of stavudine at the end of 12 hrs. It is shown in figure 5a.The cumulative percentage drug release for SM1, SM2, SM3, SM4 and SM5 coated CPOP tablets were 81.23, 82.98, 84.98, 87.52 and 96.06 respectively of stavudine at the end of 18 hrs.It is shown in figure 5b.

Table 4a. Post compression evaluation tests for core tablets

Formulation code (FC)	Thickness of tablet (mm)[a]± S.D	Hardness (kg/cm^2)[a] ± S.D	Friability (%)[b] ± S.D	Average weight of 1tablet(mg)[b] ± S.D	% Drug content (%)[a]± S.D	Diameter (mm)[a]± S.D
SM1	3.1± 0.03	6.8± 0.12	0.32± 0.08	402.1± 1.12	98.45±1.06	8.1±0.04
SM2	2.9± 0.02	6.5± 0.13	0.37± 0.06	403.2± 1.13	101.23±1.05	8±0.02
SM3	3.1± 0.03	7.2± 0.06	0.24± 0.05	401.3± 1.06	100±1.01	7.±.03
SM4	3.12± 0.04	7.4± 0.07	0.18± 0.11	401.2± 1.08	99.07±1.09	8.2±0.06
SM5	3.15± 0.06	7.9± 0.09	0.13± 0.12	400.6± 1.06	99.69±1.07	8±0.01

N.B. All values are expressed as mean S.D, [a] n = 10, [b] n = 20

Table 4b. Post Compression evaluation tests for coated tablets

Formulation code (FC)	Thickness of tablet (mm)[a]±	Coat thickness (um)[a]+S.D	Hardness (kg/cm^2)[a] ±S.D	Friability (%)[b] ± S.D	Average weight of 1tablet(mg)[b] ± S.D	% Drug content (%)[a]± S.D	Diameter (mm)[a]± S.D
SM1	3.15 ± 0.09	500.4 ± 3.6	6.9 ± 0.12	0.02 ± 0.08	402.13 ± 1.19	98.45 ± 1.06	8.1 ± 0.04
SM2	3.11 ± 0.08	401.9 ± 3.8	6.6 ± 0.13	0.07 ± 0.06	404.2 ± 1.15	101.23 ± 1.05	8.2 ± 0.08
SM3	3.21 ± 0.07	300.6 ± 3.4	7.3 ± 0.06	0.04 ± 0.05	402.3 ± 1.07	100 ± 1.01	8.1 ± 0.06
SM4	3.22 ± 0.05	201.1 ± 3.1	7.5 ± 0.07	0.02 ± 0.11	402.8 ± 1.05	99.07 ± 1.09	8.3 ± 0.07
SM5	3.25 ± 0.04	100.2 ± 3.3	8.1 ± 0.09	0.01 ± 0.12	401.7 ± 1.06	99.69 ± 1.07	8.2 ± 0.05

N.B. All values are expressed as mean S.D, [a] n = 10, [b] n = 20

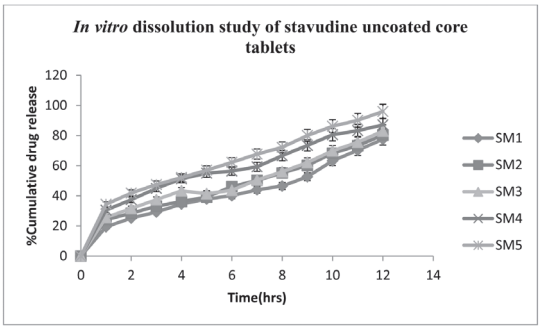


Fig. 5a. *In vitro* release profiles showing stavudine core tablets release from various fabricated formulations SM1-SM5(n=3)

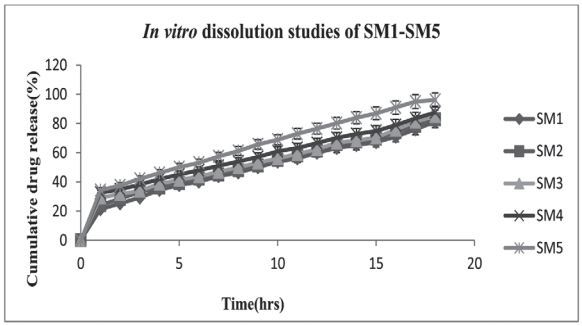


Figure 5b: *In vitro* release profiles showing stavudine release from various fabricated formulations SM1-SM5 (n=3)

Vol. 11 (4) 357-369 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

***Kinetic model*:** All the formulations showed to be best expressed by Higuchi kinetics as the plots showed high linearity. The regression values (R^2) of Higuchi were higher for all the formulations comparing to other kinetic models. It can be concluded that all the developed formulations follow Higuchi kinetics. The release exponent n value of Korsmeyer-Peppas model was more than 0.45 in SM1 formulation which indicated a non-Fickian diffusion mechanism of drug release and SM2, SM3, SM4 and LM5 show Fickian diffusion mechanism. It is shown in table 5

***Effect of osmogen concentration*:** The various batches of stavudine were developed with various concentrations of osmogen. It was observed that osmogent enhances the drug release of drug and thus had a direct effect on drug release. The drug release profile was shown in figure 5(a,b).

***Effect of pore former concentration*:** For uncoated tablet there was no membrane and no pore former, but the drug releases upto 12 hrs (Figure 5a).The core formulations were coated with various concentration of sorbitol with compared to CA. It confirms that as the level of pore former increases the membrane becomes more porous after coming contact with aqueous environment resulting in faster drug release. Release profile of various batches was shown in figure 5b.

***Effect of membrane thickness*:** For uncoated tablet there was no membrane, but the drug releases upto 12 hrs (Figure 5a).Release profiles of stavudine from various batches varying the coating thickness were evaluated. It was clearly evident that drug release was inversely proportional to coating thickness of the semi permeable membrane. It is shown in figure 5b.

***Effect of osmotic pressure*:** The drug release for SM5 was found to be 90.11% for 30 atm, 84.56% for 60 atm and 81.23% for 90 atm respectively. Hence it was concluded that drug release was inversely proportional to the osmotic pressure of release media. It is shown in figure 6.

Table 5. Fitting of IVDR data in various mathematical models

Models	Zero order		First order		Higuchi		Korsmeyer-Peppas			Hixson-Crowell	
Batches	R^2	K_0	R_1^2	K_1	R_H^2	K_H	R_K^2	Kkp	n	R^2	Ks
SM1	0.956	3.618	0.962	0.0737	0.985	17.87	0.975	18.323	0.475	0.975	0.09
SM2	0.949	3.643	0.958	0.0783	0.981	18.02	0.963	20.701	0.439	0.972	0.093
SM3	0.933	3.588	0.936	0.0829	0.970	17,80	0.929	23.768	0.392	0.956	0.095
SM4	0.917	3.623	0.943	0.0898	0.975	18.18	0.937	26.977	0.365	0.960	0.101
SM5	0.928	4.230	0.906	0.1473	0.984	21.19	0.952	28.641	0.393	0.963	0.143

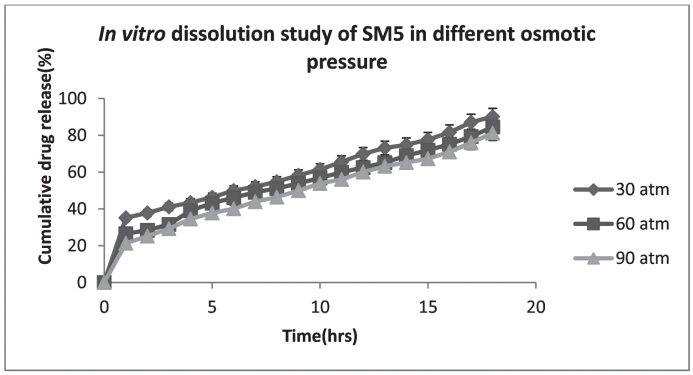


Fig. 6. *In vitro* release profiles showing stavudine release from best SM5 in different osmotic pressures.

Vol. 11 (4) 357-369 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

***Effect of pH*:** The optimized formulation SM5 was evaluated for in vitro drug release studies in buffers with different pH like 0.1NHCl (pH 1.2), phosphate buffer pH 6.8 and phosphate buffer pH7.4. It was concluded that there was no significant difference in the release profile, demonstrating that the developed formulation showed pH independent release. It is shown in figure 7.

***Effect of agitation intensity*:** The optimized formulation of SM5 batch was carried out in USP dissolution apparatus type-II at varying rotational speed(50,100 and 150rpm).It showed that the release of stavudine from core was independent of agitation intensity and the release from the developed formulation was independent of the hydrodynamic conditions of the absorption site. It is shown in figure 8.

***SEM analysis*:** Fig.9a suggesting that there is no evidence of development of pores in the membrane before dissolution study of SM5.On the other hand figure 9b shows that more pore formation after dissolution. When comparison was studied of the membranes containing different levels of porogen,it was observed that the membrane that contained a higher level of porogen became more porous after dissolution studies.

Stability studies: From short term stability studies of optimized formulation SM5, it was confirmed that there was no significance changes in physical appearance, weight variation, %friability, drug content and % drug release. It is shown in table 6. *In vitro* release profiles showing stavudine release from best SM5 in accelerated stability conditions is shown in fig. 10.

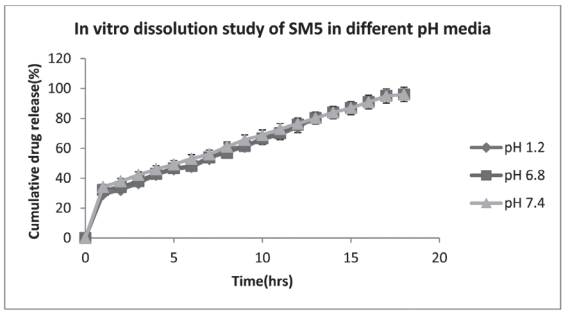


Fig. 7. In vitro dissolution study of best formulation SM5 in various pH media

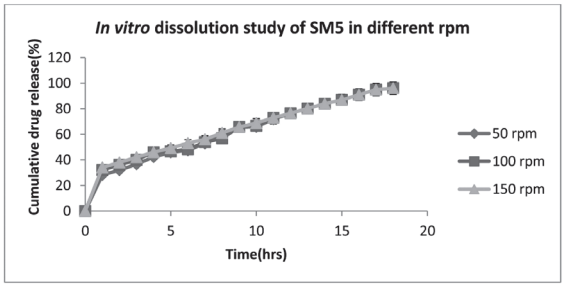


Fig. 8. *In vitro* dissolution study of best formulation SM5 in various agitation speed

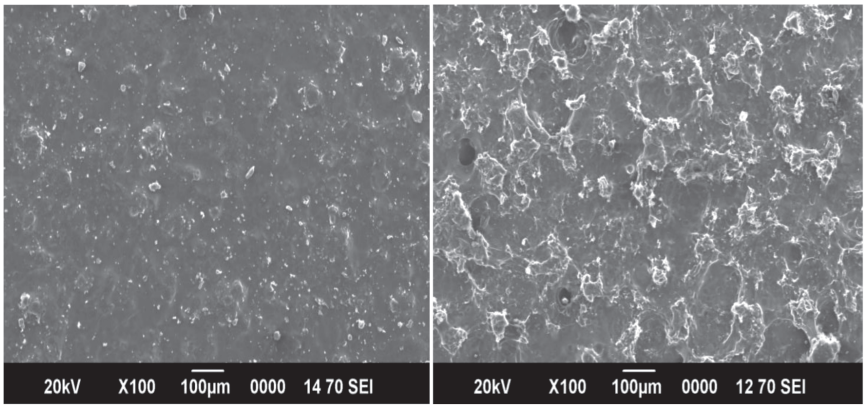


Fig. 9a. SEM micrograph of SPM before dissolution)SEM micrograph of SPM after dissolution

Table 6. Comparative physicochemical characterization of SM5 at accelerated conditions

Parameters	Initial	After 30 days	After 60 days	After 90 days
Physical appearance	Pale white, circular,concave smooth surface without any cracks	No change	No change	No change
Thickness(mm)[a] ± S.D	3.25 ± 0.04	3.25 ± 0.06	3.24 ± 0.06	3.23± 0.07
Hardness(kg/cm^2)[a] ± S.D	7.9 ± 0.09	7.9 ± 0.09	7.8 ± 0.09	7.8 ± 0.03
Friability(%)[b] ± S.D	0.13 ± 0.12	0.13 ± 0.12	0.15 ± 0.12	0.16 ± 0.11
Weight variation(mg)[b] ± S.D	401.7 ± 1.06	401.7± 1.06	400.9 ± 1.05	400.8 ± 1.02
Drug content(%)[a] ± S.D	99.69 ± 1.07	99.69 ± 1.07	99.08 ± 1.02	98.67 ± 1.05
Diameter(mm)[a] ± S.D	8.2 ± 0.05	8.2 ± 0.05	8.1 ± 0.03	8.0 ± 0.01

N.B. All values are expressed as mean S.D, [a] n = 10, [b] n = 20

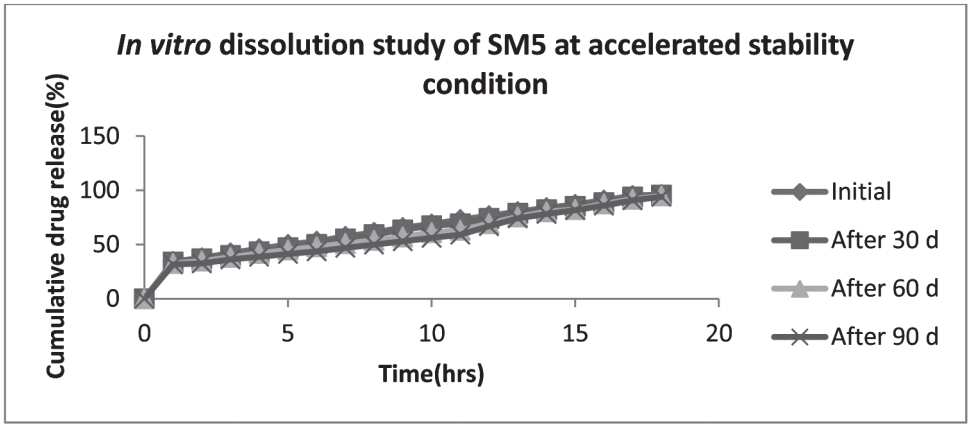


Fig. 10. *In vitro* release profiles showing stavudine release from best SM5 in accelerated stability conditions

Conclusion

The desired release of stavudine from CPOP was gained through careful monitoring of the selected formulation variables. It was evident that increase in concentration of osmogen the drug release from the system was found to be increased. The optimized formulation SM5 releases stavudine which is independent of pH, agitation intensity. Hence it was observed that the release of stavudine can be significantly controlled from the controlled porosity osmotic delivery system.

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Optimization of Variables for Lactase Production from Isolated *Bacillus subtilis strain*VUVD001 Through Submerged Fermentation

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Abstract

Lactase enzyme is commercially important and is generally used for lactose hydrolysis in milk and whey. To date, it has been isolated from various sources. In this study, different strains of isolated bacteria were evaluated for their lactase productivity, but *Bacillus subtilis* VUVD001 resulted with the highest production. Therefore, optimal physical conditions were determined in batch fermentation process using one-variable-at-a-time approach for the production of lactase. The influence of some physical conditions namely pH, incubation temperature and time, inoculum size on enzyme production were studied for higher yield. Maximum activity of lactase in shake flask culture was found 15.27 U/ml at optimized conditions of incubation period 36 h, temperature 37 ^{o}C, pH 7.0 and inoculums size of 5%.

Keywords Lactase, *Bacillus subtilis*VUVD001, Shake Flask Culture

Introduction

Lactase is widely used in food and pharmaceutical industries due to its ability to hydrolyze lactose in milk and its by-products. This enables reduction of lactose intolerance problem (1, 2). Previously it has been reported that different bacterial species were used for production of lactase because of various factors such as simple fermentation, high enzyme activity and stability (3, 4). It is also reported that various microbes such as *Bacillus circulans*, *Bifidobacterium adolescentis*, *Lactobacillus reteri, L. plantarum* and *B. infantis* have shown lactase producing activity (5-7). The dairy industry uses bacterial genus *Lactobacillus* and *Bifidobacterium* for the production of lactase enzyme (8, 9). These *bacilli* are generally regarded as safe (GRAS) and thus, the lactase secreted by them can be consumed without excessive purification by lactose intolerance patients. Reduction in lactase activity may cause lactose intolerance (10). The yeast species, *Kluyveromyceslactis*, *K. marxianus* and *Saccharomyces fragilis* and molds such as *Aspergillus* and *Rhizomuor* species were also used in commercial production of lactase (11-13). Enzyme production was influenced by various factors namely strain type and cultivation conditions like temperature, pH, agitation speed and incubation time (14, 15). The aim of this study was to assure the production of high yield of lactase from *Bacillus subtilis* VUVD001, through optimization study. The physical conditions such as fermentation time, pH, temperature and inoculum percent were studied for improving production.

Material and Methods

Microorganism and shake flask fermentation

The organism *B. subtilis* strainVUVD001 was isolated from the effluents of Sangam Dairy,

Vadlamudi, Guntur District. India. The culture was maintained in the laboratory at room temperature and preserved at 4°C on nutrient agar medium. The original fermentation medium consisted of 4 g/L of lactose, 4 g/L of yeast extract, 1g/L of $MgSO_4 \cdot 7H_2O$ and 0.1g/L of Tryptophan. The shake-flask fermentation was performed with 100mL of production medium.

Effect of culture conditions on enzyme production

Inoculum size : The influence of inoculum size on production of lactase was studied by changing the percent of inoculum (1-6%, v/v) to production medium.

Incubation time : The effect of incubation time on production of lactase was determined by incubating the bacterial strain at 37 °C in broth medium at different time intervals.

Temperature : Optimum temperature for the production of enzyme was determined by incubating inoculated culture medium at different temperatures namely 27°C, 37°C and 47°C for 36 h.

pH : The best possible pH for production of lactase was optimized by using broth medium and determined by adjusting pH (5-8) of medium using 1M NaOH and 1M H_2SO_4. The inoculated culture flasks were kept for incubation for a period of 36h.

Lactase assay : Lactase activity was determined using ortho-Nitrophenyl- β-galactoside (ONPG) as substrate. The ONPG solution was prepared with phosphate buffer and used for assay. The assay system contained 0.5mL of enzyme source with 2.0mL of substrate and incubated for 30 min. The reaction was stopped with the addition of 0.5 mL of 1 M Na_2CO_3 and absorbance was recorded at 420 nm. Activity of lactase was determined from ONP standard graph. One unit of activity is defined as amount of enzyme that liberates 1 micromole of ONP from the substrate per minute under assay conditions (16).

Results and Discussion

Effect of incubation time : The changes in enzyme activity were observed during incubation periods from 12 to 48 h. This represents the change in state of population number from log to stationary phase. The maximum enzyme activity of 15.13 U/ml was found after 36 hours of incubation beyond this the activity declined due to depletion of nutrients (Fig.1). Qian et al (17) reported that the highest lactase activity was found at 36 h of incubation time through fermentation in

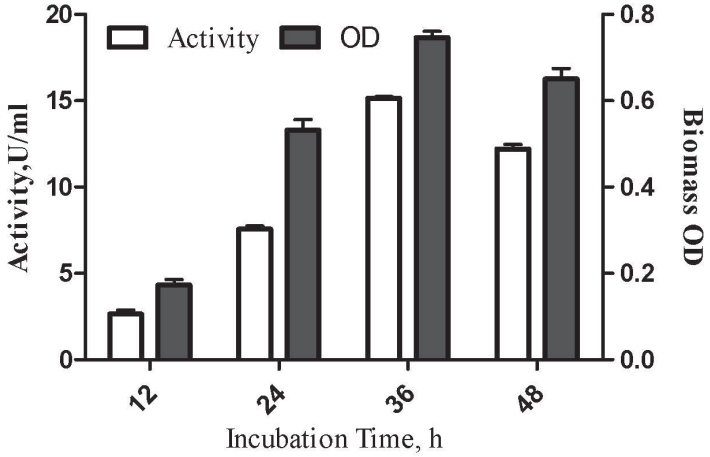

Fig. 1. Effect of incubation time on enzyme production

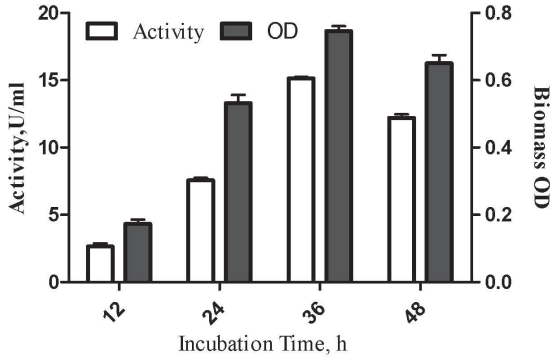

Fig. 2. Effect of temperature on production of enzyme

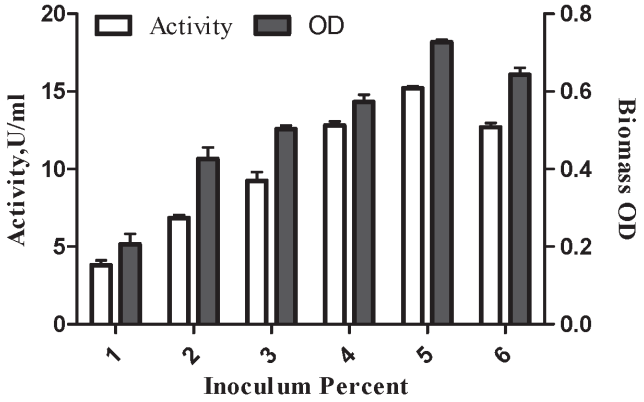

Fig. 4. Effect of size of inoculum on production of lactase

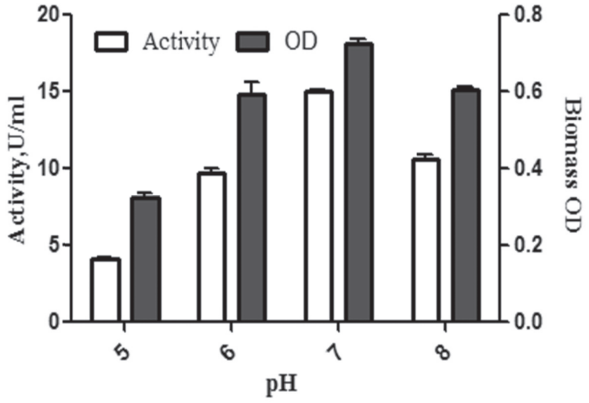

Fig. 3. Effect of pH on enzyme production

Lactase Production Through Submerged Fermentation

shake flasks with a thermotolerant strain. Similarly, Mukesh Kumar et al (18) observed the maximum lactase activity with 48 hours of incubation period in submerged fermentation process by using *Bacillus* sp. MPTK121. Findings of Jayashree Natarajan et al (19) also show that the optimum incubation period for lactase production was 48 h.

Effect of temperature : The submerged fermentation process showed that the enzyme activity increases with an increase in incubation temperature up to 37 °C and then enzyme activity slows down beyond this temperature. Thus, 37 °C was found optimum temperature for lactase production by *B. subtilis* strain VUVD001 (Fig.2). Murad et al (20) stated that lactase production was increased by *Lactobacilli* strain when the cultivation temperature was maintained at 30 °C. Tryland and Fiksdal (21) reported that 35 °C was the maximum temperature for lactase activity and beyond this temperature the enzyme activity was decreases up to 44 °C. Roopashri and Varadaraj (22) reported that highest lactase activity of 10.6 U/ml was obtained with *Lactobacillus plantarum* MTCC5422 in the soy whey based medium at 37 °C.

Effect of pH on enzyme production : Enzyme activity gradually increased with changes in the pH of the medium from 5.0 to 7.0 and the activity slowly decreased with an increase in pH. The highest enzyme activity was found 15.12 U/ml at pH 7.0 (Fig.3). The intracellular lactase activity was high at pH 6.8 through cultivation of *L. delbrueckii* spp.*bulgaricus* ATCC11842 and *B.animalis* spp.*lactis* Bp12 (23). Cherabarti et al (24) proved that relative activity of lactase was higher at pH 7 through submerged fermentation using the strain *B. poymyxa*. Sangwan *et al (25) reported that the pH 6.8 is the optimum for the production of lactase through the submerged fermentation from Streptococcus thermophilus*.

Effect of inoculum size : Inoculum size of bacterial culture has an important effect on the production of enzyme quantity. Maximum lactase activity of 15.20 U/ml was observed with an inoculum size of 5%, and minimum with 1% inoculum, respectively (Fig.4). Gowdhami et al (26) observed the highest lactase production by *L. bifermentans* at 2% v/v inoculum. Anisha and Prema (27) found the highest lactase activity when the fermentation medium was inoculated with 10% (v/v) of 72 h grown inoculum. Mahalakshmi et al (28) reported that the higher lactase enzyme production was achieved with 1% inoculum of *L. amy lophilus* GV6.

Conclusion

It is concluded that the highest biocatalytic activity could obtained by using *B. subtilis* strainVUVD001. The maximum lactase activity was found 15.27 U/ml at optimum conditions of 5% (v/v) inoculum size, pH 7, 37 °C temperature and incubation time 36 h. Based on the results reported in this paper, it is predicted that the crude enzyme extract lactase from *B. subtilis* will have possible application in the food and pharmaceutical industries.

Acknowledgement

The authors acknowledge the Vignan's Foundation for Science, Technology and Research University, Vadlamudi, Guntur, Andhra Pradesh, India for providing necessary facilities to carry out this work.

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Validated Area Under Curve UV Spectrophotometric Method for the Determination of Glimepiride in Tablet Formulations

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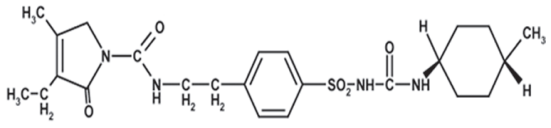
Abstract

A novel Area under curve method was developed for the quantification of Glimepiride using analytical grade methanol as solvent. Glimepiride obeys Beer's law in concentration range 20-40 µg/ml in area between 227nm - 233nm.The recovery studies ascertained the accuracy of proposed method and result was validated according to ICH guideline. The result of analysis has been validated statistically by recovery studies. This method was successfully carried out for the estimation of Glimepiride in tablet dosage form without the interference of common excipients.

Keywords: Glimepiride, Area under curve method, Spectrophotometric method, UV determination.

Introduction

Glimepiride is used with a proper diet exercise program to control high blood sugar in people with type 2 diabetes . It may also be used with other diabetes medications. Controlling high blood sugar helps prevent kidney damage, blindness, nerve problems, loss of limbs, and sexual function problems. Proper control of diabetes may also lessen your risk of a heart attack or stroke. Glimepiride belongs to the class of drugs known as sulfonylurea. It lowers blood sugar by causing the release of your body's natural insulin (2).



Glimepiride is 1-[[p-[2-[3-ethyl-4-methyl-2-oxo-3-pyrrolinepyrroline-1-carboxamido] ethyl]-phenyl]-sulfonyl]-3-[trans-4-methylcyclohexyl] urea (1)

Review of Literature : Based on the literature survey, it was found that different methods for determination of Glimepiride have been reported. Analysis of Glimepiride by HPLC [3], RP-HPLC method for the determination of Glimepiride [4-7], Estimation by UV spectrophotometric method [8]. However, there is no area under curve found for estimation of Glimepiride. There for a new method was developed and reported.

Materials and Methods

***Materials*:** Shimadzu 1800 spectronic UV Spectrophotometer with 1cm matched quartz cells was used for data collection and analysis. Methanol was used as a solvent for drug substance.

***Preparation of standard stock solution*:** Standard stock solution of Glimepiride was prepared by dissolving accurately weighed quantity of Glimepiride 25mg in 100 ml of methanol and

Vol. 11 (4) 376-381 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

transferred it to 100ml volumetric flask. Volume was made to the mark with methanol for obtaining stock solution up to 1000µg/ml conc. Further dilution made to get the concentration of 100µg/ml. Dilutions were done to get concentration of 20µg/ml.

***Determination of Area Under Curve*:** The standard solution of Glimepiride (20µg/ml) was scanned in the wavelength from 220 to 250 and absorption maximum was found to be at 230nm. There for, area from 227 to 233nm was selected for the analysis. (Figure 1)

***Stability of Drug in Selected Solvent*:** The stability of drug in selected solvent was determined by measuring the absorbance of the drug solution (20µg/ml) at different time intervals. After every 5 min. of interval the absorbance was measured the solution was found to be stable. (Table 1)

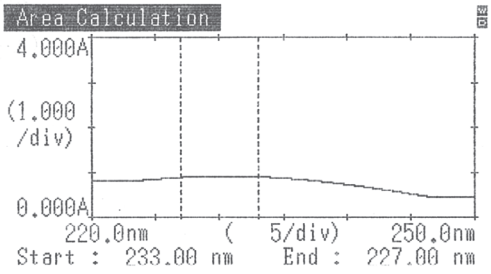


Fig. 1: Area under curve of Glimepiride in solution of methanol

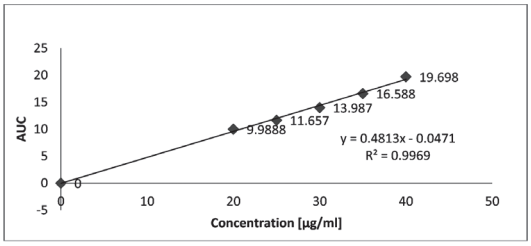


Fig. 2:: Calibration plot for Glimepiride

***Linearity*:** From the standard stock solution of Glimepiride, appropriate aliquots were pipette out into 25 ml of volumetric flask & dilutions were made with methanol to produce working standard solution of Glimepiride 20,25,30,35,40 µg/ml. The area under curve of Glimepiride was measured in area between 233 to 227 nm. The calibration plot of the drug Glimepiride was plotted. The concentration range over which the drug followed linearity was chosen as an analytical concentration range i.e. 20 µg/ml to 40µg/ml for Glimepiride. (Table 2 and Figures 2 to 7)

Validation of proposed method : A. Estimation of Drug from Dosage Form (Tablet) (Assay Study) Brand name- GLIMISTAR ^{O}-1

***Standard*:** From the standard stock solution of Glimepiride, appropriate aliquots were pipette out into 25 ml volumetric flask and dilutions were

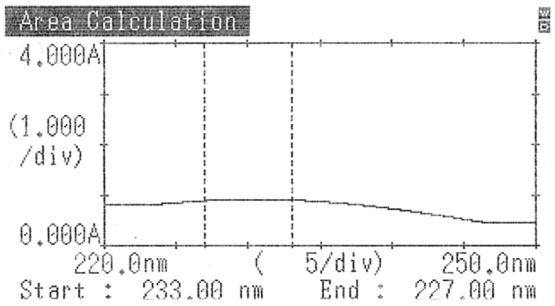


Fig. 3:: Area under curve of Glimepiride 20 µg/ml.

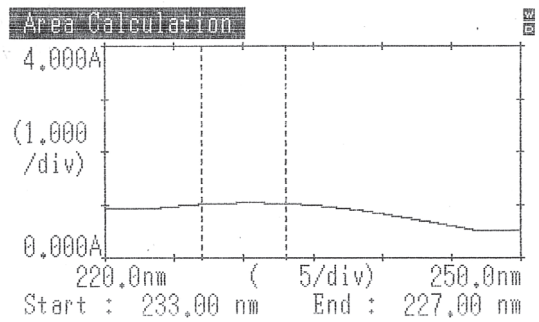


Fig. 4:: Area under curve of Glimepiride 25 µg / ml

Vol. 11 (4) 376-381 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

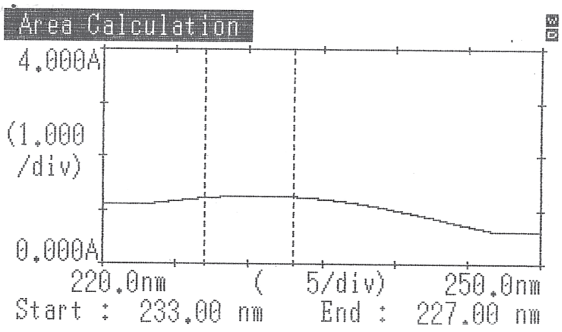


Fig. 5:: Area under curve of Glimepiride 30 µg / ml.

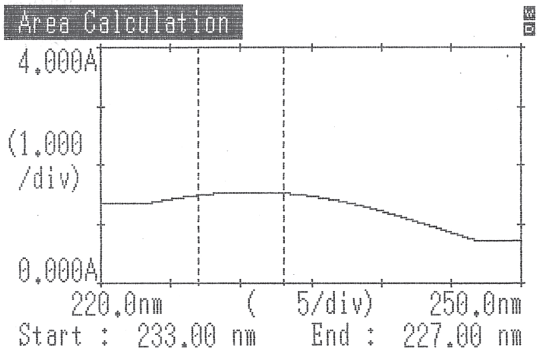


Fig. 6:: Area under curve of Glimepiride 35 µg /ml.

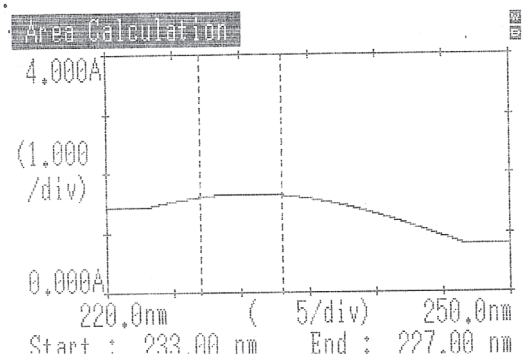


Fig. 7:: Area under curve of Glimepiride 40 µg /ml

made with methanol to obtain working standard solution of Glimepiride 40µg/ml. This concentration was scanned between wavelengths of 233nm to 227nm.

Sample: Five tablet contents of brand GLIMISTAR Ò-1 containing 1 mg of Glimepiride weighed and finally powered with the help of mortar. Each uncoated tablet contains 1 mg of Glimepiride. A quantity of powder sample of equivalent to 5 mg of Glimepiride was taken into volumetric flask. Dilutions were made to get concentration of 40µg/ml. These concentrations were scanned between of 233nm to 227nm. (Table 3)

B. Accuracy (Recovery Study) : Recovery experiments were used for study of accuracy of the method. This study was carried out by adding known amount of bulk sample to tablet and recovery was performed at three levels, 80, 100 and 120% of Glimepiride standard concentration. Samples for recovery studies were prepared according to before mentioned procedure. Three samples were prepared for each recovery level. The solutions of sample were analyzed and %

Table 1: Stability Data for Glimepiride

Sr. No.	Time(min.)	AUC
1	0	9.9838
2	5	9.9888
3	10	9.9995
4	15	10.001
5	20	10.010

Table2: Standard Calibration Table for Glimepiride

Sr. No.	Concentration (µg/ml)	Area Under Curve
1	0	0
2	20	9.9888
3	25	11.657
4	30	13.987
5	35	16.588
6	40	19.698

Vol. 11 (4) 376-381 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

recoveries were calculated by using fallowing formula.

$$\% \text{ Recovery} = \frac{\text{Observed amount of compound in sample}}{\text{Amount of all compound present in sample}} \times 100$$

The recovery values are summarized in following tables 4.

C. Precision : Four independent samples of Glimepiride were used for the evaluation of precision. The intermediate precision (inter day precision) of the method was also evaluated using four different analysts in the same laboratory. The values obtained by four analysts were summarized in table 6.

Results and Discussion

The standard solutions of Glimepiride in Methanol were subjected to scanning under the area from 227nm to 233nm. For the area under curve method Shimadzu 1800 spectronic UV-Visible spectrophotometer was used. The method is also simple, rapid and economical method which gives reproducible results on the instrument used. The reported method is an economical method in which only Methanol solution is used as the solvent and does not require the use of costly reagents.

The calibration curve of Glimepiride was found to be linear at conc. Range 20 to 40 µg/ml at area between 233to 227 nm. There for, it was clear that Glimepiride can be determined in presence of methanol with no intervention of any irrelevant substance in pharmaceutical products. With the intention of determining the practicability of the developed technique for the assessment of commercially available brand (GLIMISTAR Ò-1) of medicinal formulations, the technique was initially

Table 3: Assay for Glimepiride Tablet Formulations

Brand Name	Label Claim (mg/tablet)	Amount Found (mg/tablet)	% of Label Claim	Mean	SD	CV
GLIMISTAR Ò-1	1	1.05	105	101.08	0.0311	0.0308
	1	0.99	99			
	1	0.98	98			
	1	1.03	103			
	1	1.04	104			

Table 4: Accuracy parameters of Glimepiride (Brand Name- GLIMISTAR Ò-1)

Label % recovery	Amount present (mg/tablet)	Amount of Standard added (mg/tablet)	Amount Recovered (mg/tablet)	Total % recovery	%mean recovery	SD	CV
80	1	80	78.32	97.9	98.58	0.7280	0.0073
80	1	80	79.48	99.35			
80	1	80	78.81	98.51			
100	1	100	99.86	99.86	99.86	0.32	0.0032
100	1	100	99.54	99.54			
100	1	100	100.18	100.18			
120	1	120	120.45	100.37	99.98	0.3453	0.0034
120	1	120	119.64	99.7			
120	1	120	119.87	99.89			

Table 5. Determination of Precision of Glimepiride

Sample Number	Assay of Glimepiride as % of Labelled amount(inter – day precision) Analyst 1	Analyst 2	Analyst 3	Analyst 4
1	99.98	100.08	100.15	99.87
2	100.04	99.89	100.78	99.46
3	99.85	100.20	99.67	100.03
4	99.74	100.09	99.88	100.18
Mean	99.90	100.06	100.12	99.88
SD	0.1342	0.1287	0.4818	0.3103
CV	0.0013	0.0012	0.0048	0.0031

attempted on bulk drugs in their synthetic mixture sample as well as concentrations were estimated.

Then the technique was subjected to the assay of in marketed dosage forms and satisfactory results were attained within the appropriate limits as per the content of the label claim for Glimepiride.

The newly developed method was validated as per the international guidelines and parameters. The novel method for the quantitative investigation of Glimepiride was subjected to different validation parameters like specificity and selectivity in presence of formulation additives and excipients, studied for Linearity and range at different levels of concentrations and calibration standards where the determination range was optimized, accuracy was proved by recovery studies at different concentration levels, precision was established through inter day precision studies, where the samples were subjected to changed conditions other than optimized parameters.

Conclusion

From the above experimental studies it is concluded that area under curve method developed for estimation of Glimepiride was suitable for the routine determination of Glimepiride. The proposed method for the selected drug Glimepiride was found to be precise and accurate. The most important striking features of spectrophotometric methods are their rapidity and simplicity.

The newly developed method is alternative to HPLC methods and zero order UV spectrophotometric methods. Results of validation parameters demonstrate that these performed analytical procedures are suitable for its intended purpose and meet the criteria defined in ICHQ2A/B guidelines.

Conflict of Interest: None.

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UV-visible Spectrophotometric Estimation of Montelukast and Fexofenadine by Simultaneous Equation Method in Bulk & Combined Tablet dosage form

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Abstract

A simple, rapid, accurate and precise spectrophotometric method has been developed for simultaneous estimation of Montelukast Sodium and Fexofenadine HCl in Pure and tablet dosage form. Proposed method involves formation of 'simultaneous equations' at 259.60nm for Fexofenadine Hydrochloride and 283.00 nm for Montelukast Sodium using methanol as a solvent. The linearity was observed in the concentration range of 30-120 mg/ml for Fexofenadine HCl and 6-20 mg/ml for Montelukast Sodium. The correlation coefficient was found to be 0.9927 for fexofenadine HCl and 0.9985 for Montelukast Sodium. Thus the proposed method is reproducible which can be suitably applied for the estimation of FEXO &MONT in combined dosage forms. The results of analysis have been validated statistically and by recovery studies.

***Key-Words**:* Fexofenadine HCl (FEXO), Montelukast Sodium (MONT), Simultaneous equation method, spectrophotometry

Introduction

Fexofenadine hydrochloride (FEXO) (Figure 1)(*RS*)-2-[4[1-Hydroxy-4-[4-(hydroxy-diphenyl methyl)-1-piperidyl] butyl]phenyl]-2-methyl-propanoic acid is used to relieve the allergy symptoms of seasonal allergic rhinitis (hay fever), including runny nose; sneezing; and red, itchy, or watery eyes; or itching of the nose, throat, or roof of the mouth in adults (1- 2). It is carboxylic acid metabolite of terfenadine, a nonsedating selective histamine H1 receptor antagonist. This drug contains an asymmetric carbon in its chemical structure and is administered clinically or is used as a *P*-glycoprotein probe as a racemic mixture of *R*-and *S*-enantiomers (3-4).

Montelukast sodium (MONT) (Fig. 2) is chemically (S, E)-2-(1-((1-(3-(2-(7-chloroquinolin-2-yl) vinyl) phenyl)3-(2-(2-hydroxypropan-2-yl)phenyl)propylthio)methyl)cyclopropyl) acetic acid (5) which is a leukotriene receptor antagonist used in the treatment of chronic asthma and allergic rhinitis (6-7).

Literature survey reveals that fexofenadine hydrochloride is estimated individually or in combination with other drugs by UV spectrophotometry (8–10). The aim was present investigation to develop and validate spectroscopic methods (UV- Spectrophotometer) which are Accurate, Sensitive, Precise, and Economical method for simultaneous determination of Fexofenadine Hydrochloride and Montelukast Sodium in bulk drug and pharmaceutical tablet dosage form.

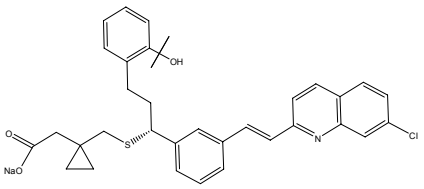

Fig. 1. Structure of Fexofenadine Hydrochloride

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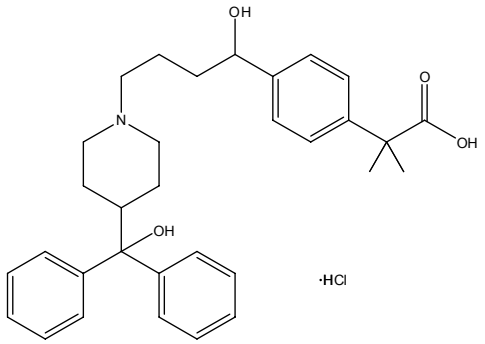

Fig. 2. Structure of Montelukast Sodium

Materials and Methods

Apparatus: A Shimadzu UV -2450 Double beam spectrophotometer has software UV-Probe 2.21 with path lenth 10 mm and variable Slit width was used for the absorbance measurements. All the solutions were freshly prepared using Methanol A.R. Grade.

Preparation of standard stock solutions: Accurately weighed Fexofenadine Hydrochloride and Montelukast Sodium were separately dissolved in sufficient quantity of methanol, then further diluted with methanol to give concentration of 1000µg/ml respectively for Fexofenadine Hydrochloride and Montelukast Sodium. These solutions were used as standard stock solution for the further analysis.

Selection of analytical wavelength: From the sample and standard stock solutions appropriate dilutions of both the drugs were made to obtain final concentration each containing 120 µg/ml of Fexofenadine Hydrochloride and 20 µg/ml of Montelukast Sodium. Solutions of both drugs were scanned in the wavelength range of 200 – 400 nm.

The wavelengths selected should be such that where each wavelength absorptivity difference between two components should be as large as possible. Fexofenadine Hydrochloride shows maximum absorption at wavelength (ëmax) 259.60nm and Montelukast Sodium shows

Reagents and Solutions	
Fexofenadine Hydrochloride	(Gaurav Enterprises, Indore, India),
Montelukast Sodium	(Melody Healthcare Pvt. Ltd., India),
Methanol AR grade	(S.D Fine chem. Ltd, India),
MONTEMAC- FXFexofenadine Hydrochloride, Montelukast Sodium	(Macleods Pharmaceuticals Ltd., India)

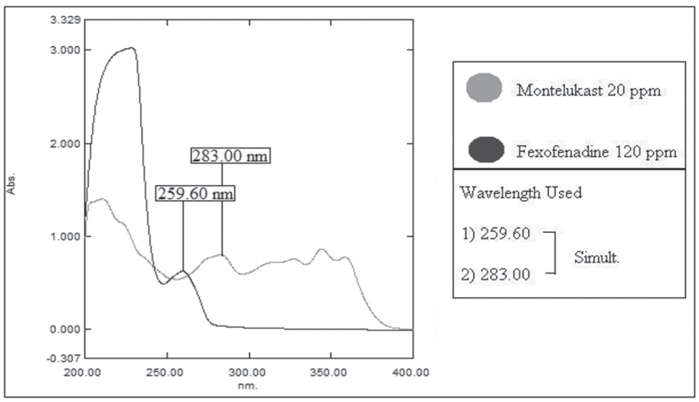

Fig. 3. Overlay spectra of Fexofenadine and Montelukast

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maximum absorption at wavelength (ëmax) 283.00 nm. Hence, the range for AUC method for Fexofenadine Hydrochloride was 259.60 ± 10 and for Montelukast Sodium was 283.00 ± 10 (Fig. 3).

Result and Discussion

Method validation for Simultaneous Linearity study : For each drug appropriate aliquots were pipette out from standard stock solutions into a series of 10 ml volumetric flasks. The volume was made up to the mark with methanol to get a set of solutions. The Absorbance of each of these solutions were measured at the selected wavelength i.e. 259.60nm and 283.00 nm for Fexofenadine and Montelukast respectively and plotted against concentration (Fig. 4-5 and Table-1 and 2). The concentration range over which the drugs obeyed Lambert-Beer's law. The range was found to be 30-120 mg/ml for Fexofenadine and 6-20 mg/ml for Montelukast.

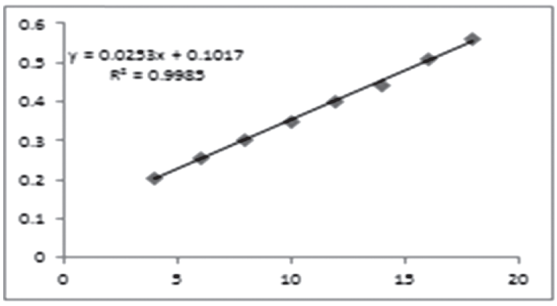

Figure 4: Calibration Curve of Montelukast Sodium

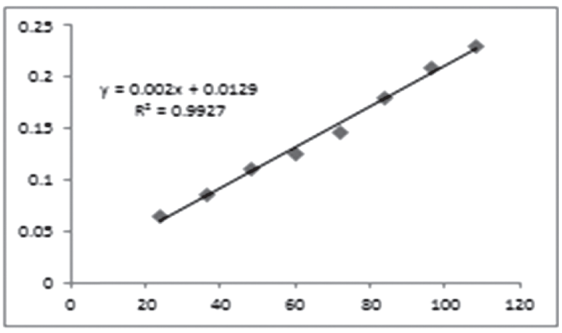

Fig 5. Calibration Curve of Fexofenadine HCl

Determination of absorptivity at analytical wavelength : For each drug appropriate aliquots were pipette out from standard stock solution and a series of dilutions of different concentrations were made for Fexofenadine and Montelukast in the concentration range 30-120 mg/ml for Fexofenadine and 6-20 mg/ml for Montelukast. Absorbances were then divided by concentration gm/L to get absorptivities values. These values are noted in table no.1 and 2.

Precision

Repeatability of method was established by analyzing various replicate standards of Fexofenadine and Montelukast. All the solutions were analyzed thrice, in order to record any intra-day and inter-day variation in the result. % RSD calculated from 3 replicate readings of absorbance values at each concentration confirm the precision of the method. The results of precision are given in Tables 3 and 4.

Analysis of Standard mixture

Standard Mix was made in the ratio of 120:20(FEXO:MONT) from the working stock solution of Fexofenadine and Montelukast (1000 µg/ml), 1.2 ml Fexofenadine and 0.2 ml of Montelukast were taken in 100 ml volumetric flask to Prepare 120 mg/ml of Fexofenadine and 10 mg/ml of Montelukast (Table 5). The standard mixtures prepared were then scanned over the range of UV i.e. 200 to 400nm.The absorbances were measured at selected wavelengths i.e. 259.60 and 283.00 nm. The concentrations of Fexofenadine and Montelukast were calculated by putting the absorbance value in equation.

Amount of each drug was calculated using following formulae,

$$C_X = \frac{A_2\, a_{y1} - A_1\, a_{y2}}{a_{x2}\, a_{y1} - a_{x1}\, a_{y2}}$$

$$C_Y = \frac{A_1\, a_{x2} - A_2\, a_{x1}}{a_{x2}\, a_{y1} - a_{x1}\, a_{y2}}$$

Table 1. Linearity of Montelukast sodium

S. No.	Concentration (µg/ml)	Absorbance of Montelukast sodium at ëmax 259.60nm
1	2.4	0.065
2	3.6	0.086
3	4.8	0.111
4	6.0	0.127
5	7.2	0.148
6	8.4	0.18
7	9.6	0.211
8	10.8	0.231

Table 2: Linearity of fexofenadine

S. No.	Concentration (µg/ml)	Absorbance of fexofe nadine at ëmax 283nm
1	4	0.205
2	6	0.257
3	8	0.302
4	10	0.352
5	12	0.405
6	14	0.446
7	16	0.51
8	18	0.562

Where,

Cx and Cy: - Concentration of FEXO and MONT (gm/L) respectively.

A_1 and A_2: - Absorbances of mixture at 259.60 and 283.00 nm respectively.

ax_1 and ax_2 : - Absoptivity of FEXO at 259.60 and 283.00 nm respectively.

ay_1 and ay_2 : - Absoptivity of MONT 259.60 and 283.00 nm respectively.

Analysis of tablet formulation : Ten tablets were weighed accurately and powdered. Powder equivalent to 120 mg of Fexofenadine & 10 mg Montelukast was weighed and Spiking was done using 10 mg of Montelukast. Whole content was transferred to 100 ml volumetric flask, dissolved in 50 ml of methanol by shaking the flask for 20 min with the help of Sonicator and volume was made up to the mark with methanol. The solution was filtered through Whatmann paper no.41. An aliquot of working stock solution was made by diluting 0.1 ml of standard stock solution to 10ml of methanol to get concentration 120ìg/ml of Fexofenadine and 20ìg/ml of Montelukast. The results of tablet analysis are given in Table 6.

Recovery studies : Recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 80 %, 100 % and 120 %. In recovery study for Fexofenadine amount of standard drug solution added was 48 mg/ml, 60 mg/ml and 72 mg/ml in 80 %, 100 % and 120 % respectively. In recovery study for Montelukast amount of standard drug solution added was 8 mg/ml, 10 mg/ml and 12 mg/ml in 80 %, 100 % and 120 % respectively. The mixed sample solutions were analyzed to get the spectrum, the absorbance measured at 259.60 nm and 283.00 nm and the concentration of each drug was determined using the equations. At each levels of the amount, three determinations were performed. The results for recovery studies are given in Table 7.

Accuracy: The low values of S.D, %RSD, and 95% confidence interval indicate that method is precise. % recovery within limits indicates the non interference from the formulation excipients and confirms the accuracy and precision of the method.

UV spectrophotometric method : The method for the estimation of Fexofenadine and Montelukast was carried out by using a UV-Visible double beam spectropho-tometer (Shimadzu), model no. UV-2450.

Simultaneous Method for estimation of Fexofenadine and Montelukast : The method was found to be simple, accurate, reproducible and rapid, for routine analysis of the formulations.

Table 3. Results of method precision (Intra-day) for simultaneous equation method

Parameters	Standard Deviation		% R.S.D.		Standard Error	
	FEXO	MONT	FEXO	MONT	FEXO	MONT
Low	0.00107	0.006164	0.0122	0.0234	0.0006177	0.00355
Mid	0.00754	0.1177	0.0589	0.3278	0.004353	0.06795
High	0.005431	0.2416	.0293	0.5252	0.003135	0.1394

% R.S.D. = Relative Standard Deviation

Table 4: Results of method precision (Inter-day) for simultaneous equation method

Parameters	**Standard Deviation**		**% R.S.D.**		**Standard Error**	
	FEXO	MONT	FEXO	MONT	FEXO	MONT
Low	0.001326	0.0091	0.01535	0.03432	0.0007656	0.03408
Mid	0.0083267	0.006807	0.06699	0.018925	0.004807	0.003930
High	0.01124	0.00866	0.0615	0.0189	0.00648	0.005

% R.S.D. = Relative Standard Deviation

Table 5. Results of standard mixture analysis (no of determinations = 3)

Parameters	**Components**	
	Fexofenadine	**Montelukast**
Conc.of drug (µg/ml)	120	10
Drug content % ± SEM	101.26±0.3692	100.18±1.027
%RSD	0.6337	1.81135

RSD= relative standard deviation, SEM – Standard error of mean.

Table 6. Results of commercial formulation analysis (no of determinations = 3) for simultaneous determination method

S. No.	**Labeled Claim(mg)**		**Amount Found (ig/ml)**		**% of Labeled Claim**	
	FEXO	MONT	FEXO	MONT	FEXO	MONT
1.	120	10	119.5	9.91	99.58	99.10
2.	120	10	119.8	9.95	99.83	99.5
3.	120	10	119.2	10.03	99.33	100.3
Mean					99.58	99.6
±Standard Deviation					0.25	0.06123
%RSD					0.00251	0.006144
±SEM					0.1443	0.3536

% R.S.D. - Relative Standard Deviation ; SEM - Standard error mean

The reproducibility, repeatability and accuracy of the method were found to be good which is evidenced by low values of standard deviation and percent relative standard deviation. The standard error at 95% confidence level 0.001357 & 0.001798 for Fexofenadine and Montelukast respectively shows the precision of the method. The percent recovery obtained indicates non-interference from the excipients used in the formulation. High Molar absorptivity 28047.002 & 2981.43 for Fexofenadine and Montelukast respectively and low Sandell's sensitivity 0.0173862 & 0.219023 for Fexofenadine and Montelukast respectively for the method reveals that the method is highly

Table 7. Recovery studies of Fexofenadine and Montelukast for Simultaneous estimation method (no of determinations = 3)

Level of % Recovery	**% Recovery***		**% RSD**		**±Standard Error**	
	FEXO	MONT	FEXO	MONT	FEXO	MONT
80	101.3	99.08	0.0002	0.0015	0.01200	0.0869515
100	101.4	102.56	0.0002	0.0062	0.01154	0.3622402
120	99.33	99.79	0.0005	0.0057	0.300231	0.3338337

% R.S.D. - Relative Standard Deviation

Table 8. Simultaneous Estimation of Fexofenadine HCl

S. No	Concentration (µg/ml)	259.60 nm	283.00 nm	Absorptivities	
				259.60 nm	283.00 nm
1.	36	0.086	0.05	23.89	13.89
2.	48	0.111	0.054	23.125	11.25
3.	60	0.127	0.057	21.16	9.5
4.	72	0.148	0.06	20.55	8.33
5.	84	0.18	0.065	21.42	7.73

$Ay_1 = 22.029$ $Ay_2 = 10.14$

Table 9. Simultaneous Estimation of Montelukast Sodium

S. No	Concentration (µg/ml)	283.00 nm	259.60 nm	Absorptivities	
				283.00 nm	259.60 nm
1.	6	0.284	0.257	473.5	428.33
2.	8	0.402	0.302	502.2	377.5
3.	10	0.48	0.352	480	352
4.	12	0.564	0.405	470	337.5
5.	14	0.636	0.446	454.2	318.5

$Ax_1 = 475.98$ $Ax_2 = 362.766$

sensitive. The LOD 5.94 & 2.023 for Fexofenadine and Montelukast respectively and LOQ 18 & 0.6678 for Fexofenadine and Montelukast respectively. Range found between 24-120 µg/ml for Fexofenadine and 6-20 µg/ml for Montelukast with correlation coefficient and respectively. Hence, this method can be successfully applied for the estimation of Fexofenadine and Montelukast in pharmaceutical formulation (Table 8 and 9).

Conclusion

The methods have wider linear range with good accuracy and precision. Hence, the data presented in the manuscript by spectrophotometry thus can be extended for routine analysis of Fexofenadine and Montelukast succinate in pharmaceutical industries, hospitals and research laboratories. Methods were validated as per regulatory guidelines. The results of recovery studies, LOD and LOQ for each of the methods were found to be satisfactory. Intra and inter-day variation of UV method was well within limit with % RSD < 2 %. Statistical analysis of all the method was done according to one-way ANOVA.

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Production, Characterization and Evaluation of thrombolytic activity of Staphylokinase of *Staphylococcus hominis*

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Abstract

Staphylokinase isolated from *Staphylococcus hominis* has therapeutic function to dissolve the blood clots. *Staphylococcus hominis* was isolated from curd. *Staphylococcus* spp was confirmed by morphological, biochemical and molecular techniques such as 16s rDNA sequencing. Satoh's medium was used for the production Staphylokinase. Cells were separated from culture broth by centrifugation and the supernatant fluid was added to 3 volume of acetone. After centrifugation of the mixture, the resultant precipitate was purified by ion exchange column chromatography (DEAE Cellulose). Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) was done by using 10 to 20% gradient polyacrylamide gel and a 4% stacking gel at 4°C. The purity of the Staphylokinase was determined by SDS-PAGE and HPLC. Finally the thrombolytic activity of Staphylokinase was evaluated by Radial Caseinolytic assay and Heated Plasma agar assay. It was suggested that 0.18 of enzyme is sufficient to dissolve the blood clot.

Key words: Staphylokinase, *Staphylococcus hominis*, SDS-PAGE, Caseinolytic, blood clot.

Introduction

Thrombolytic disorders have emerged to be one of the main causes of human mortality worldwide (1). A blood clot (thrombus) developed in the circulatory system can cause vascular blockage leading to life threatening consequences. A healthy homeostatic system suppresses the development of such blood clots in normal circulation. However, reacts extensively during vascular injury to prevent blood loss (2). The failure of the system to produce the bodily clot lysine such as tissue plasminogen activator (t-PA) and Urokinase, leads to stroke, pulmonary embolism, deep vein thrombosis and acute myocardial pathologies. The clinical intervention to cure these disorders is carried out by the external administration of thrombolytic agents (3).

Staphylokinase is a bacterially derived protein that has been used effectively as a plasminogen activator (4). Staphylokinase could be relatively inexpensive when compared to that of other thrombolytic agents and scaled up into large amounts for industrial production (5). Apparently, the only limitation with this thrombolysis is its bacterial origin that could raise undesired immune responses. Considering a thrombolytic agent for industrial scale development, the present work is undertaken to isolate the mature Staphylokinase from a new source.

Staphylococcus hominis is a coagulate-negative member of the bacterial genus of

Staphylococcus, consisting of Gram-positive, spherical cells in pairs or tetrads. *S. hominis* tends to colonize in areas with numerous porcine glands, such as axillae and the pubic region.

Materials and Methods

***Sample preparation*:** Staphylokinase producing *Staphylococcus* spp was isolated from curd and was mixed with the mixture of dall and fermented curd and it was allowed for incubation for 24 hours.

***Bacterial isolation and sub culturing*:** Serially diluted fermented sample were inoculated by spread plate method over nutrient agar and allowed for incubation at 35°c for 24 hours. Each single strain was isolated by repeated streaking on nutrient agar medium and blood agar medium. The individual colonies were characterized and ensured the purity. Three bacteria were isolated and isolates were maintained on nutrient agar and blood agar plates.

Screening of Staphylokinase producing *Staphylococcus spp*.

***Screening by heated plasma agar plate** (6):* The isolated samples were analyzed for Staphylokinase production by heated plasma agar plate assay.

***Identification*:** The following tests were carried out to identify the bacteria. Gram staining, spore staining, motility, haemolysis test, antibiotic sensitivity test, Oxidase test, Catalase test, carbohydrate fermentation test and MRVP test.

Molecular screening by 16s rDNA sequencing: By 16s rRNA sequencing, the bacterial isolates were identified. The genomic DNA was extracted from pure culture and amplified by Polymerase Chain Reaction (PCR) by using *Taq* DNA polymerase, the universal primers forward (i.e. 5'-AGAGTTTGATCMTGGCTCAG) and reverse (i.e. 5'-AAGGAGGTGWTCCARCC).

***Culture Conditions*:** Nutrient broth and solid medium were used for propagation of *Staphylococcal* strains. Blood agar medium (contains a base similar to nutrient agar) was added with 5% of human blood cells and used for discriminating the collected microbes.

***Enzyme production*:** *Staphylococcus hominis* was grown on Satoh's medium (7). One ml of uniformly prepared suspension of *staphylococcus hominis* was inoculated and incubated at 35°C and 150 rpm in an orbital shaker. Cells were removed at 48th hour by centrifugation and supernatant was collected.

Enzyme assay and characterization

***Enzyme assay*:** Fibrinolytic activity of crude enzyme was determined by heated plasma agar assay (8), Radial caseinolytic assay: It includes Caseinolytic Agar [5] and Skim Milk Agar.

***Enzyme purification*:** Collected supernatant was added to 3 volumes of acetone which was allowed to stand at 4°C for 1 day. Later the mixture was centrifuged at 10,000rpm for 15 min and the resultant precipitate was purified by ion exchange column chromatography (DEAE Cellulose) followed by Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis (SDS-PAGE) with 20% gradient polyacrylamide gel and 4% stacking gel at 4°C.

***Ammonium salt precipitation for purification of enzyme** (7):* The Staphylokinase enzyme was also purified by ammonium sulfate saturation. The fraction of the protein was precipitated with 85% ammonium sulfate. Ammonium sulfate was found to enact the fibrinolytic movement after dialysis. Staphylokinase enzyme was partially purified by using anion exchange column chromatography (DEAE Cellulose, MERK) and affinity chromatography (8).

Protein Estimation: The purity of Staphylokinase was confirmed by SDS-PAGE. Protein expressions were analyzed by running on 15% SDS-PAGE. 40 ml of separating gel and 10 ml of stacking gel was prepared to run 15% SDS-PAGE. Low molecular protein marker was used here. HPLC was also carried out to check the purity of the enzyme.

***Estimation of protein by Lowry's Method** (9):* The relative activity and quantitative estimation of

fibrinolytic enzymes were estimated by Lowry's method spectrophotometrically at 600 nm. BSA was used as standard.

Modified Holmstorm Method *(10):* This is one of the important methods to test the thrombolytic activity of the enzymes. In this method we used the purified enzyme. 1ml of human blood sample was taken in eppendroff tube and allowed the blood to clot. After the blood clot completely, purified enzyme were added at a concentration of 10 to 100 µl. 10, 20, 40, 60, 80, 100 of purified enzyme were added. After that we have observed the time taken by the enzyme to liquefy the clots. Then we have calculated Enzyme units utilized (units/ml of clotted blood).

Results and Discussions

Bacterial isolation and Sub Culturing**:** The three isolates were named as MS1, MS2 and MS3 and sub cultured on nutrient agar and blood agar to screen for production of staphylokinase. Colonies on the plate, Gram staining and pure culture on nutrient agar and blood agar plates are shown in figure 1-4.

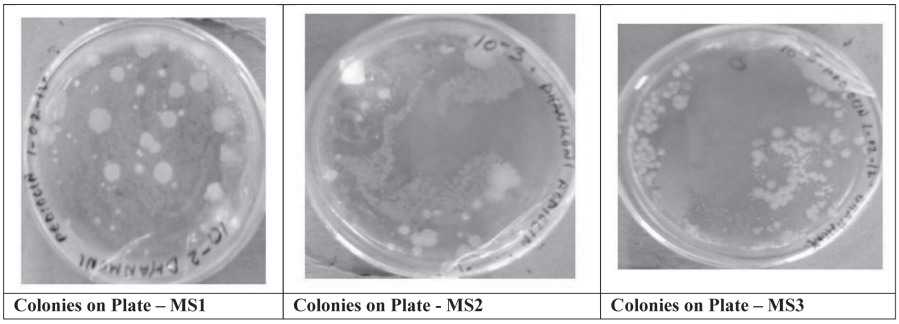

Fig. 1. Cultural characteristics of isolates

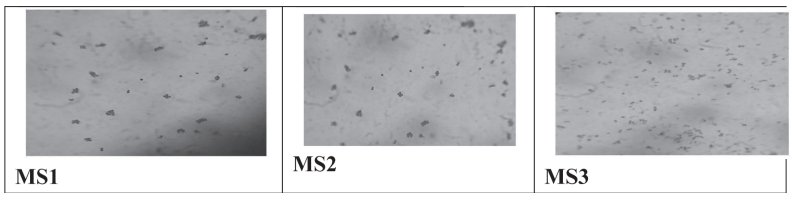

Fig. 2. Gram's staining

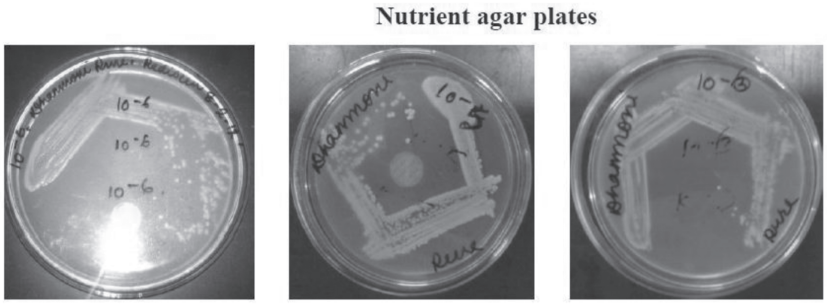

Fig. 3. Pure culture of MS1, MS2 and MS3 from left to right

Screening of Staphylokinase Producing *Staphylococcus* spp: The halo zones around the colonies indicate the positive result for this test. Out of the three strains isolated two have shown positive outcome.

Haemolysis test: MS3 was found to be non hemolytic remaining two were hemolytic (Fig. 4).

Further exploration and confirmation of thrombolytic activity of Staphylokinase, casein hydrolytic and skim milk agar assays were carried out and out of three strains, two strains showed positive results (fig 5 & 6). These two positive isolates were further experimented.

***Molecular screening by 16s rDNA Sequencing*:** Outcome of DNA sequencing of two

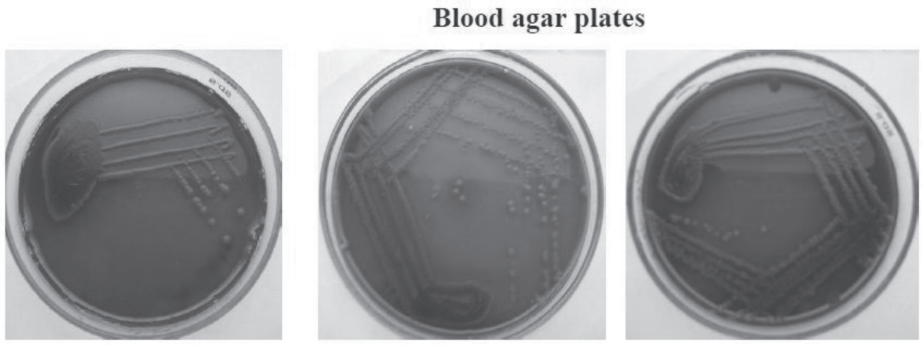

Fig. 4. Pure culture of MS1, MS2 and MS3 from left to right

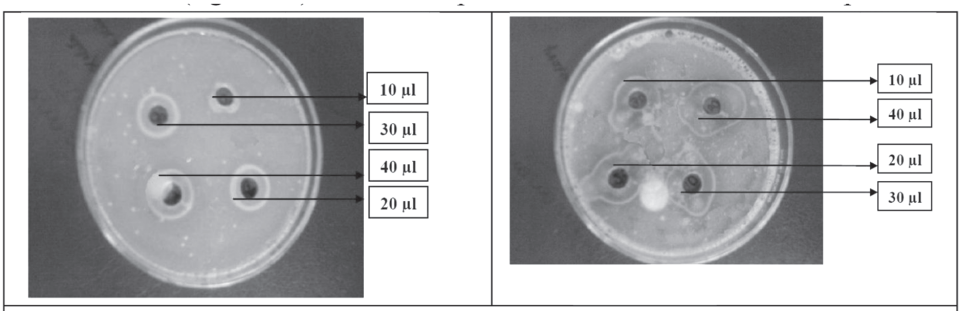

Fig. 5. Caseinolytic Agar Assay of MS1 and MS2

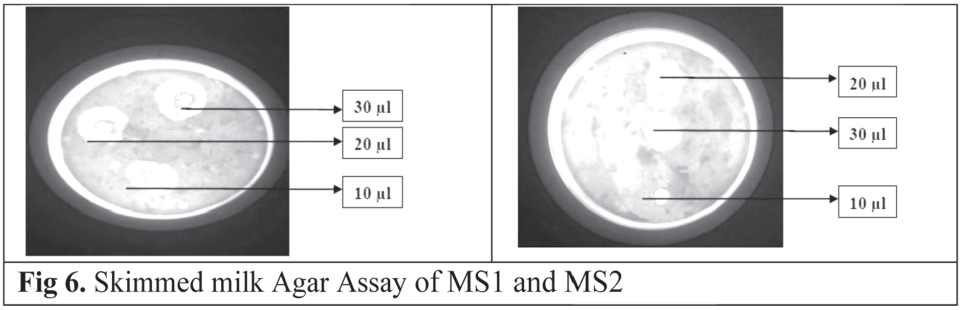

Fig 6. Skimmed milk Agar Assay of MS1 and MS2

isolates MS1 and MS2 confirmed that the identified organism as *Staphylococcus hominis* and further compared with NCBI gene library and determined closest homologous.

Enzyme Production **(**11): Satoh's medium was prepared for the production of enzyme. It was prepared by mixing of 1% nutrient broth (Difco Laboratories, Detroit, Mich.), 0.3% yeast extracts (Difco), 0.5% sodium chloride, and 1% glycerol with pH adjusted to 7.4. The pH was adjusted to 7.4 with 2 M acetic acid and 2 M NaOH. Medium was sterilized by autoclaving at 121°C for 35 min and cooled to room temperature. One ml of uniformly prepared suspension of *staphylococcus hominis* was used as an inoculum; incubated at 35°C and 150 rpm in an orbital shaker. After 2 days of fermentation, cells were removed by centrifugation.

Enzyme assay and characterization *:* Fibrinolytic activity was determined by the following methods. i) Heated plasma agar assay, ii) Radial caseinolytic assay: It included a) Caseinolytic Agar and b) Skim Milk Agar

Heated Plasma agar assay (11): The isolated samples were analyzed for Staphylokinase production by heated plasma agar assay method. 10, 20, 40µl of enzyme sample was added into the wells and the plates were incubated at 37°C. After incubation, halo zones around the wells were observed over night which indicated the positive result for this test. Diameter of the halo around the well was measured to check the functional activity of the proteins fig. 7.

Enzyme Purification

Ammonium Sulphate Precipitation Method**:** The enzyme staphylokinase was purified by ammonium sulphate precipitation method by using dialysis membrane. Ion exchange chromatography and affinity chromatography were used to purify the enzyme. After purification by these two chromatographic techniques, sample was collected in fractions of 1 ml. UV-VIS spectroscopic reading was taken to know the protein concentration.

Protein Estimation

SDS-PAGE**:** Protein expressions were analyzed by running on 15% SDS-PAGE and a very clear 15.5 KD and 15 KD protein band was identified against a low molecular weight protein marker (Fig. 8).

HPLC**:** The purity of the Staphylokinase was determined by HPLC and the retention time of the enzyme was 5.41 (Fig. 9) (12).

Modified Holmstrorm Method (13) : The enzyme was added at a concentration of 10 to 100µl and

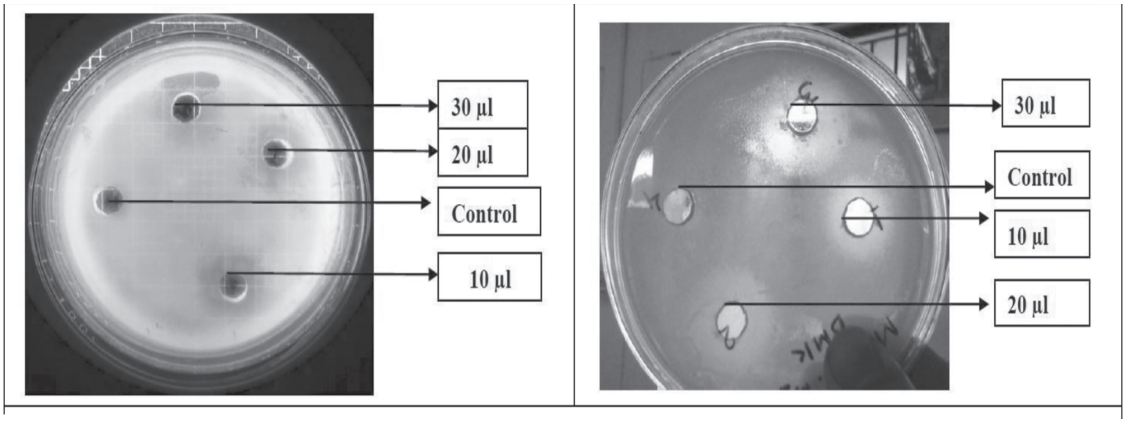

Fig. 7. Heated Plasma Agar Assay of MS1 and MS2

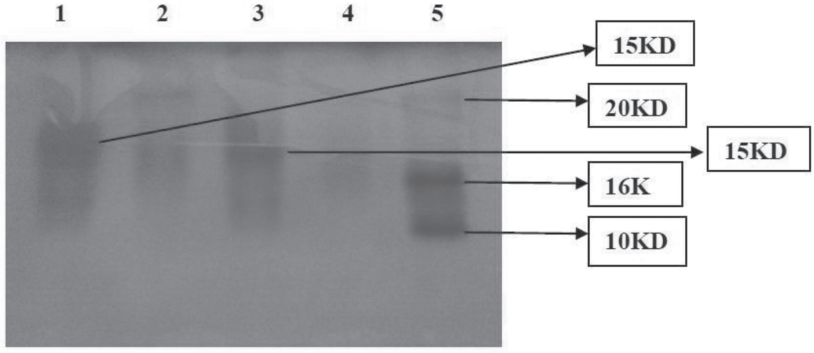

Fig. 8. SDS-PAGE of protein fractions collected from chromatography
1. Fraction 1, **2.** Fraction 2, **3.** Fraction 3, **4.** Fraction 4 and **5.** Protein marker

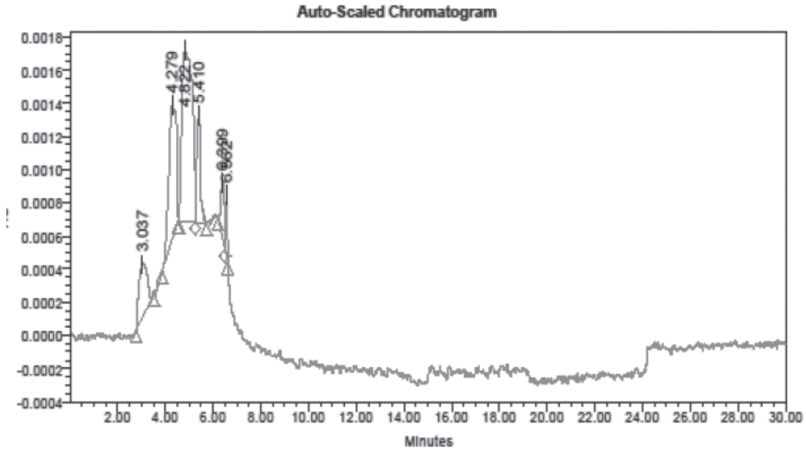

Fig. 9. HPLC of Staphylokinase extracted from Staphylococcus hominis

the enzyme concentration 10, 20, 40, 80, 100 showed positive results to liquefy the clots. The time taken by the enzyme to liquefy the clots was 20 to 30 minutes. Dubey *et al.* (14) announced that 55 µl of protein created by β-haemolytic streptococci is required to break down or lyse the coagulation. However here we have better outcomes similarly that of 10 µl of catalyst somewhat lysed the coagulations (14). But in between 40 to 60 µl the clot was lysed completely by the enzyme (Fig. 10).

Conclusion

Previously Dubey *et al.* (14) reported that 55il of enzyme was required for complete lysis of blood clot. But with reference to our study, some better results compared to that. The results of modified Holmstrorm method concluded that at very low concentration like 10, 20 and 40 µl of enzyme or 0.18, 0.36, 0.72 unit of enzyme could

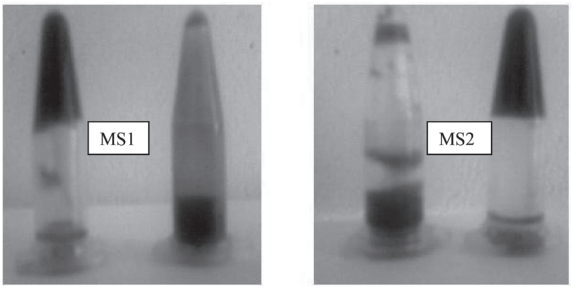

Fig. 10. Blood liquefied by staphylokinase produced by *Staphylococcus hominis (*MS1 and MS2)

lyse the clots. 10 µl of enzyme can partially lyse the clot, but in between 40 to 60 µl we observed complete lysis of the blood clot.

Acknowledgement

We express our sincere gratitude to Dr M R Hulinaykar, Honorable Managing Trustee, Sri Shridevi Charitable Trust, Tumakuru, Karnataka, India and Dr Gurulingappa M Patil, Principal, Shridevi Institute of Engineering and Technology, Sira Road, Tumkur, Karnataka, India for their support, encouragement and extending all the necessary facilities in the college for the successful completion of this project.

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Engineering Rice for Abiotic Stress Tolerance: A Review

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Abstract

Rice (*Oryza sativa* L.) is widely grown as a major staple food crop providing calories for about half of the world's human population and is grown on 160 million hectares worldwide. It is being cultivated in India and China for several thousands of years. A significant yield loss in this crop is due to various biotic and abiotic stresses including insect pests, diseases, drought, salinity, adverse temperature and submergence. Development of abiotic stress tolerant rice genotypes with high grain yield is the major objective now in rice breeding and genomic research. Despite many concerted efforts all over the world, conventional breeding approaches are resulting in slow progress in developing abiotic stress tolerant rice genotypes. One of the approaches to rectify this is to introduce genes of interest that confer tolerance to abiotic stress *via* genetic engineering methods. Besides, with the availability of high quality rice genome sequence (IRGSP, 2005), there has been rapid accumulation of functional genomic resources including many known cloned and characterized genes particularly for abiotic stress tolerance, genes/full-length cDNA from global expression profiles and resequences of the rice genotypes. The identified genes are successfully transferred into rice to produce transgenics with promising traits. Transgenics are now being evaluated under field conditions in different countries. The objective of this review is to provide an overview of recent developments on the production of various transgenic lines in rice that are highly promising for abiotic stress tolerance.

Keywords : Genetic engineering, Rice, Abiotic stress

Introduction

Rice is the most important cereal crop, leading as food crop for more than half of the world population (IRRI, 2006). The global rice area cultivation has increased from about 129 million hectares in 1968 to about 159.4 million hectares in 2010. India has the largest farm area under rice production in 2009 at 44 million hectares. China and India are the largest rice producers followed by Indonesia, Bangladesh, Vietnam, Myanmar and Thailand.

Abiotic stress has a negative impact on plant growth and productivity. It leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (10). Generally, plants are able to cope with moderate levels of stress. However, when the stress exceeds the threshold level, the physiological mechanism imparting tolerance breaks down causing death of the plants. Among abiotic stresses, drought, cold and salinity, are the most pervasive that limit the yields of crop plants including rice. Understanding the mechanism of stress tolerance that involves a plethora of genes involved in stress signaling network is vital for

crop improvement (Fig. 1). Since large portions of rice-growing areas are affected by abiotic stress conditions, it would be difficult to meet the future food demands of ever increasing world population (11). Efforts involving conventional breeding methods for improving traits that confer tolerance to the abiotic stresses have met with limited success (12). Therefore, to meet the food demands of the growing world population, conventional breeding methods need to be combined with tools such as molecular markers and genomics. Several biotechnological approaches are adopted to increase quality and quantity of rice as well as its resistance to pests, diseases and environmental stresses (13). Ye et al. (14) produced transgenic golden rice with high source of provitamin-A (â-carotene) by manipulating the biosynthetic pathway using tissue specific promoters. They introduced three genes, *phytoene synthase*, *lycopean cyclase* and *phytoene desaturase* (2 genes

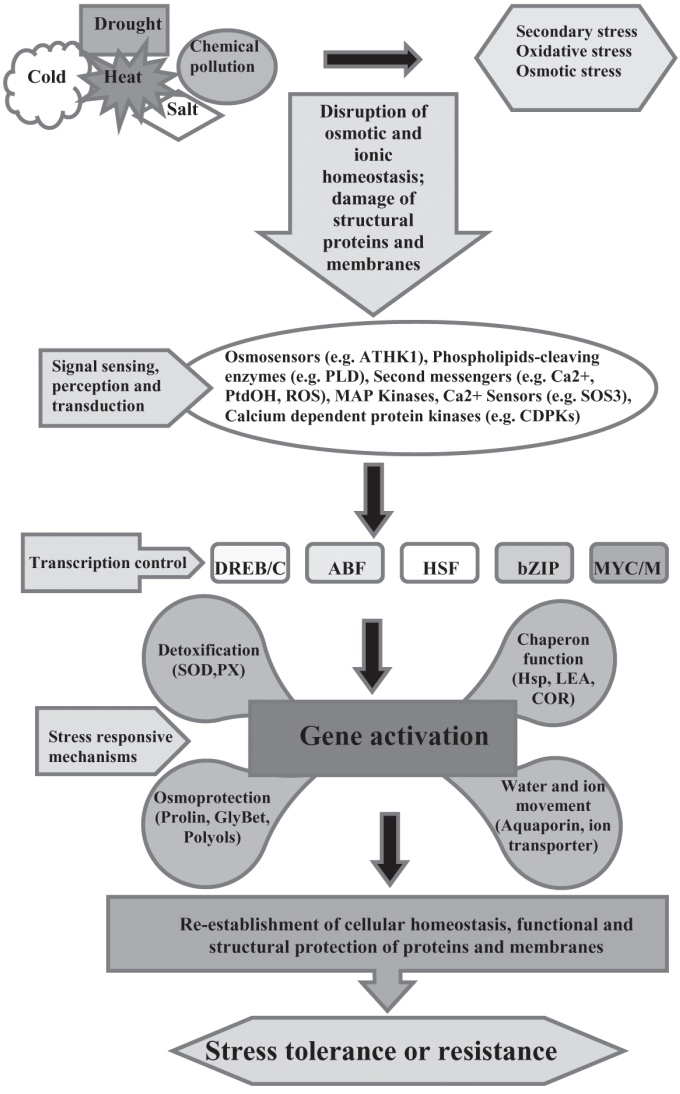

Fig. 1. Schematic representation of developing abiotic stress tolerant plants

from Daffodil and 1 gene *Erwinia uredovora* respectively) and expressed them in the endosperm. The development of recombinant DNA technology allowed the investigators a deeper understanding of transcriptional regulation of genes and facilitated overproduction of endogenous or foreign proteins in plants, besides unraveling the biochemical and molecular processes. A large body of literature on genetic engineering of rice is now available. An attempt was made here only to review the literature concerned with abiotic stress.

Genetic engineering for abiotic stress tolerance : The strategies for plant genetic engineering for abiotic stress tolerance rely on the expression of genes that are involved in signaling and regulatory pathways or genes that encode proteins conferring stress tolerance or enzymes present in pathways leading to the synthesis of functional and structural metabolites. Many genes and gene products have been identified which get induced upon exposure of plants to various abiotic stresses. Therefore, stress inducible genes have been utilized to improve stress tolerance through gene transfer. Although gene transformation in *japonica* rice is performed routinely in several laboratories, transformation in *indica* rice is comparatively difficult. Therefore, relatively large number of transgenic plants must be developed in *indica* species in order to select desirable transformants as well as to study the expression of introduced genes. Since the last two decades, a large number of genes were isolated and cloned which are involved in signal transduction, transcription regulation, ion transporters and metabolic pathways. Recently, some genes of calcium- signal and nucleic acid pathways have been reported to be up-regulated in response to both cold and salinity stresses indicating the presence of cross talk between these pathways. The role of calcium as an important signaling molecule in response to various stress signals has also been reported (16).

Genes for abiotic stress tolerance

Transcription factors : Genes that have been utilized for transformation of rice till date are listed in the Table 1. Plant stress response is regulated by multiple signaling pathways that activate gene transcription and its downstream machinery. Transcriptome analyses using microarray technology, together with conventional approaches have revealed that dozens of transcription factors (TFs) are involved in plant response to abiotic stress. *Arabidopsis* dedicates about 5.9% of its genome coding for more than 1,500 TFs (18).

Osmolyte biosynthetic genes : Osmolytes are synthesized in response to osmotic stress and do not interfere with normal cellular biochemical reactions. There are several examples of accumulation of osmolytes contributing to the relatively high water content necessary for growth and cellular metabolism (57). Osmolytes include proline, sugars (fructans and trehalose), polyols (mannitol and D-ononitol), quaternary ammonium compounds (glycine betaine) and tertiary sulfonium compounds. Trehalose is a nonreducing disaccharide of glucose that functions as a compatible solute in the stabilization of biological structures under abiotic stress in bacteria, fungi, and invertebrates. These findings demonstrated the feasibility of engineering rice for increased tolerance to abiotic stress and enhanced productivity through tissue specific or stress dependent overproduction of trehalose. *OsTPP*1 and *OsTPP*2 are two major *trehalose*-6-*phosphate phosphatase* genes over-expressed in vegetative tissues of rice that transiently induced tolerance in transgenic rice in response to chilling and other abiotic stresses (9). Accumulation of trehalose in transgenic *indica* rice using bifunctional fusion enzyme of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase of *E. coli* has resulted in osmoregulation, removal of free radicals and stabilization of the hydrated structure of proteins to maintain membrane integrity and protein stability under various stress conditions (39). Trehalose helps in maintaining individual cell structure and functions during severe environmental stress conditions. It affects sugar metabolism and imparts osmoprotection. The enzyme arginine decarboxylase (adc), associated with putrescine biosynthesis is involved in minimal chlorophyll loss under salt stress in *O. sativa*. Accumulation of proline in dehydrated plants is caused both by activation of biosynthetic pathway enzymes and by inhibition of its degradation. It has been

demonstrated that overproduction of proline results in increased tolerance to salinity in transgenic rice (40).

Heat shock proteins : Heat shock proteins (Hsps) and molecular chaperones, as well as late embryogenesis abundant (LEA) protein families, are involved in abiotic stress tolerance (41). Hsps and LEA proteins help protect against stress by controlling the proper folding and conformation of both structural (i.e. cell membrane) and functional (i.e. enzymes) proteins. Overexpression of *HSP101* from *Arabidopsis* in rice plants resulted in significant improvement in growth performance during recovery from heat stress (24). Overexpression of LEA proteins was correlated in several cases with desiccation tolerance, although the actual function of these proteins is still unknown (42). Recently, overexpression of *HVA1*, a group 3 LEA protein isolated from barley (*Hordeum vulgare* L.), conferred dehydration tolerance in transgenic rice (43).

Detoxification genes : During stress, electrons that have a high energy state are transferred to molecular oxygen ($^1O2$) to form reactive oxygen species (ROS) (44). ROS, such as singlet oxygen ($^1O2$), superoxide ions (O2) and peroxides, the most widely distributed being hydrogen peroxide (H2O2) are toxic molecules (45). ROS target high molecular mass molecules, such as membrane lipids or mitochondrial DNA. The toxic effects of ROS in plants are counteracted by inducing enzymatic as well as non-enzymatic antioxidative system such as: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbic acid (AsA), tocopherol, glutathione, phenolic compounds etc. Chloroplast transformation of tobacco with *E. coli catalase* gene (*kat E* gene) with tomato rbcS3C promoter efficiently improved plant resistance to photo-oxidation caused by drought stress at high light intensity, despite the inactivation of APX in the chloroplast (46). Same gene *kat E* when over expressed under CaMV 35S promoter in *japonica* rice, conferred tolerance to 250 mM NaCl and enhanced the catalase activity to 1.5 to 2.5 times more than non-transgenic plants (47). Overexpression of *glyoxalase I* (*gly I*) and *glyoxalase II* (*gly II*) genes together have conferred improved salinity tolerance in transgenic tobacco plants (35) and thus offered another effective strategy for manipulating stress tolerance in crop plants. These findings further have established the potential of manipulation of the *glyoxalase* pathway involving enzymes as a probable candidate gene for increased salinity tolerance (NaCl) without affecting yield in crop plants. The *glyoxalase* II cDNA cloned from rice (*Osgly* II) encoding a polypeptide of 336 amino acids was overexpressed in rice that displayed tolerance to various abiotic stresses (48). Thus, the multi-stress response of *Osgly* II gene documents its future utility in developing tolerance to various stresses in crop plants.

Engineering transcription factors for abiotic stress tolerance : Transcription factors (TFs) are proteins that act together with other transcriptional regulators, including chromatin remodeling/modifying proteins, to employ or obstruct RNA polymerases to the DNA template (49). TFs interact with *cis*-elements in the promoter regions of several stress-related genes and thus up-regulate the expression of many downstream genes resulting in imparting abiotic stress tolerance (50). Transcriptome data in *Arabidopsis* and in numerous other plants suggest that there are several pathways that independently respond to environmental stresses (in both ABA dependent- and independent- manner), suggesting that stress tolerance or susceptibility is controlled at the transcriptional level by an extremely intricate gene regulatory network (Fig. 2) (51, 52). Few of the TFs and their utility in engineering stress tolerance for crop improvement programs are given in Table-2 (53 - 77).

ABA dependent : The phytohormone ABA is the central regulator of abiotic stress particularly drought resistance in plants, and coordinates a complex gene regulatory network enabling plants to cope with decreased water availability (78, 79). ABA-dependent signaling systems have been illustrated as pathways that mediate stress adaptation by induction of at least two separate regulons (a group of genes controlled by a certain TF): (1) the AREB/ABF (ABA-responsive element-binding protein/ ABA-binding factor) regulon; and (2) the MYC

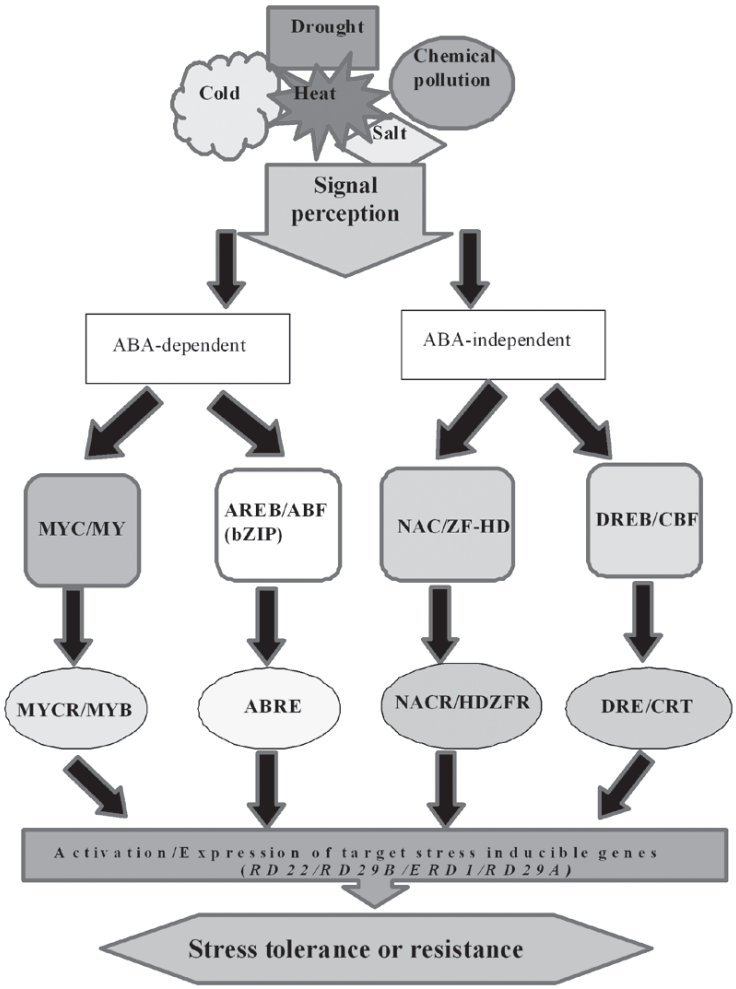

Fig. 2. A schematic representation of transcriptional regulatory networks of cis-acting elements and transcription factors involved in abiotic-stress-responses. Transcription factors are shown in boxes cis-acting elements are shown in ellipses and target stress inducible genes are shown in long rectangular box at the bottom.

(myelocytomatosis oncogene)/MYB (myeloblasto sis oncogene) regulon (80). The AREB or ABFs are bZIP (basic leucine zipper) TFs that bind to the ABRE motif and activate ABA-dependent gene expressions. They were first isolated in a yeast one-hybrid screening (81). A conserved *cis*-element named as ABA-responsive element (ABRE; PyACGTGG/TC) was identified from the promoters of ABA-inducible genes (82). Subsequently, it was revealed that ABA-responsive gene expression needs multiple ABREs or the combination of an ABRE with a coupling element (CE) as a functional promoter (83). Both in the ABA-deficient aba2 and in ABA-overexpression, improved salinity tolerance was recorded in rice (84). Overexpressing *OsbZIP23,* a member of AREB/ABF subfamily significantly improved drought and high salinity resistance in transgenic rice at the reproductive stage 47). Enhanced tolerance to drought and heat was observed in *35SOsAREB1* transgenic *Arabidopsis* plants (55).

ABA independent regulons : ABA-independent well known regulons are: (1) NAC (NAM, ATAF and CUC) and ZF-HD (zinc-finger homeodomain) regulon and (2) CBF/DREB regulon. However, in addition, several studies have identified the existence of both ABA-dependent and independent pathways of stress response that function through AP2/EREBP (ERF) family members (85).

1.The NAC (NAM, ATAF and CUC) and ZF-HD (zinc-finger homeodomain) regulon : The NAC family of plant-specific TFs is one of the largest in the plant genome, with 106 and 149 members in Arabidopsis and rice, respectively (86, 87). NAC family TFs contains a highly conserved N-terminal DNA-binding domain and a diversified C-terminal domain (88). A rice NAC gene, *ONAC045* was induced by drought, high salt, low temperature, and ABA treatment in leaves and roots (106). The *SNAC1* overexpressing in rice seedlings showed significantly higher survival rate than wild type under drought treatment and significantly enhanced salinity tolerance as well (70). A rice *R2R3-MYB* gene (*UGS5)* containing putative NACRS in the promoter region was also induced in the *SNAC1*-overexpressing plants (70). Many abiotic and biotic stress responsive genes were upregulated in the *OsNAC6* transgenic plants, and the transgenics were tolerant to dehydration, and high salt stresses (71). Similarly, *ONAC045* overexpressing rice plants showed enhanced tolerance to drought and salt treatments (72). Hence, NAC TFs play an indispensable role in physiological adaptation for successful plant development under abiotic stress conditions.

2. CBF/DREB regulon : The dehydration responsive element binding proteins (DREBs) are important APETALA type (AP2/ERF) TFs that induce a set of abiotic stress-related genes, thus imparting stress tolerance to plants. These play an important role in the ABA-independent pathways that activate stress responsive genes. *OsDREB1A* and *OsDREB1B* were induced early (within 40 min) after cold exposure but not on ABA treatment. *OsDREB1A* was induced within 5 h of salinity stress whereas *OsDREB1C* showed constitutive expression (89). A detailed study of all five rice *OsDREB2s* showed that *OsDREB2A* expressed to the highest levels under the control condition, and its expression was increased to some extent by high temperature, drought and high salinity, but not by low temperature treatments. Expression of *OsDREB2B* was markedly increased after 20 min of high and 24 h of low temperature stress. While the transcript levels of *OsDREB2C, OsDREB2E* and *OsABI4* were low under the control condition and were transiently induced by the abiotic stresses (67). Transgenic *Arabidopsis* and rice plants over expressing *OsDREB1A* displayed tolerance to low temperatures, high salinity and drought (90). Likewise, the constitutively over expressing *CBF3/DREB1A* and *ABF3* transgenic rice showed better drought and salinity tolerance without any growth inhibition or phenotypic anomalies (59). Overexpression of *OsDREB1F* greatly enhanced the tolerance of plants to high salinity, drought, and low temperature both in rice and *Arabidopsis,* thus playing a significant role in plant stress signal transduction (68).

Function of *AtDREB1A* : *AtDREB1A* protein consists of its characteristic AP2 domain that binds to DRE/CRT cis acting element present in promoters **(**Fig. 3**).** The tertiary structure of *AtDREB1A* (Fig. 4) consists of a three-stranded â-sheet and one á-helix running almost parallel to it that contacts DNA via Arg and Trp residues located in the â-sheet (91). Two conserved functional amino acids (valine and glutamic acid) at 14th and 19th residues respectively, exist in the DNA binding domain, which are crucial sites for the binding of DREBs and DRE core sequences (92). An alkaline N-terminal amino acid region that serve as a nuclear localization signal (NLS) and a conserved Ser/Thr rich region responsible for phosphorylation near the AP2/ERF DNA binding domain are also mostly present (92, 93). The proteins contain an acidic C terminal region which might be functional in *trans*-activation activity (94). The activation of these transcripts is organ-specific and comparative to the extent of the stress given. *AtDREB1A* was induced within 10 min at 4 ºC Liu *et al* (95). *AtDREB1A* gene expression in response to abiotic stresses is activated by signal sensing,

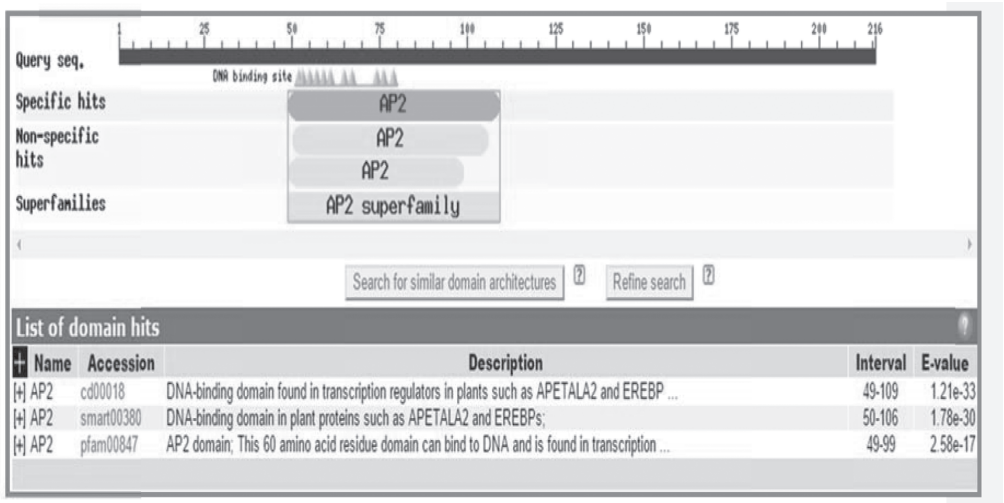

Fig. 3. Graphical representation of AtDREB1A protein AtDREB1A protein showing the location of AP2 domain and the DNA- binding sites (indicated by ?).

perception and transduction through abscisic acid (ABA) - independent manner.

Overexpression of *Arabidopsis CBF3/DREB1A* and ABF3 in transgenic rice showed tolerance to abiotic stress without stunting growth (59). The Arabidopsis gene *CBF3/DREB1A* has been used to improve abiotic stress tolerance in *japonica* rice (*Oryza sativa* cv. Nakdong) by constitutive expression (59) and in *indica* rice (*O. sativa* cv. BR29) by inducible expression (96).

Studies under field conditions : Plant productivity should be taken into consideration while evaluating the plants for abiotic stress tolerance. Also, drought and heat stresses occur concurrently in the field. Therefore, the results obtained under greenhouse/laboratory conditions are incomparable to that of the observations made under field conditions. Field trials thus play a critical role for the analysis of stress-tolerant transgenic crops. Drought tolerant transgenic rice constitutively overexpressing *OsLEA3-1* gene that encodes the proteins which accumulate in high quantity in water-stressed tissues and the plants expressing stress-inducible promoter transgene under field conditions was analyzed by Xiao et al.

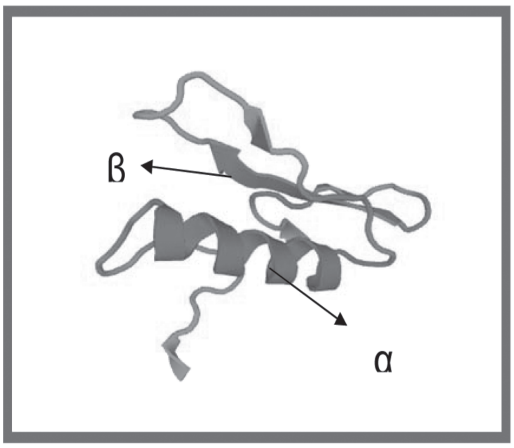

Fig. 4. Tertiary structure of AtDREB1A

(97). Xiao et al. (98) also examined drought tolerance of transgenic rice overexpressing seven well-reported stress related genes namely CBF3/DREB1A, an AP2/ERF-type transcription factor; SOS2, a serine/threonine protein kinase; NCED2 and LOS5, enzymes involved in ABA biosynthesis; NPK1, a mitogen activated protein kinase kinase kinase; ZAT10, a C2H2-type zinc finger transcription factor; and NHX1, a vacuolar Na^+/H^+ antiporter with an actin promoter under field conditions. Drought stress in the field decreased grain yield in these transgenics whereas the same

in LOS5, ZAT10 and NHX1 overexpressors were less affected. Since absolute grain yields under normal growth conditions were less in the transgenic rice over expressing CBF3/DREB1A, SOS2, NPK1, LOS5, ZAT10, and NHX1 with the stress-inducible promoter in comparison with untransformed controls, further improvement is required for practical application.

Field grown transgenic rice plants overexpressing SNAC1, a NAC type transcription factor subjected to two different levels of drought stress treatments of severe stress with 15% soil moisture and moderate stress with 28% soil moisture given at the anthesis stage has resulted in increased spikelet fertility in the transgenic plants whereas agronomic traits like plant height, panicle number, spikelet number, spikelet fertility, and grain yield, were similar between transgenic plants and the controls under non-stressed conditions (70). Exposure to drought stress by draining surface water and halting irrigation until leaves were rolled carried out during the panicle heading stage in the drought resistant transgenic rice overexpressing OsNAC5, OsNAC9/SNAC1 and OsNAC10 under the control of root-specific promoter have shown similar results among all the three transgenic rice lines. The grain yield decreased under drought conditions and the grains were significantly smaller in all three transgenic lines than those observed in their non-transgenic counterparts (99, 100 and 101). You et al. (102) studied the drought tolerance of transgenic rice plants overexpressing OsOAT gene that codes for OsOAT protein, an enzyme that increases proline content and is a direct target gene of the stress-responsive NAC transcription factor SNAC2, under field conditions. In the field, exposure to drought was carried out by stopping irrigation at the flowering stage in a refined paddy field covered with a movable rain-off shelter. Transgenic rice plants have shown slower wilting, fewer withered leaves and a higher rate of seed-setting than the non-transgenic lines. Increased grain yield was observed, when the transgenic rice plants overexpressing the *AP37* gene, an AP2/ERF-type transcription factor were given drought stress in the field at the panicle heading stage by draining the surface water and halting irrigation until leaves were rolled (103). The drought treated transgenic plants showed increase in grain yield which was due to the higher grain-filling rate compared to that of the drought treated non transgenic plants. The field evaluation of transgenic rice plants overexpressing EDT1/HDG11, a homeodomain-leucine zipper transcription factor, has also been performed by Yu et al. (104). The field grown transgenic rice subjected to drought by stopping the irrigation until the seed maturation stage, has shown higher grain yields with larger panicle sizes and higher tiller numbers than those observed in the drought treated non-transgenic rice.

Future prospects in the enhancement of abiotic stress tolerant transgenic rice : Although there are many reports of transgenic rice plants with enhanced abiotic stress tolerance during field trials, further research is required to reveal the regulatory mechanisms of stress response and tolerance under field conditions. The discovery of new genes that elevate stress tolerance without yield loss under abiotic stress is very much needed. Other approaches to new gene investigation is to study stress tolerance mechanisms of stress-adapted extremophiles such as desert plants, halophilic plants, cold water fishes and thermophilic bacteria (105). The functions of 18-38% of total proteins in a well characterized species remain unknown (106) and the explanation of which will be helpful in discovering new genes. Uga et al. (107) reported that the QTL Deeper Rooting 1 (DRO1) enhanced the growth angle of root in rice showing high-yield performance under drought stress conditions. This study shows that the modification of root architecture also plays an important role in the development of abiotic stress-tolerant rice plants. The development of submergence-tolerant rice cultivars and studies of submergence-tolerance mechanisms that have improved considerably (108, 109, 110, 111 and 112) signifies the exceptional properties of drought-tolerant rice plants with submergence-tolerant cultivar backgrounds which can survive under low as well

Table 1. Genetic engineering of functional genes used so far for abiotic stress tolerance

Nature of gene	Gene	Function	Source	Response	Reference
	cbf1	CRT/DRE binding factor	*A . thaliana*	Transformants showed regulation of several *cor* genes at the same time and showed freezing tolerance.	20
Transcription factor	*DREB1A*	DRE-binding protein	*A. thaliana*	Transformants showed enhanced expression of various stress-induced genes and showed tolerance to freezing and	21
	cbf3	CRT/DRE binding factor	*A . thaliana*	Transformants showed regulation of several *cor* genes at the same time and showed freezing tolerance.	22
	Cys2/His2 type STZ (salt-tolerance zinc finger protein)	Zinc- finger transcriptional repressor	*A. thaliana*	Transformants exhibited dehydration, high - salt, cold stress and ABA tolerance to drought stress	23
	*abi*3	Abscisic acid-induced protein	*A. thaliana*	Marked increase in expression of low temperature-induce freezing tolerance.	24
	cazfp1	Transcriptional repressor	yeast	Showed tolerance to drought stress and resistance against bacterial infection in *Arabidopsis*.	25
	alfin1	Member of Zn finger family proteins	*Medicago sativa*	Transformants overexpressing *alfin1* showed salinity tolerance.	26
	s coff-1	Soybean cold-inducible factor-1	*A. thaliana*	Transformants showed induction of *cor* genes and enhance cold tolerance.	27
	*tsi*1	Tobacco stress induced gene 1	*N. tabacum*	Transformants showed marked tolerance towards salinity and salicylic acid.	28
Fructan	*sac B*	Fructosyl transferase (fructan synthesis)	*Nicotiana tabacum*	Enhanced growth of seedling under osmotic stress.	29
Osmolytes Trehalose	*OsTPP*1 & *OsTPP*2	Trehalose biosynthesis	Rice	Tolerance towards chilling and other abiotic stress	30
Glycine betaine	*bet* B	Betain aldehyde dehydrogenase	*N. tabacum*	Transformed plants showed better growth under osmotic stress conditions.	31
Glycine betaine	*cod*A	Choline oxidase A (glycine betaine synthesis)	*A. thaliana, Oryza sativa* & *Brassica juncea*	Transformed tolerant to salt and cold. Enhanced tolerance to salt and low temperature stress. Enhanced capacity to germinate under salt stress.	32, 33, 34
Polyamines	*adc*	Arginine decarboxylase (putrescine biosynthesis)	*O.sativa*	Minimal chlorophyll loss under salt stress.	35
D-oninotol	*imt* 1	Myo – insitol-o-methyl transferase	*N.tabacum Arabidopsis thaliana*	Transformants were better adapted to water and salt stress. Enhance seed germination under salinity	36, 37
Mannitol	*mlt* D	Mannitol-1 phosphate dehydrogenase	*N. tabacum A. thaliana*	Enhanced tolerance to methyl viologen induced oxidative stress. Enhanced salt tolerance.	38a, 38b, 39
Proline	*p5cs*	Δ^1 -pyrroline 5-carboxylate synthase	*N. tabacum O. sativa*	Enhanced biomass under salt stress. Enhanced tolerance to salt and water stress.	40,41
Signal	*at-df2*	Cell cycle regulated phosphoprotein	*N. tabacum*	Transformants showed striking tolerance to heat, salt, cold and osmotic stress upon over expression	29

transductio n	*Atgs k1*	Arabidopsis homologue of GSK3/Shaggy like kinase	*N. tabacum*	Transformants showed induced expression of NaCl stress responsive genes	42
	Cnb1	Calcineurin B1	*N. tabacum*	Transformants showed substantial NaCl tolerance by co expression of catalytic and the regulatory subunits.	43
	Os cdpk7	Calcium dependent protein kinase	*O. sativa*	Overexpression showed induction of some stress responsive genes in response to salinity/drought and cold.	44
Heat shock proteins	*hsp 101*	Heat shock protein 101	*O.sativa*	Transformants showed enhanced tolerance to high temperature.	45
	hsp 101	Heat shock protein 101	*A.thaliana*	Transformants showed tolerance to sudden shifts to extreme temperature.	46
	hsp 17.7	Heat shock protein 17.7	*Daucus carota*	Transformants showed increased termotolerance.	47
	hsp 17. 6A	Heat shock protein 17.6A	*A.thaliana*	Transformants were tolerant to Osmotic stress but not heat stress.	48
Detoxificati on genes	*apx3*	Ascorbate peroxidase	*N. tabacum*	Transformants showed increased protection against oxidative stress.	49
	hvapx1	Ascorbate peroxidase	*A. thaliana*	Transformants were more tolerant to heat stress.	50
	gst/gpx	Glutathione-S-transferase and glutathione peroxidase	*N. tabacum*	Transformants showed better seedling growth under chilling and salt stress.	51
	Sat	Serine acetyl transferase	*N. tabacum*	Transformants were resistant to oxidative stress.	52
Detoxificati on genes	*sod*	Superoxide dismutase	*N. tabacum* *M. sativa*	Transformants showed increased regrowth after freezing stress. Transformants showed higher photosynthetic activity during	53, 54
	fe-sod	Fe-superoxide dismutase	*N. tabacum*	Transformants showed enhanced protection against superoxide radicals	55
	gly1 & *glyII*	Glyoxalase pathway genes	*Brassica juncea* *Pennisetum glaucum*	Improved salinity tolerance in tobacco	56

as excessive soil water content conditions. Global climate changes may alternately expose crops to abiotic stresses like drought, salinity, flooding etc. Attempts to develop rice cultivars that exhibit abiotic stress tolerance would be of great help to overcome the adverse conditions in the present climatic conditions for increasing the rice crop production so as to meet the growing food demands.

Conflict of interest The authors declare that they have no conflict of interest.

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Table 2. Over expression of stress-responsive transcription factors in transgenics

Gene	Family	Source	Abiotic stress tolerance	Reference
*ABF*2	bZIP	*Arabidopsis*	Drought	25
*GmbZIP*78	bZIP	*Arabidopsis*	Salinity, Freezing	53
*OsbZIP*23	bZIP	Rice	Drought, Salinity	54
*OsAREB*1	bZIP	Rice	Drought, Heat	55
*MYB*15	MYB	*Arabidopsis*	Drought, Salinity	56
*OsMYB*4	MYB	Tomato	Drought	57
*OsMYB*3R-2	MYB	Arabidopsis	Drought, Salinity,cold	58
AtDREB1A	CBF/DREB	Rice	Drought, Salinity	59
AtDREB1A	CBF/DREB	Potato	Salinity	60
AtDREB1A	CBF/DREB	Peanut	Drought	61
AtDREB1A	CBF/DREB	Wheat	Drought	62
*AtDREB*2ACA	CBF/DREB	*Arabidopsis*	Drought	63
*AtCBF*2	CBF/DREB	Tomato	Freezing	64
*BNCBF*5	CBF/DREB	*Brassica napus*	Freezing	65
*AtDREB*2C	CBF/DREB	*Arabidopsis*	Thermotolerance	66
*OsDREB*2B	CBF/DREB	Rice	Drought, Thermo tolerance	67
*OsDREB*1F	CBF/DREB	Rice, *Arabidopsis*	Drought, Salinity, Freezing	68
*OsDREB*1G	CBF/DREB	Rice	Drought	69
*SNAC*1	NAC	Rice	Drought, Salinity	70
*OsNAC*6	NAC	Rice	Drought, Salinity	71
*ONAC*045	NAC	Rice	Drought, Salinity	72
*OsWRKY*89	WRKY	Rice	UV irradiation	73
*OsWRKY*45	WRKY	*Arabidopsis*	Drought, Salinity	74
*OsISAP*2	ZFP	Onion	Salinity	75
*ZPT*2-3	ZFP	Petunia	Drought	76
Others	HARDY	Rice	Drought, Salinity	77

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Screening, Production and Partial Characterization of Xylanases from Woodchips Fungi with Potential Application in Bioethanol Production

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Abstract

The objective of this present study was to isolate, identify and screen for potential fungal isolates from local wood chips with respect to xylanase production. The fungal strains were isolated from degrading wood chips. All the fungal strains were screened for their ability to produce xylanase by the plate screening method using Congo red as indicator. Two isolates were further selected among them and screened for the production of the enzymes in liquid medium and identified by 18S rRNA. *Phialophora alba*, had the highest xylanase activity of 24.43 U/ml with temperature optima at 50°C and 90°C respectively. Enzymatic hydrolysis of pre-treated sugarcane bagasse using crude enzyme resulted in the production of 0.36 g/ml reducing sugars after 48 hours. This is the first report of a thermophilic xylanase from *Phialophora alba*. These results suggest that the application of this xylanase in bioethanol production may be very promising.

Keywords: Xylanase, *Phialophora alba*, Screening, Cultural conditions, Bioethanol production

Introduction

Xylanases (EC 3.2.1.8) catalyse the hydrolysis of internal β (1, 4) glyosidic linkages connecting xylopyranosyl units in xylan. This polysaccharide is the most abundant among the hemicellulosic materials and contributes to cohesion and integrity of plant cell walls, at the interface between lignin and cellulose (1, 2). Diverse forms of these enzymes exist, showing varying folds, mechanisms of action, substrate specificities, hydrolytic activities (yields, rates and products) and physicochemical characteristics (3).

Interest in xylanolytic enzymes has intensified in the past two or three decades due to their potential industrial application in the food, feed, and pharmaceutical industries and for sustainable production of fuels and chemicals (4). Also, they can be applied in some processes in which cellulolytic activity must be absent, to preserve vegetal fibres, in the pulp and paper industries (1), and in the processing of flax (5), hemp and jute in the textile industries (6). For commercial purposes, many xylanases have been highly expressed in heterologous systems, such as *Escherichia coli*, *Bacillus* spp. and *Pichia pastoris* (7, 8). The most widely used xylanases are from the fungal genera *Trichoderma*, *Aspergillus* and *Penicillium*, and these enzymes are generally highly active over a temperature range of 40–60° C (9). At these temperatures, complete saccharification of biomass polysaccharides requires a long reaction time with high contamination risks (10). Therefore, high-temperature active xylanases are needed to enhance the mass transfer and reduce the substrate viscosity (11).

When a new efficient xylanase-producing microorganism is isolated, it is essential to purify and characterize the enzymes to know the action towards substrates of each component of a xylanolytic complex, its regulation and biochemical properties in order to develop more competitive processes. In the current study, several fungal strains were isolated from Eucalyptus spp. woodchips and tested for their ability to produce xylanases. Among 46 strains isolated (Table 3.1), a strain producing a thermostable and thermoactive xylanase was identified using PCR amplification and sequencing to be *Phialophora alba*.

Materials and Methods

Isolation and initial screening of microbial strains: A primary screening was conducted among microbial strains for xylanase production from wood chips collected from Sappi Saiccor, Umkomaas, KwaZulu-Natal, South Africa. Microorganisms were screened for xylanase activity on nutrient agar plates supplemented with 1% birchwood xylan (Sigma-Aldrich, Switzerland) and 1% low sulfonate lignin (Sigma-Aldrich, Switzerland), respectively. The pure cultures were incubated at 50°C to select for thermophilic microorganisms. Zones of clearing which were indicative of enzyme activity were visualized upon staining with 0.1% Congo Red and destaining with 1 M NaCl. The diameters of the zones of hydrolysis were subsequently measured. Fungal isolates were selected based on differences in macroscopic characteristics the size of the zone of hydrolysis and subjected to further analysis. These strains were maintained on potato-dextrose agar plates with subculturing every three months.

Production of xylan-degrading enzyme: Enzymes were produced by cultivation of the fungal isolates using 50 ml medium in 250 ml shake flasks on an orbital shaker at 200 rpm using modified mineral media (pH 5) for the cultivation of fungi as described by Gomes et al. (12). One litre of media contained 5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.05 g $CaCl_2$. $2H_2O$, 5 g NH_4NO_3, 1% Birchwood xylan and 1 ml trace-element solution [g.l-1: $MnCl_2.4H_2O$ (0.03), $ZnSO_4.7H_2O$ (1.4), H_3BO_3 (0.3), $CoCl_2.6H_2O$ (0.2), $CuCl_2.2H_2O$ (0.01), $NiCl_2.6H_2O$ (0.02), $NaMoO_4.H_2O$ (0.0277).$6H_2O$, $CoCl_2.6H_2O$]. Samples were withdrawn periodically under aseptic conditions and assayed for xylanase activities. One millilitre of culture medium was removed from each flask and centrifuged at 13 000 rpm for five minutes. Time course for optimal enzyme production was determined for six isolates using the cell-free supernatant. The amount of reducing sugars liberated was determined using the DNS (dinitrosalicylic acid) assay. All experiments were done in triplicate.

Enzyme assays: Xylanase activity was assayed spectrophotometrically at 540 nm by the dinitrosalicylic acid (DNS) method as described previously (13) using birchwood xylan (1%) as substrate. Xylanase activity was expressed as nkat.ml-1 where one unit of enzyme activity (nkat) was defined as the amount of enzyme one nmol of xylose liberated per second, under the assay conditions.

Total protein concentration was determined using the Bradford assay (14) using bovine serum albumin (BSA) as the standard. Specific activity was determined by dividing xylanase activity (nkat.ml-1) by protein concentration (mg.ml-1) and expressed as U, where one U is equivalent to one $nkat.mg^{-1}$.

Temperature and pH stability: The optimum temperature of crude xylanase was determined by incubating the culture filtrates in a water bath at various temperatures (from 40 to 90°C) for 15 min before the reaction was stopped. The optimum pH of crude xylanase was determined at optimum temperature by carrying out the enzyme assay at different pH values (pH 4-9) using the following buffers: using the DNS assay as described by Bailey et al. (13) 50 mM citrate phosphate (pH 4.0, 5.0 6.0, 7.0, 8.0 and 9.0).

Native PAGE gel electrophoresis: A 10% Native PAGE gel supplemented with 1% Beechwood xylan (250 µl) was done as previously described by Ninawe et al. (15). Briefly, a substrate gel was

prepared containing 1% Birchwood xylan. The PAGE gel was placed onto the substrate gel and incubated for 1 hour at 50°C. After incubation, the protein gel was stained and destained. The activity gel was stained for 2 hours with 0.1% Congo Red and destained with 1 M NaCl. The gel was fixed by washing with 0.5% acetic acid. The crude enzyme was loaded, separated by electrophoresis and subsequently stained with Congo red for 1 hour and destained with 1M NaCl

Molecular identification of Fungal isolates: Genomic DNA extraction: The fungal isolates were cultivated in malt extract broth (MEB) (Sigma-Aldrich, Switzerland) and incubated at 30°C for 3 days. After incubation, cultures were centrifuged at 10 000 × g for 3 minutes and the pellets were re-suspended in 200 µl of sterile distilled water. Both fungal and bacterial DNA extraction was performed using a ZR soil microbe DNA kit (Zymo Research, USA). DNA samples were stored at -20°C.

PCR amplification of the 18S ribosomal ITS region of the 18S rRNA gene: The primer pair ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4R (5'-CCTCCGCTTATTGATATGCTAAG-3') were used to amplify the ITS1-5.8S-ITS2 region of the fungal isolates F1 and F10. The universal bacterial primers 63F (5'-CAGGCCTAACA CATGCAAGTC-3') and 1387R (5'-GGGCGG(A/T)GTGTACAAGGC-3') were used for the amplification of a segment of the bacterial genomic DNA. In the PCR reaction mixture 10 mM buffer (5 µl), 25 mM MgCl2 (2 µl), 2.5 µM each of the forward and reverse primers (2.5 µl), 250 U Super-ThermTaq polymerase (Fermentas, South Africa)) (0.5 µl), 10 mM dNTPs (5 µl) were added. Five microlitres of template DNA was added to the reaction mixture and brought up to volume with sterile double distilled water. Amplification of a 50 µl PCR mix was performed using the following thermal cycling conditions: initial denaturation at 95°C for 5 minutes, followed by 31 cycles of denaturation (95°C) for 1 minute; annealing (55°C) for 1 minute and extension (72°C) for 1 and a half minutes. A final extension step was conducted at 72°C for 5 minutes.

PCR products were visualized by electrophoretic analysis on a 1% agarose gel. Once the presence of the PCR amplicon was confirmed, they were sent for sequencing to Inqaba Biotech (Pretoria, South Africa). BLAST searches of the amplified regions were conducted to identify the isolates.

Enzymatic hydrolysis of pre-treated sugarcane bagasse: Enzymatic hydrolysis was performed using 1% (w/w) substrate in 50 mM citrate buffer (pH 5) with a final reaction volume of 50 ml as described by Adsul et al. (16). Briefly, the sugar cane bagasse (SCB) was dried in an oven overnight at 60°C. The dried samples were milled to particles of 0.5-1 cm in size and passed through 0.5 mm and 1 cm screens. The milled SCB (20 g) was mixed with 450 ml of distilled water and served as no pretreatment control. Another experiment was set up in which the milled SCB was pre-treated with alkali by adding 20 g of the milled substrate to an Erlenmeyer flask containing 450 ml of 2N NaOH. The flask was incubated at 30°C for 20 hours followed by several washing steps with distilled water to neutralize the pH of the preparation. Thermal and pressure pretreatment were conducted simultaneously by autoclaving (121°C, 0.103 MPa) an Erlenmeyer flask containing 20 g of substrate and 450 ml of distilled water for 90 minutes. All preparations (untreated, thermal/pressure and alkali) were filtered through 0.45 µm filters to remove the liquid phase. The remaining SCB was used in subsequent hydrolysis studies. Enzymatic hydrolysis was performed using 1% (w/w) substrate in 50 mM citrate buffer (pH 5) with a final reaction volume of 50 ml. The thermally inactivated crude enzyme and partially pure xylanase served as a control for each pretreatment process and the untreated SCB. Hydrolysis of the pretreated SCB was carried in duplicate for each type of treatment. All flasks were incubated at 50°C with shaking at 150 rpm. Samples (1 ml) were withdrawn after 0, 24 and 48 hours. All samples were filtered using 0.45 µm filters and the supernatant assayed for residual sugars using the DNS assay. All

experiments and analyses were carried out in triplicate.

Results and Discussion

Enzyme production: Amongst 46 microorganisms isolated from *Eucalyptus* spp. woodchips, 46% were positive for xylanase activity. Furthermore, mixed population of bacterial and fungal species were isolated from *Eucalyptus* spp. woodchips. Selection of isolates was based on differences in morphology, macroscopic characteristics and the size of the zone of hydrolysis. Only the fungal isolates were selected for further characterization. (Table 1). Fungal isolates F1, F2, F6 and F10 displayed relatively large zones of clearing (Table 1) in comparison to the bacterial isolates (data not shown). No ligninase activity was observed for the fungal isolates.

Among the selected microorganisms, fungal isolates F2 and F10 produced optimal quantities of xylanase after 5 days while F1 and F10 required a cultivation period of 7 days in order for optimal amounts of xylanases to be achieved. F1 and F10 demonstrated highest enzyme activity amongst the fungal isolates with specific activities of 12.25 U and 24.43 U, respectively. This is in agreement with enzyme titres obtained in studies conducted by Gomes et al. (12) and Palaniswamy et al. (17). Based on the desirable activities displayed by selected isolates, further characterization in terms and pH and temperature optimum, stability, molecular identification and molecular weight was determined

Enzyme Characterization

Xylanase production and optimization of fermentation parameters: Preliminary studies were conducted in 0.1 M sodium citrate buffer, pH 5 containing birchwood xylan as the primary carbon source for production of xylanase at under shaking conditions (200 rpm). For xylanase activity, the DNS method (18) is used under standard conditions. The fungal isolates F1, F2, F6 and F10 produced high levels of xylanase. Highest levels of production were observed for F1 and F10 with specific activities of 12.58 U and 24.23 U, respectively after 7 days of cultivation (Fig. 1). Enzyme activity in relation to protein concentration was low for the remaining fungal isolates in comparison to F1 and F10.

The pH optima determined for the two fungal

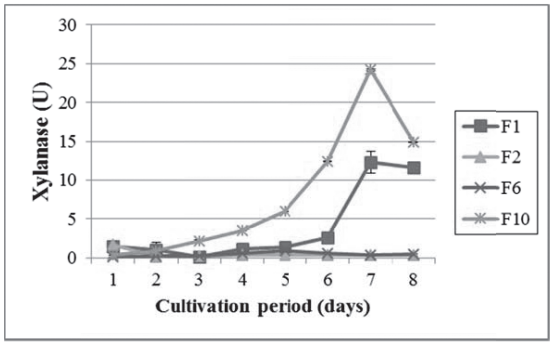
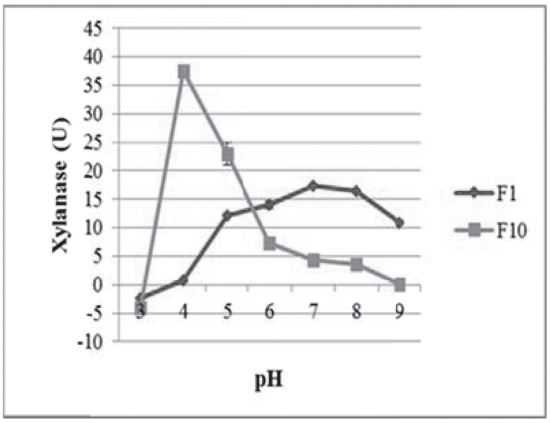


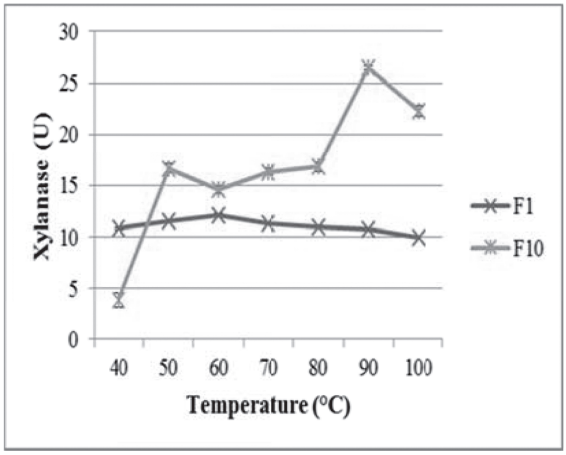
Fig 1. Time course for optimal xylanase production for fungal isolates F1, F2, F6 and F10 isolated from woodchips.

(F1 and F10) xylanases, F1 xylanases displayed a neutral optimum pH with a corresponding specific activity of 17.33 U. However, F10 xylanases displayed an acidic pH optimum of pH 4 and a corresponding specific activity of 37.51 U (Fig. 2a & b). F1 displayed a broad optimum temperature within the range of 40-100ºC with a slight increase at 60ºC (specific activity of 12.15 U). However, F10 displayed two distinct peaks



2a. pH optima of F1 and F10 xylanases.

within its temperature profile at 50ºC and 90ºC with corresponding production levels of 16.68 U and 24.5 U.



2b. Temperature optima of F1 and F10 xylanases
Fig. 2. pH and temperature optima studies of fungal isolates F1 and F10.

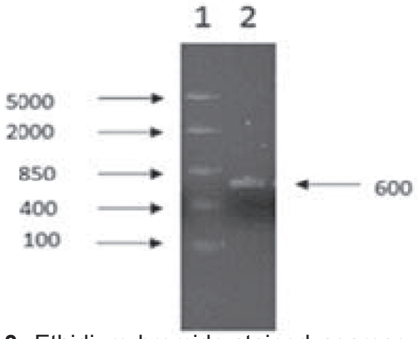


Fig. 3. Ethidium bromide-stained agarose get containing PCR products 18S rRNA amplicon of F10 fungal DNA lane I 100 bp Fast Ruler middle range DNA ladder, lane 2 18S PCR amplicon

Molecular identification of fungal isolate: Substantial xylanase activity of 37.51U was detected in the culture supernatant of F10 strain when birchwood xylan (1%) is used as substrate. The crude enzyme was precipitated using 20% ammonium sulphate to homogeneity and native PAGE and is performed. Native PAGE analysis

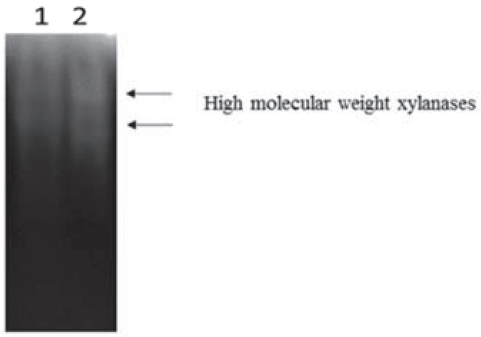


Fig. 4. 10% Native PAGE gel supplemented with 1% beechweed xylan showing the isozymes present in the crude enzyme.
lane 1 : crude enzyme, *lane* 2:10+ concentrated crude enzyme. Each *lane* was loaded with 15 ug of crude enzyme

(Fig. 4) indicated the presence of more than three high molecular weight xylanase isozymes.

Bioethanol production using crude enzyme: The enzymatic hydrolysis of SCB was established using the crude enzyme and partially pure xylanase preparations from *P. alba*. The milled SCB was subjected to two different types of pretreatment conditions prior to enzymatic saccharification (alkali and temperature/pressure). The reducing sugars liberated after pretreatment with alkali and high temperature and pressure was difficult to determine as the high NaOH concentrations in the alklali-treated SCB interfered with the DNS assay. Sugar concentrations below 0.4 g/ml were present after 24 and 48 hours (Fig 5) in the untreated control in the absence of enzyme. The addition of crude enzyme preparation to the control produced tenfold higher reducing sugar levels after 48 hours.

(N: untreated+buffer; NP: untreated+partially pure xylanase; NC: untreated+crude enzyme; T: thermal treatment+buffer; TP: thermal treatment+partially pure xylanase; TC: thermal treatment+crude enzyme; A: alkaline treatment+buffer; AP: alkaline treatment+partially pure xylanase; AC: alkaline treatment+crude enzyme). (All data points with bars are means ± standard deviation (n=3)).

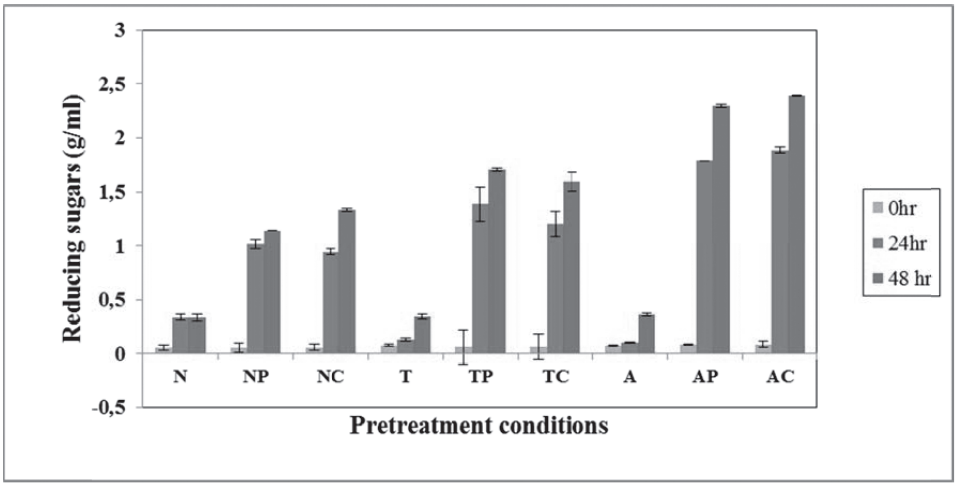


Fig 5. Enzyme hydrolysis of sugarcane bagasse using partially pure xylanase and crude enzyme (15 IU/g) from *P. alba*

Discussion

Woodchips are known to contain a plethora of microorganisms with diverse characteristics and enzymatic capabilities. Bacterial and fungal isolates have been isolated from such environmental samples (19). The enzymatic capabilities of these microorganisms have also been exploited for various applications in industrial biotechnology. In the current study, of the 32 microorganisms isolated from *Eucalyptus* spp. woodchips, 46% were positive for xylanase activity. Of the xylanase producers, 8 isolates were selected for further enzyme characterization. Selection of isolates was based on differences in morphology, macroscopic characteristics and the size of the zone of hydrolysis. The time required for optimal enzyme production was determined. Among the selected fungal isolates, F2 and F10 produced optimal quantities of xylanase after 5 days and F1 and F10 demonstrated highest enzyme activity with specific activities of 12.25 U and 24.43 U, respectively. Similar enzyme titres were obtained in studies conducted by Gomes et al. (12) and Palaniswamy et al.(17). Based on the desirable activities displayed by the above-mentioned isolates, further characterization in terms and pH and temperature optimum, stability and determination of protein size was conducted.

All isolates displayed a pH optima of 7 except for F10 which exhibited an acidic pH optimum of 4. Most xylanases have pH optima ranging from 4.5-7; however, reports of a xylanase produced by *Talaromyces emersonii* with an acidic pH optimum have been made (20). Good stability at their optimum pH was observed for the crude xylanases which indicates that their application in the paper and pulp industry is promising. Biopulping requires thermostable, alkaline xylanase (21) while bioleaching requires thermostable, acidic xylanases (22).

High xylanolytic activity was observed for crude fungal xylanases isolated from wood chips. F1 remained stable for 105 minutes at optimum pH with greater than 99% of activity being retained. Desired stability was exhibited by the crude xylanases at pH 5 as 98% of activity was retained for 90 minutes. A similar trend was observed for F10. Approximately 98% of xylanases activity was retained at pH 4 and 96% at pH 5 for 75 and 60 minutes. The xylanase activity displayed by these isolates is consistent with

what has been reported in literature. However, in most instances such activities have been observed after optimization of media composition by different carbon sources such as wheat straw, maize straw and oat hay as inducers of enzyme activity in order to minimize cost by manipulating media components. (23).

F10 displayed temperature optima at two peaks at 50ºC and 90ºC respectively (Fig 2b). This suggests that more than one type of xylanase may be present each with its own optimum temperature and specific activity which agrees with reports that multiple xylanases may be produced by a single microorganism (2). As a result of genetic redundancy fungi like *Aspergillus niger* and *Trichoderma viride* are known to produce fifteen and thirteen xylanases, respectively (24). The complexity of xylan requires more than one type of enzyme to ensure penetration into the cell. Furthermore, desirable activity was observed at 100ºC for both fungal isolates indicative of the production of thermoactive enzymes necessary for application in bioprocessing. Fungal xylanases are typically more stable at elevated temperatures than bacterial xylanases. There have been reports of fungal xylanases which retained 100% of activity between 65ºC and 80ºC for 30 minutes after optimization of growth media (25). Thermostability can be attributed to the presence of a thermostabilising domain, expressed either as discrete entities or as their natural fusions with the catalytic module have capacity to bind various carbohydrates and potentiate hydrolytic activity (26). Among the fungal isolates in current study, differences in thermal stability were observed as F1 was more stable than F10 xylanase. Such differences in stability could be attributed to the presence of co-factors and salts in the media which tend to elicit varying effects on proteins in general.

The thermophilic fungus in the present study was identified as *P. alba* by 18S rRNA amplicon of F10 fungal DNA. Further, edited sequence were used to determine the identity of the fungus against a database of known microorganisms. The fungus was thus, identified as *P. alba* (HM 116755) with a 99% homology.

Initial electrophoretic analysis yielded no visible bands. To enhance the visibility of the bands, the crude extracts were concentrated 10× and bands were only visualized for F10 although clearing in the native substrate PAGE gels indicate fairly high specific activity for several isozymes. Therefore, in the near future, the crude extracts need to be further purified to enhance visibility of bands for electrophoretic analysis. Once bands have been visualized, zymogram analysis may be carried out to determine molecular weight of xylanases.

In the present study the effect of two pretreatment strategies (viz., high temperature, pressure and alkali pretreatment) of SCB in conjunction with enzymatic hydrolysis was determined. The effect of pretreatment alone on SCB could not be established as the high NaOH concentration interfered with the DNS assay and precluded determination of sugars released. In the current study, the additive effect of enzyme treatment (partially pure xylanase and crude enzyme preparation) with pretreatment strategies was assessed (Fig 5). Milling was responsible for some degradation of the SCB as low baseline levels of reducing sugars were detected in all samples at 0 hours. Whilst, both enzyme preparations had a moderate effect on the milled SCB as less than 0.35 mg/ml reducing sugars were produced after 48 hours. According to Corrales et al. (27), the pretreatment of lignocellulosic residues including SCB is essential in order to achieve efficient hydrolysis to monomeric sugars. Untreated SCB represents the fully intact polymer complex of lignocelluloses.

Under thermal pretreatment conditions, the crude enzyme produced the maximum amounts of reducing sugars after 48 hours with sugar concentrations of 1.2 g/ml and 1.6 g/ml detected at 24 and 48 hours, respectively. Thus the reaction time determines the amount of reducing sugars liberated. This is in agreement as reported by Saores et al. [28], where the amount of reducing

Table 1. Microorganisms isolated from woodchips (Sappi Saiccor) and their respective cellulase, ligninase, and xylanase activity

Pure Isolates	Species	Phyla Affiliation	Best Match database (Gene Bank Accession No.)	Similarity (%)	Xylanase
F1	*Paecilomyces* sp.	Ascomycota	AB217858.1	99	+++
F2	*Aspergillus fumigatus*	Ascomycota	GU992275.1	100	++
F3	*Phanerochaete chrysosporium*	Basidiomycota	AF475147.1	100	-
F4	*Paecilomyces formosus*	Ascomycota	GU968673.1	99	++
F5	*Paecilomyces formosus*	Ascomycota	GU968664.1	99	-
F6	*Geosmithia arqillacea*	Ascomycota	GU165722.1	98	++
F7	*Penicillium verruculosum*	Ascomycota	HM469420.1	99	+
F8	*Acremonium implicatum*	Ascomycota	FN706553.1	98	+
F9	*Aspergillus fumigatus*	Ascomycota	GU566217.1	100	++
F10	*Phialophora alba*	Ascomycota	HM116755.1	99	++
F12	*Curvularia* sp.	Ascomycota	HQ631061.1	100	+

- : absence of zones; + : 1-18 mm; ++ : 19-29 mm; +++: 30-40 mm

sugars from steam pretreated SCB were liberated over a 72 hour period.

Alkaline pretreatment coupled with enzyme hydrolysis proved to be the most effective SCB preparation resulting in the highest production of reducing sugars. A similar study by Hernandez-Salas et al. (29) using a cocktail of enzymes showed a higher concentration of reducing sugars with alkali treated SCB compared to either acid hydrolysed or steam pretreated bagasse. Approximately 11-20% reducing sugars were generated over a 4 hour period at 55°C.

The results obtained in the current study are consistent with reports that fungi produce xylanases with higher activity than bacteria (30, 31).The fungal xylanases have auxiliary application in biopulping and bioethanol production.

Conclusions

The current study presented evidence for the presence of thermostable and thermoactive xylanolytic enzymes produced from woodchip-inhabiting microorganisms. The desirable activity, pH and thermostability of F1 and F10 are indicative for their potential application in a range of bioprocesses. Potential applications include bioethanol production, biobleaching and biopulping which are evidenced by the high activity observed at pH 5, pH 4 and pH 9. As robust biocatalysts, F1 and F10 xylanases show potential to withstand the elevated temperatures and extremes in pH during industrial processing. Furthermore, xylanase activity observed at 90ºC indicates that these enzymes can be used in the degradation of hemicellulose-rich sugarcane bagasse and agricultural wastes for biofuel production following a thermal pre-treatment step. Therefore, the cooling step which is usually implemented after thermal pre-treatment may be eliminated as the enzymes function at the same temperature.

P. alba isolated in this study would be more useful for enzyme production for industrial applications. Further purification to homogeneity, analysis of the characteristics of xylanase produced by *P. alba* will be needed in order to apply the results to industry.

Conflict of interest

The authors declare no conflict of interest.

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L-Asparaginase a Biotherapeutic for Acute Lymphoblastic Leukemia – A Molecular Perspective

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Abstract

L-asparaginase (L-asparagine amino hydrolase) is an enzyme which was clinically proved as an antitumor agent to treat acute lymphoblastic leukemia. It catalyzes L-asparagine hydrolysis to L-aspartate and ammonia, and the depletion of asparagine causes cytotoxicity to leukemic cells. Microbial L-asparaginase (ASNase) production has attracted good attention regarding its cost effectiveness and eco-friendliness. The focus of this review is to provide a discussion regarding the microbial ASNase production, purification, its mechanism of action, sources, therapeutic side effects and focusing on the future prospects like protein engineering, recombinant microorganisms to develop a efficient therapeutics with significantly less side effects. This study is also focusing on the production of ASNases from new sources with improvement in the availability as a drug, and issues related to reducing the cost of the drug by improving the pharmacokinetics, pharmaco-dynamics and toxicological profiles in producing the ASNase enzyme.

Key words: Microbial L-asparaginase production, Biopharmaceutical drug, Acute Lymphoblastic leukemia.

Introduction

L-asparaginase (ASNase) is an enzymatic drug used in chemotherapy against diseases such as acute lymphoblastic leukemia (ALL), lymphosarcoma, Hodgkin's disease (1). Tumor cells, more specifically lymphatic tumor cells, requires high amount of asparagine to survive with their rapid malignant growth. This drug depletes L-asparagine (Asn) in to L-aspartate and ammonia in blood, blocking protein synthesis in T-cells and inhibiting DNA and RNA synthesis in cancer cells. As a result, cell functions are impaired resulting in apoptosis. However, normal cells are capable to synthesize their own Asn and are less affected by its depletion by treatment with ASNase. Nonetheless, when the drug was used for long-term treatment, it may cause hypersensitivity leading to allergic reactions such as respiratory disorders, skin rashes, low blood pressure, loss of consciousness (2). Various ASNase preparations from *Escherichia coli* [native and PEGylated form] or *Erwinia chrysanthemi* [native form] are available on the market (3). Moreover, researchers found that *Escherichia coli* yielded preparations that inhibited tumors, compared to the other bacterial ASNases are very less active or completely inactive state (4). Subsequently, the native *E. coli* ASNase was then used for developing a drug in the market.

Recently the interest in using ASNases to treat ALL in adults, specifically young adults has been increased (5). Among 4000 ALL cases diagnosed every year in the USA, approximately two-thirds are children and adolescents, making

ALL the most common cancer among this age group (6). Around 80% of the children are reported to have a long-term improvement and an overall survival rate was 90%, where as in adults the figures are reduced to 38% and 50% respectively (7-8). In recent years, there has been reportedly good progress in leukemia treatment. ASNase is found widely among many different sources in nature, found not only in microorganisms, but also in plants and tissues of various animals like mammals, birds and fishes. However, microbes are known to be a better source compared to animals or plants, due to their ability to grow easily on simple and inexpensive substrates. Furthermore, they offer easy optimization of culture conditions for enzyme over production, easy genetic modification to increase the yield, commercially viable upstream and downstream techniques, good stability and consistency (9).

Many of the l-asparaginases are not suitable for therapeutic purposes, so many homologous ASNases have been selected are cloned and characterized to potentially reduce the side effects and less toxicity (10). Hence the ideal enzyme could persist for a longer time in the circulatory system with reduced antigenic properties. In order to meet these challenges, many trials have been made to solve the problem by attaching the ASNase enzyme with chemicals like polyethylene glycol (11-12), encapsulation to RBC's (13), deimmunization of T-cell epitope removal by neutral drift (14), trypsin resistance ASNase with increased stability was achieved by immobilization technique (15). When ASNase was used repeatedly due to its short half-life and instability leads to more serious side effects on the patients. However, due to chemical modification of the enzyme reduces the activity of the enzyme. So, to increase the stability of the enzyme thermo tolerant ASNase was cloned, purified from *E.coli* has been reported (16). Taking into account all this scenario, the main aim of this review is to provide a thorough discussion on microbial ASNase production. More particularly, it focuses on microbial related productions, recombinant microorganisms that are likely feasible for a better cost in the market.

Mechanism of Action

L-Asparaginase produced by different sources has different half –lives. Different half-lives of the ASNases preparations lead to the different durations of depletion of the asparagine. The action of L-Asparaginase on the leukemic cells makes them deprive of the asparagine by causing the hydrolysis of asparagine in to asparatic acid and ammonia (Fig.1).

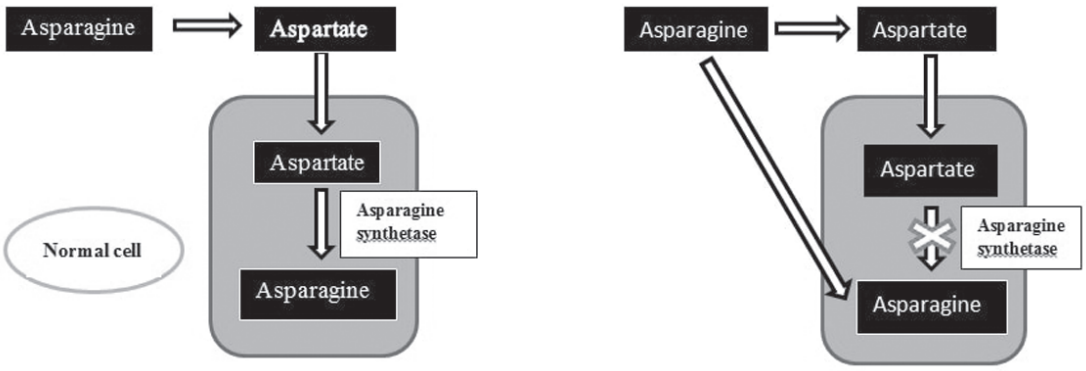

Fig 1. Mechanism of action of ASNase on normal and tumor cell

Normal cells contain the asparagine synthetase enzyme to fulfill the requirement of asparagines in their diet and asparagine is a non-essential amino acid for normal cells. This enzyme leads to the changes in the characteristics of asparatic acid by adding an amine group from the glutamine and hence leads to the production of asparagine. Asparagine is an essential amino acid for the tumor cells as they do not have the self-producing capability of asparagine due to the lack of asparagine synthetase (17). Protein and RNA synthesis is inhibited in the absence of asparagine (18) and as a consequence cell cycle arrest and apoptosis is induced in leukemia cell lines (19). To achieve the complete asparagine depletion in the human circulation, the L-Asparaginase activity level in serum must be > 100 IU/L (20). It has been reported that tumor cells can develop the potential to synthesize L-Asparagine intracellularly, which enables them to resist the action of the enzyme. The expression of asparagine synthetase is regulated by methylation of cytosine residues, which is responsible for the synthesis of asparagine synthesis. This offers the tumor cells a safe and confirms the exit from the action of L-Asparaginase (21).

Sources of L-asparaginase : Microorganisms have been proved to be very efficient and inexpensive sources of L-asparaginase. Large number of bacteria, fungi, yeast, actinomycetes and algae are reported as potential source of L-asparaginase (22).

Bacterial source : From *E.coli* L-asparginase II enzyme was isolated by Howard cedar and James H. Schwatrz (23). The deamidation of L-asparagine from *Escherichia coli*, due to the presence of L-asparaginase II was first reported and identified by Tsuji. Normally ASNase was produced under aerobic conditions but a significant higher yield of the enzyme was found under anaerobic conditions by using media enriched with high concentration of different amino acids .Optimum aerobic conditions have been proved useful for the greater yield of the enzyme compared to the turbulent conditions showed high amount of the biomass resulting in reduced yield of the enzyme. Ammonium sulfate precipitation and ethyl alcohol precipitation, purification techniques are proved to gain 40 folds amount of the enzyme.

Hymavathi et.al. (24) optimized the conditions for the production of ASNase from a strain *Bacillus circulans* MTCC 8574 by solid state fermentation, by using the agricultural waste as the suitable nutrient source. Incubation temperature, moisture content, glucose, L-asparagine, inoculum level are the conditions which affect the yield of the enzyme.

Production of recombinant *Erwinia caratovora* L-asparaginase II in *E.coli* cells by fed-batch cultures. Using the fed-batch technique with already determines exponential feeding rates; the bioreactor culture yielded 30.7 g of dry cell weight and 0.9 g of soluble rErAII protein per liter of culture broth (25).

Researchers produced a stable L-asparaginase enzyme which was tolerant at 45^0c. The cloning and expression of ASNase enzyme from thermo-tolerant strain *Escherichia coli* (KH027) was isolated from camel dung and could grow at 45^0c. Expression of recombinant asparaginase was performed by fusion of ASNase gene to pelB leader sequence and 6His residues at C-terminal under the inducible T7 promoter in DH5 cells. The protein that purified through nickel affinity chromatography showed optimum conditions at temperature of 43^0c and pH 6**.** Different other sources of bacteria which produce the enzyme are shown in (Table 1).

Yeast and Fungal sources : The production of Asparaginase from various strains of fungi has been reported using a different range of media. The production of ASNase by filamentous fungi such as *Aspergillus tamarii* and *Aspergillus terreus* has been reported with highest L-asparaginase production level in 2% proline medium from *A. terreus* (26). The production of asparaginases from fungi by various methods has been reported and L-asparaginase producing fungus, *Aspergillus terreus* was isolated and various parameters for

Table 1. L-asparaginase production by various Bacterial speices.

S. No	Bacterial sp.	Substarte/Media	Operating conditions	Fermentation	Activity reported	Reference
1.	E.coli	Yeast extract – 4%, Peptone – 2%, L-Asparagine – 0.1%	37°C, 220 rpm, 12 h	SMF(submerged fermentation)	60.8 IU/mL	(42)
2.	Erwinia aroideae NRLL-B	Lactose – 1%, Yeast extract – 1.5%	24°C, pH – 7.5, 200 rpm, 12 h	SMF	4 IU/mL	(43)
3.	Pseudomonas aeroginosa 50071	Casein hydrolysate – 3.11%, Corn steep liquor –3.68%	37°C, pH – 7.9, inoculum – 1%, 4 days	SSF(solid state fermentation)	142.2 IU/mL	(44)
4.	Pseudomonas aeroginosa	Peptone 1%, yeast extract –0.5%, NaCl – 1%, glucose –0.1%	37°C, 200 rpm, 24 h	SMF	$210*10^3$ U/mg	(45)
5.	Recombinant E.coli BL 21	TB media with ampicillin100 µg/ml	37°C, 220 rpm, pH – 7.2, 24 h	SMF	22 IU/mL	(46)
6.	Zymomonas mobilis cp4	Molasses – 10%, Yeast extract – 0.2%	30°C, inoculum – 10%, 21 h	SMF	16.55 IU/mL	(47)
7.	Staphylococcus sp	Ammonium chloride and glucose – 1:0.75	39°C, pH -7.5,100 rpm, 12 h, inoculum – 3%	SMF	55.6 IU/mL	(48)
8.	Pectobacterium carotovoram	Yeast extract – 2.08 %, Tryptone – 0.5 %, Monosodium glutamate – 9.89%, L-asparagine – 1%, Galactose – 0.9 %	30°C, pH – 6, inoculum – 5%, 120 rpm	SMF	3.25 IU/mL	(49)
9.	Bacillus circulans MTCC 8574	Red gram husk – 5 g, Glucose – 1.17 g, LA sparagine – 1.24%, Moisture – 99.5%	36.3°C, inoculum – 2.8 ml	SSF	2322 U/g	(50)
10.	Pectobacterim carotovoram	Glucose – 0.2%, L-Asparagine – 0.4% along with yeast extract and peptone	30°C, inoculum – 2%, rpm – 120 SMF (I/E)	SMF	14.56 IU/mL	(51)

ASNase production through solid state fermentation were optimized (27). L-proline is the best nitrogen source for the production of ASNase from *A. terreus* for maximum asparaginase activity using latin square design (28). The *Penicillium* sp. from the soil producing ASNase with anti-oxidant properties has been found (29).

The ASNase enzyme was purified to homogeneity from *Penicillium* sp. that was grown on submerged fermentation. This purified enzyme showed 13.97 IU/mg specific activity and 36.204% yield. The enzyme showed maximum activity at 7 pH and 37°C. This shows that the enzyme is independent on pH particularly from this organism (30).

Abha Mishra et.al., (31) was the one who reported higher yield of the enzyme from a different isolate of *Aspergillus niger*, agro waste from the leguminous crops as a source. She followed the process of solid state fermentation (SSF). Bran of Glycine max was used as a main source of nutrients gave highest yield of enzyme, which was further followed by the *Phaseolus mungo,* and *Cajanus cajan.*

Actinomycetes sources : The first L-asparaginase from actinomycetes was reported in *Nocardia* spp. (32). Production of intracellular and extracellular asparaginases from *Streptomyces* spp. was also studied (33, 34). Saleem et.al. (35) is the first to report on the production and partial purification of L-asparaginase from marine actinomycetes isolated via solid state fermentation (SSF). In the final purification step, the enzyme showed a specific activity of 662.61 IU/mg, which is approximately 2-fold purity. Optimum pH was found to be 7.5, which is close to blood pH, compared to L-asparaginases from other bacterial sources such as *Serratia marcescens*, *Mycobacterium* spp. and *Pseudomonas* spp. showed optimum pH in the range of 8.0 to 8.5. At 50 °C, the enzyme showed its optimum activity.

A potential extracellular ASNase was characterized from the *Streptomyces griseus* NIOT-VKMA29. Box-Behnken based optimization was used to determine the culture medium components to enhance the L-asparaginase production. In this report the authors have performed molecular characterization ad design of the ASNase gene. Further ASNase biosynthesis gene (*ans*A) from *Streptomyces griseus* NIOT-VKMA29 was heterologously expressed in *Escherichia coli* M15 and the enzyme production was increased threefold (123 IU mL”1) over the native strain (36). Few more actinomycetes sources with the enzyme activity are given in (Table 2).

Plant sources : A few variety of plant species are described with significant amount of asparaginase. Green chillies (*Capsicum annum* L.) and tamarind (*Tamarindus indica*) contain certain amount of ASNase and the enzyme was purified using ammonium sulphate precipitation, sephadex gel filtration and affinity chromatography (37). Enzyme that was isolated from the green chillies was purified up to 400-folds by various methods and it was observed that enzyme exist in two forms and only one of them showed the anti-tumor activity. Enzyme had a pH of 8.5 and a temperature optimum of 37°C. Gene encoding for ASNase enzyme was isolated from plant, *Lupinus angustifolius* (38). The low temperature inducible cDNA sequence that encodes ASNase was isolated from soybean leaves and cloned expressed in *E.coli* with almost has 3 times increased activity (39). *Withania somnifera* is the potential source of enzyme ASNase on the basis of high specificity of enzyme. The ASNase producing micro-organisms from *Ocimum sanctum L* were screened and characterized. ASNase from *Withania somnifera* was cloned and over expressed in *E. coli* with anti-cancer properties (40).

Algal sources : ASNase from a marine C*hlamydomonas* spp. has been purified in 1982. This L-asparaginase has shown limited antitumor activity in anti-lymphoma assay *in vivo.* Properties of this L-asparaginase varied with those of asparaginase from prokaryotic and eukaryotic microorganisms (41).

Table 2. L-asparaginase production by various fungal speices.

S.No	Fungal sp	Substrate/ Media	Operating conditions	Fermen-tation	Activity reported	Reference
1.	Aspergillus niger	Bran of glycine max,Moisture- 70%	30°C, pH – 6.5, 96 h	SSF	40.9 U/g	(52)
2.	Aspergillus sp	L-Asparagine – 2%,Glucose – 1%, Ammonium sulphate – 1%	30°C, pH – 6.5, 160rpm, inoculum – 2%,48 h	SMF	19.5 IU/mL	(53)
3.	Aspergillus niger	Sesame cake	32°C, Aeration – 0.4vvm, Bed height 22cm	PBR-SSF	344.21 IU/g	(54)
4.	Aspergillus terreus	Carob pod with 65%moisture content,30 mm bed depth, particle size 2 mm	35°C, pH – 35°C, pH – 4.5, 72 h 4.5, 72 h	SSF	6.05 IU/g	(55)

Disadvantages of L-asparginase production from bacteria : Bacterial sources are better described for commercial production of L-asparaginase. Properties of ASNase vary from microorganism to microorganism and contrasted with those of prokaryotic and eukaryotic sources (56). ASNase from bacterial sources causes hypersensitivity in the long-term use, leading to allergic reactions and anaphylaxis. It has been observed that eukaryotic microorganisms like yeast and filamentous fungi genera have a potential for ASNase production with less adverse effects than prokaryotic microorganisms. The search for other ASNase sources, like eukaryotic microorganisms, can lead to an enzyme with less adverse effects (57).

Mode of optimization and production process : Researchers performed submerged fermentation for the production of the enzyme ASNase. They have taken the soil sample of *Bacillus* spp., culture conditions were optimized for producing the higher yield of the enzyme (58). The carbon sources like maltose and glucose were used for the production of the enzyme. The most adopted method of production of ASNase enzyme is from submerged fermentation, which has been performed throughout the world. Some limitations of process were observed in later research and to overcome those disadvantages method of solid state fermentation has been adopted. It has several advantages compared to submerged fermentation like low capital cost, higher yield of the product, low energy consumption, usage of less water, simple fermentation media (59-60).

In SSF agricultural waste can be used as source of nutrients which is cost effective and environment friendly (61). SSF holds high potential for the production of secondary metabolites and has been increasingly applied in recent years (62). Abha et.al., reported higher yield of the enzyme from a different isolate of *Aspergillus niger*, agro waste from the leguminous crops as a source. An attempt was made to study the optimized production of L-Asparaginase by *Fusarium equiseti* using soya bean meal under solid state fermentation (SSF) by Hosamani et. al., (63). Solid state fermentation has emerged as a potential technology for the production of microbial products utilizing the cheaply available raw materials. Soya bean meal proved to be one of the best substrate

for L-Asparaginase production. In the present study production of L-Asparaginase started at 24 hours and reached maximum at 48 hours and then decreased significantly with increase in the incubation time.

Optimization of production level of L-Asparaginase from *Erwinia carotovora* was done by Vaibhav et.al., (64). A Central composite Rotatable Design (CCRD) of Response Surface Methodology (RSM) was used to determine the combined effect of the three variables viz. Yeast Extract, Maltose and L-asparagine which were identified earlier using 'one-factor-at-a time' approach by them. The significant variable was Yeast Extract among three variables.

Extracellular L-asparaginase was produced using a fungi isolated from soil. The effect of various physical and chemical parameters was optimized for extracellular L-asparaginase production under submerged fermentation. The maximum L-asparaginase activity of 19.5 U/mL was obtained using MCD medium containing 2% (w/v) L-asparagine as substrate, 1% glucose as carbon source, 1% ammonium sulphate as an additional nitrogen source. The optimum process parameters for maximum L-asparaginase production were: Incubation time 96 h, incubation temperature at 30ºC, initial pH 6.5 and inoculum level 20% (v/v) with 48 h old inoculum (65). The filamentous fungi *Bipolaris* spp. isolated from brown rice in Thailand was identified to produce extracellular L-asparaginase. The maximum L-asparaginase activity of 6.3 U/mL was obtained using MCD media containing 1% L-asparagine and 0.4% glucose at 30ºC in 72 h of incubation. L-asparaginase from *Bipolaris* sp. was proved to be non-cytotoxic when tested against Vero cell lines and has potential application in food industry.

L-Asparaginase production by *Aspergillus* sp. under solid state fermentation was studied using different agro-industrial wastes such as rice bran, green gram bran, wheat rawa, wheat bran, bombay rawa, black gram bran, barley, saw dust, jowar flour, rice flour, castor oil cake, groundnut oil cake, coconut oil cake and sesame oil cake as substrate. Of all the substrates studied that supported growth and enzyme formation by the fungi, groundnut oil cake showed the highest L-asparaginase production. The maximum production of L-asparaginase (60 U/gds) was achieved by using groundnut oil cake. The optimum process parameters for maximum L-asparaginase production were: Incubation period of 5 days, initial moisture content of solid substrate 90%, 1:10 (v/w) ratio of salt solution to weight of groundnut oil cake, inoculum level 20% (v/w), incubation temperature at 30°C and initial pH 6.5.

Enzyme in recombinant form : Due to very high cost of medicinal drug to be used against cancer and side effects of L-asparaginase isolated from *E. coli,* the enzyme is cloned in other vectors to improve the characteristics of the enzyme and reduce the side effects of the enzyme. As the enzyme is mainly used as anti-cancer drug, therefore, the recombinant techniques must be a part of the process adopted for the production of Asparaginase, which can help in reducing the allergic reaction produced by the enzyme and also the cost of the final product as well as the treatment. Considering the importance of implementation of recombinant techniques, Priscila Lamb Researchers have adopted a work plan:

a) Cloning of *E. Carotovora subsp. Atroseptica* L-Asparaginase II *era* gene,
b) Protein expression in *E. coli* cells,
c) Purification of the recombinant enzyme and
d) Measurement of Asparaginase activity and kinectic characterization of this enzyme

Another attempt for generating the recombinant form of the enzyme was made by the Harry et.al. They constructed the whole genomic library of *Erwinia chrysanthemi* in bacteriophage A1059 and purified, isolated anti-Asparaginase IgG were used to detect the recombinants expressing the enzyme. The gene was subcloned in pUC9 and sub-cloning was done to get the actual position of the gene. Recombinants were not observed to repress glycerol as their sole source rather they repress

glucose. Recombinants cells of *Erwinia carotovora* resulted in increased yield of the enzyme (66). To increase the production level of the enzyme, overproducing L-Asparaginase strains through protoplast fusion technique between two highly L-Asparaginase- producer local isolates, i.e., *Bacillus subtilis* and *B. cereus* was developed by Wafaa et.al., (67). *B. subtilis* was found to be sensitive to rifampcin (Rifs) and could utilize L-Asparagine as a sole source of nitrogen while *Bacillus cereus* was resistant to (Rifr), does not grow on minimal medium and cannot utilize L-asparagine.

Treating the cells with 1 mg/ml lysozyme for three hours in SMM buffer caused the protoplast fusion. Protoplast regeneration was successfully obtained on sodium-succinate medium where protoplast regeneration rates were 39.8 and 25.6% for *Bacillus subtilis* and *B. cereus*, respectively. Protoplast fusion was performed between the two parental protoplasts in the presence of 40% PEG 6000. Among forty five fusants, 18 showed significant higher L-Asparaginase activity, they produced approximately 2.5 fold more L-Asparaginase.

L-Asparaginase production by *Aspergillus* sp. under solid state fermentation was studied using different agro-industrial wastes such as rice bran, green gram bran, wheat rawa, wheat bran, bombay rawa, black gram bran, barley, saw dust, jowar flour, rice flour, castor oil cake, groundnut oil cake, coconut oil cake and sesame oil cake as substrate. Of all the substrates studied that supported growth and enzyme formation by the fungi, groundnut oil cake showed the highest L-asparaginase production. The maximum production of L-asparaginase (60 U/gds) was achieved by using groundnut oil cake. The optimum process parameters for maximum L-asparaginase production were: Incubation period of 5 days, initial moisture content of solid substrate 90%, 1:10 (v/w) ratio of salt solution to weight of groundnut oil cake, inoculum level 20% (v/w), incubation temperature at 30°C and initial pH 6.5 (68).

Different patterns of expressions of recombinant L-Asparaginase in different *E. coli* hosts were studied by Gustavo et.al. Mutant strains of *E. coli* were constructed by genetic manipulations with the help of recombinant tools. After amplification of the L-Asparaginase gene from *Erwinia carotovora*, the gene was cloned into the expression vector pET30a (+) and used to transform different *E. coli* strain by electroporation method. A control was kept to verify the results. The *E. coli* strains used were: BL21 (DE3) NH, BL21 (DE3) Star, C41 (DE3), C43 (DE3), Rosetta (DE3), and BL21 (DE3). The cultures were maintained on TB medium at 37°C. As a control, the *E. coli* strain was transformed with the plasmid lacking the L-Asparaginase gene. For expression analysis, 1 mL of the cultures were submitted to electrophoresis on SDS-PAGE. All *E. coli* strains tested showed the higher expression of the L-Asparaginase at 37°C. At the moment, the cultures are being tested at 30°C in order to observe if the temperature will influence in the expression of the protein. The results were promising and can be used for the scale up process to increase the production level of the enzyme (69).

Purification of Enzyme : Juan et.al., *(*70) discovered L-Asparaginase in a significant active form when lysine is over produced in cultures of *Corynebacterium glutamicum*. Purification measures adopted by them include:

- Protamine sulphate precipitation,
- DEAE-Sephacel anion exchange,
- Ammonium sulphate precipitation and
- Sephacryl S-200 gel filtration

98-fold purification was adopted by these methods and the purified enzyme was eluted from gel, when subjected to PAGE, in an active form. The enzyme, sometimes, produced intracellularly.

Purification of L-Asparaginase from *Acinetobacter calcoaceticus* was done by precipitation with streptomycin, chromatography on DEAE-cellulose and CM-cellulose, gel filtration on agarose and chromatography on phosphocellulose. The enzyme catalyzed the

deamination of L-glutamine to about the same extent as L-asparagine and showed a weak tumor inhibitory power (71).

Bacterial L-asparaginases catalyze the conversion of L-asparagine to L-aspartate and ammonia. Kotzia et.al., (72) reported the cloning and expression of L-asparaginase from *Erwinia chrysanthemi* 3937 (ErLASNase) in *Escherichia coli* BL21(DE3) and purification of the enzyme was done by a single-step procedure involving cation exchange chromatography on an S-Sepharose FF column which showed comparatively high activity.

L-Asparaginase (Isozyme II) from *E. coli* was cloned and expressed extracellularly. The resulting recombinant protein was purified by a single step using Ni-NTA affinity chromatography which gave an overall yield of 95 mg/L of purified protein, with a recovery of 86%. This is approximately 8-fold higher to the previously reported data in literature (73). Gladilina et.al., (74) reported the cloning and expression of recombinant protein from *Helicobacter pylori* J99 in *E. coli* (BL21) which was first purified up to 1.8 fold, then sonicated for the preparation of cell free extract and the enzyme was purified from the soluble fraction of cell free extract by chromatography on SP-Sepharose which gave more than 60% yield.

Statistically based experimental design was done to assess the physical process parameter was applied to maximize the production of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428 which after purification via a three step process enhanced the production and productivity of L-asparaginase by 26.39% (specific activity) and 10.19%, respectively (75).

Purification of the L-asparaginase enzyme to homogeneity from *Pseudomonas aeruginosa* 50071 cells that were grown on solid-state fermentation was done by applying different purification steps including ammonium sulfate fractionation followed by separation on Sephadex G-100 gel filtration and CM-Sephadex C50crude culture filtrate. The enzyme was purified 106-fold and showed a final specific activity of 1900 IU/mg with a 43% yield by Khushoo et.al., (74).

The recombinant L-asparaginase enzyme of *Pyrococcus koshii* was expressed in *E.coli* (BL21) and purified by anion exchange chromatography and gel filtration followed by hydrophobic interaction chromatography and ultrafiltration. L-asparaginase of *Streptomyces tendae* isolated from laterite soil samples of Guntur region and the crude enzyme was purified to homogeneity by ammonium sulfate precipitation, Sephadex G-100 and CM-Sephadex G-50 gel filtration (77).

Immobilization of the enzyme : Some of the major limitations in the use of the enzyme are its severe immunological reactions and a very short serum half-life. Modifications like formulation and immobilization of the enzyme onto a suitable matrix can greatly reduce the immunogenicity of the enzyme; increase its half-life and its therapeutic potential.

The immobilization of *E.coli* L-asparaginase into a hydrogel matrix made of poly (ethylene glycol) PEG and BSA showed a 200 fold increase in its Km value and a wider pH range for the optimal activity of the enzyme with 90% of activity at physiological pH of 7.3 as compared to 43% activity for the native form. Also the half-life of the immobilized enzyme enhanced to 50 days with 90% activity at 37 ºC as compared with the half-life of 2 days for the native enzyme. In an another experiment, they coupled *E.coli* asparaginase in a biocompatible hydrogel made of rat serum albumin and PEG and assessed for its effectiveness to deplete the serum L-asparagine *in vivo*. It was found that 85-90% of serum asparagines got depleted in 2 days with 5 units/rat and also 80% activity of the enzyme was still retained even after 10 days (78). Vina et.al., (79) and other reported the immobilization of L-asparaginase from *Erwinia carotovora* on a biologically active fructose polymer levan of different molecular mass (75 and 2000 kDa) obtained from *Zymomonas mobilis*. They employed the method of periodate oxidation of the polysaccharide followed by reductive alkylation which retained.

Immunological side effects and enzyme instability : ASNase administration can promote a number of harmful side effects including immunological responses, ranging from allergic reactions to fatal anaphylactic shock, pancreatitis, coagulation, hyperglycemia, protein synthesis inhibition and hepatotoxicity (80). As far as the immunological side effects are present, the decay of ASNase antitumoral activity is directly associated with the production of L-asparginase antibodies by the patient, which leads to the drug clearance from the bloodstream and reduces the treatment efficacy. In this respect, early studies have shown high circulating levels of ASNase by ELISA with low enzyme activity, which was initially attributed to L-asparginase denaturation (81). However, more recent studies suggest that ASNase clearance may be a result of protease cleavage (82). Immunogenic effects and Protein stability are apparently closely related. The proteolytic cleavage of ASNase may be responsible for additional epitopes exposure, which is involved in the patients' immune response . Hypersensitivity occurs more frequently when the treatment is interrupted, with children presenting less hypersensitivity and antibody production when compared to adolescents and adults (83-84).

Formulation and Modification of the Enzyme : L-Asparaginase has certain side effects and very less half life. Its dosage activates the immune response in the body. It is the need of time to reduce the immunogenicity of the drug with some modification in the structure of the drug. Various formulations and modifications has been tried to improve the influence of the drug. The enzyme from the *E. coli* was modified by Kodera et.al., (85). They manipulated the enzyme by coupling it with two types of comb shaped copolymer of polyethylene glycol derivative and maleic anhydride with multivalent reaction sites. This coupling improves the half life of the modified drug and stabilizes it. The serum retained in the body for a longer period.

Another experiment was performed by Yoshihiro et.al. They modified the enzyme from *E. coli* A-1-3 with activated polyethylene glycols with molecular weights of 750, 1900 and 5000. The modification of enzyme did not show any significant results and the retained enzymic activity was just 7% mPEG2 (2,4 bis(Omethoxypolyethyleneglycol)- 6-chloro-S-triazine) is also used to modify the enzyme by Zhang *et al.* (86), it is performed in the presence of L-asparagine and the molar ratio maintained was mPEG2/-NH2 was 10. The modified enzyme retained 33% of initial enzymatic activity with complete abolishment of immunogenicity and *in vitro* half-life get increased from 4.6 h to 33 h has been obtained (86).

Cross linkage was the technique adopted by Handschumacher et.al., to modify the enzyme to reduce its side effects. They used the dimethyl suberimidate to cross link the tetrameric form of enzyme from *E. coli*. The cross linkage cause the reduction in the activity of enzyme and only 17% of the total activity was retained after modification. Approximately 60% of the enzyme is converted to dimers and higher oligomers (87).

The modified L-Asparaginase from *E. coli* retained the activity of 8% after the modification with monomethoxypolyethylene glycol, reported by Kamisaki et.al., (88). Cyanuric acid chloride was used as a coupler in the reaction. The modified enzyme did not react with the anti- L-Asparaginase antibody in precipitin reaction. It has the same Km value for L-Asparagine and the same optimal pH as the native enzyme. The immunogenicity of the modified enzyme was substantially reduced because mouse antiserum to it showed no significant increase in hemagglutinin titer of L-Asparaginase-coated sheep red blood cells (88).

Fermentation kinetics of L-asparaginase production : Although the production of bacterial ASNases from wide range of bacterial sources has been studied extensively by various researchers during the last few decades, the kinetics of production of this enzyme has not been explored in detail. Liu et al in reported the kinetics of ASNase production by *E. aroideae* and Arrivukkarasan et.al., reported the kinetics of L-

asparaginase production by *P. carotovorum*. The fermentation kinetics and continuous production of ASNase was first studied in the bacterial source *E. aroideae*. Cell growth and ASNase production were investigated in both batch, fed-batch and continuous fermentation using yeast extract as a growth limiting substrate. The relationship between specific growth rate and substrate concentration was found to fit the Monod model. The optimum temperature for enzyme production was 24°C, though cell growth was higher at 28°C. The enzyme yield reached its maximum of 4 IU/mL during the negative acceleration growth phase which occurs just prior to stationary growth. Compared to batch fermentation, the continuous fermentation process gave a lower ASNase yield. The optimum temperature for L-asparaginase production in batch process was 24°C, which was the same as in continuous process. Increasing the temperature from 24°C to 28°C resulted in a 20% loss of L-asparaginase yield .

Kinetics of L-asparaginase production by *P. carotovorum* MTCC 1428 in submerged fermentation (SMF) was studied using yeast extract–tryptone–galactose media, keeping constant fermentation conditions at temperature 30°C, initial pH 7.0 and agitation speed of 120 rpm. The production medium was inoculated with 5% v/v of seed culture in its mid-exponential phase at 24 h. The maximum production of periplasmic and extracellular ASNase was 3.25 and 0.83 U/mL, respectively, at 30 h of fermentation. Unstructured kinetic models such as logistic model for cell growth, incorporated Luedeking–Piret model for L-asparaginase production and logistic incorporated modified Luedeking–Piret model for substrate utilization kinetics were satisfactorily described the fermentation profile of *P. carotovorum*. Kinetics of L-asparaginase production by *P. carotovorum* in submerged fermentation (SMF) was studied using YET (yeast extract–tryptone) media, keeping constant fermentation conditions at temperature 30°C, initial pH 7.0 and agitation speed of 120 rpm. The maximum intracellular and extracellular L-asparaginase activity of 2.28 U/mL and 0.58 U/mL, respectively, were obtained at the late logarithmic phase. The unstructured models predicted the cell growth and product formation profile accurately with high coefficient of determination. No report has been found on kinetics of fungal ASNase production. The information on kinetics of ASNase production was unexplored over a long period of time in spite of its increased commercial need as high value therapeutic protein. The cost of this therapeutic enzyme is high due to the lack of efficient production of this enzyme at large scale. Hence it is important to focus on the kinetic modeling of ASNase production with less adverse effect by fungal sources in submerged fermentation to develop economically viable and efficient bioprocess.

Statistical optimization of L-asparginase production : Screening and evaluation of environmental and nutritional requirements of microorganism is an important step for bioprocess development. Optimization studies involving one factor at a time approach is tedious and tends to overlook the effect of interacting factors but may lead to misinterpretations of the results. In contrast statistically designed experiments tackle the problem effectively, which involves the specific pattern of experiments, which minimizes the error in determining the effect of parameters, and the results are acquired in an economic manner (89). The statistical design of experiments is an efficient procedure for designing experiments so that the data acquired can be analyzed to yield valid and informative conclusions. The planning of experiments begins with determining the objectives and choosing the process variables for the study.

An experimental design is the complete layout of a detailed experimental plan in process of doing the experiment. A screening experiment is performed in order to determine the experimental variables that have significant influence on the response variables. Plackett–Burman (PB) design is an effective screening design when main parameters are to be considered. This is a very economical design with the run number being a multiple of four and

comprises of two-level screening designs. PB experimental design does not consider the interaction among the variables on response variable and it is based on the first-order model. This design is very useful in finding the importance of the factors affecting the product yield in bioprocesses (90).

Response surface methodology (RSM) is a statistical technique based on the fundamental principles of statistics, randomization, replication and duplication, which simplifies the optimization process by studying the mutual interactions among the variables over a range of values in a statistically valid manner. It is an efficient statistical technique for optimization of multiple variables in order to predict the best performance condition with a minimum number of experiments. These designs are used to find improved or optimal process settings, troubleshoot process problems and weak points and make a product or process more robust against external and non-controllable influence. Central Composite Design (CCD) is one of the RSM usually utilized to obtain data that fits a full second-order polynomial model. Variables are coded as ±1 for factorial points, 0 for the center points and ± 2 for axial points. The effect of process variables on response is fit into the second-order polynomial model and it is solved for optimum level of process variables to give maximum response. Statistically based experimental design was applied for optimization of a solid-state fermentation for the production of L-asparaginase by *Pseudomonas aeruginosa* 50071.

PB factorial experimental design was used for evaluation of various culture conditions for their significance on L-asparaginase production. Casein hydrolysate, corn steep liquor and pH were identified as the most significant factors for improving L-asparaginase production process. Box–Behnken design, a type of RSM was used to find the optimum value significant factors for maximum L-asparaginase activity. The maximum L-asparaginase activity of 142.8 IU was obtained at the predicted optimum conditions of pH 7.9, casein hydrolysate 3.11%, and corn steep liquor 3.68%. L-Asparaginase production by *Zymomonas mobilis* by sugarcane molasses fermentation was optimized using factorial experimental design. A model obtained by the response surface methodology was good fit of the experimental data. Maximal enzyme activity of 16.55 IU/L was predicted under the optimum conditions of molasses 100.0 g/L of total reducing sugars, yeast extract 2.0 g/L and fermentation time 21 h. The effect of fermentation process parameters for the production of L-asparaginase by isolated *Staphylococcus* spp. was evaluated using Taguchi methodology, a type of fractional factorial experimental design. The carbon source, nitrogen source, incubation temperature, medium pH, aeration, agitation and inoculum level were evaluated and it was reported that incubation temperature, inoculum level and medium pH were the major influential parameters at their individual level, and contributed to more than 60% of total L-asparaginase production .

The interaction effect of incubation temperature, moisture content, inoculum level, glucose and L-asparagine on L-asparaginase production by *Bacillus circulans* MTCC 8574 under solid state fermentation was optimized using fractional factorial central composite design. L-asparagine and incubation temperature were found to have significant linear and quadratic effect on L-asparaginase production. L-Asparaginase production was improved from 780 to 2,322 U/gds. The effect of various carbon and nitrogen sources on production of L-asparaginase by isolated *B. circulans* MTCC 8574 under submerged fermentation was studied using PB experimental design. Ammonium chloride and glucose were found to be the most significant carbon and nitrogen source respectively for L-asparaginase production. Statistically planned experimental designs were applied to maximize the production of glutaminase free L-asparaginase from *P. carotovorum* MTCC 1428 under submerged fermentation. The fermentation media components such as glucose, L-asparagine, KH_2PO_4 and $MgSO_4.7H_2O$ were identified to have significant influence on the production of L-asparaginase using the PB experimental design. The optimum

levels of glucose, L-asparagine, KH_2PO_4 and $MgSO_4$.7H2O were found to be 2.07, 5.20, 1.77 and 0.37 g/L, respectively, using the central composite experimental design. The maximum specific activity of L-asparaginase in the optimized medium was 27.88 U/mg of protein, resulting in an overall 8.3-fold increase in production. Effect of various medium components on the production of L-asparaginase under submerged fermentation by *P. carotovorum* was studied for optimal nutrient requirements. Maximum intracellular and extracellular L-asparaginase activity was obtained in the medium containing tryptone, yeast extract, monosodium glutamate, K_2HPO_4 and L-asparagine. These medium components were optimized by central composite experimental design.

Maximum intracellular and extracellular L-asparaginase activity of 2.28 U/mL and 0.58 U/mL were obtained respectively in optimized media. Yeast extract, galactose, monosodium glutamate, $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ were identified to be the most significant components on production of L-asparaginase by *P. carotovorum* MTCC 1428 under submerged fermentation. The significant components were further optimized using CCD. Maximum periplasmic and extracellular L-asparaginase product yields of 3.25 U/mL and 0.83 U/mL, respectively, were obtained using the optimized medium components of yeast extract 20.8 g/L, galactose 9.16 g/L, monosodium glutamate 9.89 g/L, $MgSO_4.7H_2O$ 0.304 g/L and $CaCl_2.2H_2O$ 0.042 g/L (91). Although there are many reports found on statistical optimization of bacterial L-asparaginase production, there is no report has been found on statistical optimization of fungal L-asparaginase production. Hence it is important to focus on statistical optimization of media components and process conditions for the production of fungal L-asparaginase to develop cost effective and efficient bioprocess.

Applications of L-Asparaginase

The enzyme ASNase has the chemotherapeutic property against the tumor cells. It is an effective therapeutic enzyme against the treatment of cancers like acute lymphoblastic leukemia. This enzyme helps in catalyzing the hydrolysis of L-asparagine into L-aspartic acid and ammonia. The principle behind the use of ASNase as an anti-tumor agent is that it takes the fact that all leukemic cells cannot synthesize the non-essential amino acid ASN on their own, which is very essential for the growth of the tumor cells, whereas the normal cells can synthesize their own asparagine; thus tumor cells require high amount of asparagine.

L-Asparaginase has a significant role also in food industry. L-Asparaginase from fungal sources is used as food processing aid in food and allied industries to reduce the formation of acrylamide. JECFA (Joint Expert Committee on Food Additives) has recommended the use of L-asparaginase to reduce acrylamide formation for its carcinogenicity and neurotoxicity during processing of high-starch food products (JECFA 2001). Acrylamide is formed as a reaction product between asparagine and reducing sugars contained in starchy food products such as potato chips, frech fries, gingerbreads, roasted coffee and wheat dough based products such as biscuits and crisp breads when heated above 120°C during baking or frying. The heat induced reaction between a reducing sugar and asparagine, which is one of the reaction pathways of the Maillard reaction, forms acrylamide. The Maillard reaction is the process that gives the brown colour and tasty flavour of baked, fried and toasted foods. Incubation of unbaked or un-fried starchy foods with L-asparaginase solution at 37ºC reduces acrylamide level in fried foods up to 90% by converting asparagine into aspartic acid and ammonia, without altering the appearance, taste and quality of the final product (92).

In biosensors: L-asparaginase is applicable in asparagine levels sensing in mammalian and hybridoma cells or the food industry . L-asparaginase used as antioxidant: capable to reduce free oxygen radicals.

Conclusion

The discovery of the fact that ASNase is responsible for the action of anti-tumor activity

against the acute lymphoblastic leukemia has set a good mark in the field of medicine. After this discovery clear information about the enzyme and its mode of action has been dug out. It has been already proved that L-Asparaginase from *E.coli* and *Erwinia carotovora* has anti-neoplastic activity against leukemia and is being used as anti-cancer drugs. But, thorough research it is observed that the action of enzyme is leading to some side effects. Moreover, the yield of enzyme from the present discovered sources was not sufficient to fulfill the demand of the drug. Solid state fermentation has more advantages compared to submerged fermentation to it is adopted at larger scale all over the world. So, there was a need to discover new sources and production techniques to enhance the yield and reduce the side effects of the enzyme. Enzyme isolated from various sources has different optimized conditions for production and activity. The structure of enzyme that was predicted from *E. coli* has four identical units. Recombinant and formulation of enzyme is already in progress, yet there is still a long way to go to increase the yield of the enzyme. Moreover, L-Asparaginase has also have its applications in food industry, as an essential ingredient in reducing the toxicity of baked food by minimizing the amount of acrylamide in food items. So, there is a need to fulfill the thirst of the enzyme because there is a lot of demand for the enzyme in medicine and food industry.

Rational protein engineering based on protein structure is another upcoming strategy to produce ASNases with improved pharmacodynamics, pharmacokinetics and toxicological profiles. Further, approaches involving site directed mutagenesis of residues in the enzymes active site were able to produce recombinant enzyme with good ASNase activity, and negligible GLNase activity. Additional procedures involving the introduction of structural disulfides and cutting of proteases cleavage sites may allow the production of more robust enzymes. There is little information on *Saccharomyces cerevisiae* ASNase and, giving the easy possibility of cultivation and possibility of genetic manipulation of this yeast, they believe that such an enzyme is possible to be better investigated as an alternative to the existing bacterial ASNases. In particular, special attention has to be paid to its better structural and kinetic characterization as well as to the rational engineering of the yeast enzymes by means of site-directed and random mutations. Another interesting technological approach that may contribute to improve the yield of ASNase production by recombinant microorganisms is the metabolic flux analysis (MFA), it is a powerful tool to postulate the metabolic state constrained by exchange of nutrient fluxes between cells and environment.

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SCIENTIFIC NEWS

Nano material cure Parkinson's disease: A metal oxide nanomaterial capable of mimicking all three major cellular antioxidant enzymes that control the level of reactive oxygen species (ROS) inside cells has been designed. These nanozymes possess therapeutic potential to prevent ROS-mediated neurological disorders such as Parkinson's disease. A team of researchers from IISc, Bengaluru has fabricated a metal oxide nanomaterial that is capable of mimicking all three major cellular antioxidant enzymes, thereby controlling the level of reactive oxygen species (ROS) inside cells. Based on *in vitro* test results, the nanomaterial appears as a promising candidate for therapeutic applications against oxidative stress-induced neurological disorders, particularly Parkinson's. Parkinson's model was tested in the lab. The researchers are trying to design an animal model in mice for *in vivo* testing. The results are encouraging and indicate that the nanomaterial is not toxic and the nanozymes have a neuroprotective effect as they prevent neuronal cells from oxidative stress.

Mimic Fish scales to design Oleophobic surfaces in water: Inspired by fish scales that exhibit excellent property to remain oil-free even when the water is contaminated with oil, scientists have developed a special kind of superior oil-repulsive (oleophobic) coating. When applied to any material - wood, glass or metal objects - the coating keeps the surface free of oil contamination under water. By tweaking the composition of the coating, the team has been able to make the coating also extremely oil-loving (oleophilic) under water. One gram of cotton ball coated with the highly oleophilic material was able to absorb over 1,000 grams of oil, irrespective of the density of the oil, the researchers found. The coating developed were stable even at extreme temperatures -up to -15 degree C and 100 degree C. It was also found to be stable for the duration of test (30 days), when the pH of water was reduced to 2 (highly acidic) and increased to 11 (highly alkaline).

Cancer's Principle method of Energy Synthesis was prevented by a Diabetes Drug: Researchers have observed that diabetic patients whose diabetes was being treated with the drug metformin had better chances of recovering from head and neck cancer than non-diabetic patients. During the course of a three-year study, which was detailed in the journal The Laryngoscope, researchers at the Sidney Kimmel Cancer Center at Thomas Jefferson University examined this unintended side effect further and learned a great deal about how metformin affects the biology of cancer cells. In a study of 39 non-diabetic cancer patients, low-dose treatment with diabetes medication metformin resulted in a significant increase in tumor cell death.Though more studies are needed before this can become a recommended cancer treatment, the results are promising as metformin produces almost no unwanted side effects. The research is certainly off to a good start, as these clinical trials showed.

Aging cells have a Reset Button: Our blood changes as we age due to epigenetics, a process by which our gene expression is silenced or activated over time, without modification of the genetic code itself. With this in mind, the team of researchers at the University of Lunds took a look at the hematopoietic stem cells (HSC) of aged mice to see if they could unlock the mysteries of how our cells age. But when the old mice were provided with induced pluripotent stem (iPS) cells - essentially a batch of fresh stem cells - something quite fascinating happened. The iPS cells served as a "reset button", reprogramming the blood stem cells and sparking a rejuvenation of sorts. Researchers observed that the progenitor HSC cells in the old mice began to produce blood cells functionally similar to those seen in younger mice.

Photosynthesis mechanism paves way for Developing Efficient Artificial Solar Panels: A natural process that occurs during photosynthesis could lead to the design of more efficient artificial solar cells, according to

researchers at Georgia State University. This study provide quantitative evidence that inverted-region electron transfer is responsible for the very high efficiency associated with solar energy conversion in photosynthesis.

DNA Folding Mechanism: A Hub of Unseen information: The folding mechanism of DNA is believed to play a large role in how genes are read by the rest of the cell. Biologists have started to isolate mechanical cues that determine how DNA is folded. Now, theoretical physicists from Leiden University in the Netherlands confirmed through computer simulations that these cues are actually coded into our DNA. The team used genomes of baker's yeast and fission yeast to find correlations between the mechanics and the actual folding structure of DNA in the two organisms. The results confirm that this second layer of information exists. This led them to conclude that genetic mutations are not just caused by a change in the sequence of codes but also by a change in the way the strands are folded. This simulation may be helpful in hiding unwanted sequences like those that cause diseases.

Nano Robots kills Cancer cells: Tiny new robots are proving to be life-saving tools in the fight against cancer. As first reported, the scientists have developed nanomachines that are capable of drilling into cancer cells, killing them within minutes. These light-activated nanobots, the size of a molecule, move so rapidly that they can burrow through cell linings of cancer. The researchers found that in order for the nanomachines to function effectively, they need to spin two to three million times per second in order to not be inhibited by objects (or what is known as Brownian motion, or the erratic movement of tiny particles in fluid). When triggered by ultraviolet light, the nanobots begin to spin, allowing them to cut through cancer cells either to destroy the cell or create space for the delivery of beneficial drugs. These nanomachines are so small that we could park 50,000 of them across the diameter of a human hair, yet they have the targeting and actuating components combined in that diminutive package to make molecular machines a reality for treating disease.

NIN develops India's first Nutrition Atlas: The Nutrition Atlas provides information and data on nutritional status of population groups at national and state levels. Hyderabad-based National Institute of Nutrition has developed a Nutrition Atlas to provide a snapshot of all relevant data and information about nutrition India currently faces twin challenges in the nutrition sector - Undernutrition manifesting in several health problems as well as increasing problem of overweight and obesity, contributing to the burden of non-communicable diseases. In order to provide a snapshot of all relevant data and information about nutrition, the Hyderabad-based National Institute of Nutrition (NIN) has developed the country's first Nutrition Atlas. The Nutrition Atlas provides information and data on nutritional status of population groups at national and state levels, along with an overview of nutrition-related deficiencies, disorders and prevalence levels in various parts of the country. In addition, it provides information on nutrients, nutrient rich foods, nutritional deficiency disorders and a host of other topics. The portal also includes information on nutrition rich foods and nutri-guide for various nutrients, minerals, essential amino-acids, fatty acids, dietary fibers and proteins, along with their biochemical cutoffs, recommended dietary allowances, signs and symptoms and dietary sources.

Tropical forests are now the trigger for Global Warming: So much of the Earth's forest has been destroyed that the tropics now emit more carbon than they capture, scientists have found. Tropical forests previously acted as a vital carbon "sink", taking carbon from the atmosphere and turning it into oxygen, but the trend has reversed: they now emit almost twice as much carbon as they consume. Scientists said ending deforestation and degradation in the tropics could reduce global carbon emissions by 8 per cent. They used a new method to assess carbon density, measuring levels not just in areas of complete deforestation but also places where more subtle losses have

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been caused by forest degradation and disturbance. Satellite images, laser technology and field measurements were used to record how vast areas of forest have been lost since 2003. If we're to keep global temperatures from rising to dangerous levels, we need to drastically reduce emissions and greatly increase forests' ability to absorb and store carbon. The study found that the tropics now contribute more carbon to the atmosphere than they take in. 862 teragrams of carbon are emitted while only 437 teragrams are consumed. More than 60 per cent of emissions in the tropics comes from Latin America, which has witnessed large-scale damage to the Amazon rainforest in recent years. 24 per cent comes from Africa and 16 per cent from Asia.

New Categories of Catastrophic to Unknown in changing Global Climate Scenario: A new study evaluating models of future climate scenarios has led to the creation of the new risk categories "catastrophic" and "unknown" to characterize the range of threats posed by rapid global warming. Researchers propose that unknown risks imply existential threats to the survival of humanity. A temperature increase greater than 3°C (5.4°F) could lead to what the researchers term "catastrophic" effects, and an increase greater than 5°C (9°F) could lead to "unknown" consequences which they describe as beyond catastrophic including potentially existential threats. The specter of existential threats is raised to reflect the grave risks to human health and species extinction from warming beyond 5° C, which has not been experienced for at least the past 20 million years.

Cultures of Whales and Dolphins are akin to Humans : Whales and dolphins live in tightly-knit social groups, have complex relationships, communicate with each other and even have regional dialects - just like human societies - a study has found. The study is first of its kind to create a large dataset of cetacean brain size and social behaviours.

Researchers, including those from the University of British Columbia in Canada and The London School of Economics and Political Science (LSE) in the UK, compiled information on 90 different species of dolphins, whales and porpoises.It found overwhelming evidence that Cetaceans have sophisticated social and cooperative behaviour traits, similar to many found in the human culture.The study, published in the journal Nature Ecology and Evolution, demonstrates that these societal and cultural characteristics are linked with brain size and brain expansion - also known as encephalisation.The list of behavioural similarities includes many traits shared with humans and other primates, such as complex alliance relationships and working together for mutual benefit.Researchers also found the social transfer of hunting techniques - teaching each other how to hunt and using tools ocooperative hunting.

NASA says Planet Nine exist in solar system

The elusive 'Planet Nine' does exist, and may be 10 times the mass of the Earth and 20 times away from the Sun than Neptune, NASA scientists say.Planet Nine could turn out to be our solar system's missing 'super Earth' — a planet with a mass higher than the Earth's, but substantially lower than the masses of ice giants Uranus and Neptune.The signs so far are indirect, mainly its gravitational footprints, but that adds up to a compelling case, they have said.Proofs that it exists

"There are now five different lines of observational evidence pointing to the existence of Planet Nine," said Konstantin Batygin, a planetary astrophysicist at the California Institute of Technology (Caltech) in the U.S."If you were to remove this explanation and imagine Planet Nine does not exist, then you generate more problems than you solve. All of a sudden, you have five different puzzles, and you must come up with five different theories to explain them," said Mr. Batygin.Six known objects in the distant Kuiper Belt, a region of icy bodies stretching from Neptune outward towards interstellar space, all have elliptical orbits pointing in the same direction, researchers have said.

Humidity, the key for colour pattern in Butterfly coccons; IISER study

The dark-branded bushbrown butterfly *(Mycalesis mineus)* is one of the most common species in South and Southeast Asia and is found throughout the year in India. These butterflies are known to produce two different coloured pupae – brown and green.Scientists at the Indian Institute of Science Education and Research, Thiruvananthapuram have found relative humidity was one of the deciding factors which caused the change of colour. Brown pupae are more common in drier conditions and develop faster than green pupae. The results were recently published in the journal *PLOS ONE.*Sixteen female butterflies were collected from the IISER Thiruvananthapuram campus and reared in lab conditions. They were released in cages with maize, wheat and ragi plants to lay eggs. Eggs were collected every two days along with the leaf blades and kept in plastic boxes to hatch out. The hatched caterpillars (larvae) were released on maize plants in insect growth chamber. In 20-25 days, the larvae transformed into a pupa.The green pupae were formed mainly under the maize leaves, whereas the brown were almost exclusively found away from the leaves on substrates such as soil. "The pupal stage is the stationary phase and they are more vulnerable to predation. So it is important to camouflage. Merging with the background avoids detection and maybe an adaptive strategy in pupae," explains Harshad Vijay Mayekar, at IISER and first author of the paper.

Novel laser technology may detect explosives

Scientists have developed a laser-based method that can accurately identify chemicals such as explosives and dangerous gases, an advance that can be used in airport security and monitoring environmental pollutants.Researchers from University of Michigan in the U.S. used a method called multi-dimensional coherent spectroscopy that uses ultrashort laser pulses to read types of gases like a bar code.To speed up the process while preserving its accuracy, researchers combined MDCS with another method called dual-comb spectroscopy. Frequency combs are laser sources that generate spectra consisting of equally spaced sharp lines that are used as rulers to measure the spectral features of atoms and molecules with precision.

NOVEL SCIENTIFIC THOUGHTS

Extra ordinary Quantum Distortion: Vacuum birefringence is a weird quantum phenomenon that has only ever been observed on an atomic scale. It occurs when a neutron star is surrounded by a magnetic field so intense, it's given rise to a region in empty space where matter randomly appears and vanishes. Now, for the first time ever, this strange quantum effect has been observed by a team of scientists from INAF Milan (Italy) and from the University of Zielona Gora (Poland). Using the European Southern Observatory's (ESO) Very Large Telescope (VLT), observed neutron star RX J1856.5-375, which is about 400 light-years from Earth.

Novel Smart Drug enhancing the Intelligence of Brain: Qualia is a 42 ingredient 'smart drug' designed to provide users with immediate, noticeable uplift of their subjective experience within 20 minutes of taking it, as well as long-term benefits to their neurology and overall physiologic functioning. The 42 ingredient supplement stack is created by the Neurohacker Collective, a group that boasts an interdisciplinary research.

Connection between Quantum word and Human Mind: Despite all the research we've done, we still know relatively little about how the human brain works, and we know even less about the mystery of "consciousness." Scientists disagree about whether consciousness exists at all outside the illusions of our own collective imagination. Some believe it exists independently although we've yet to understand its origins have brought quantum physics into the discussion. However, it was the British physicist Roger Penrose who pointed out that, observer effect aside, quantum mechanics may be involved in consciousness. More specifically, he thought it

might be possible that quantum events cause molecular structures in the brain to alter their state and trigger neurons in different ways; that literal quantum effects within the brain exist.

Unusual Intelligence emanates from Forgetfulness: A study at the University of Toronto found that having a perfect memory might have nothing to do with your intelligence. In fact, forgetting the occasional detail might even make you smarter. Traditionally speaking, the person who remembers the most things is seen to be the smartest. The study, however, found that forgetting the occasional detail is normal. In fact, remembering the big picture as opposed to little details is better for your brain and your safety, in the long run.

ACADEMIC NEWS

UGC's antiplagiarism regulation: The University Grants Commission (UGC) has released the Draft UGC (Promotion of Academic Integrity and Prevention of Plagiarism in Higher Education Institutions) Regulations, 2017. As the name suggests, the aim of the draft is to create academic awareness about responsible conduct of research and prevention of misconduct including plagiarism in academic writing. The institutes have also been instructed to implement adequate software and other mechanisms which would ensure that thesis, dissertation or any other such documents submitted are free of plagiarism. The Institutes have also been asked to form an Academic Misconduct Panel (AMP) to investigate any allegation of plagiarism and submit report to the Plagiarism Disciplinary Authority (PDA) of the concerned institute.

New UGC parameters to qualify for NET Exam: Changes have been introduced by the University Grants Commission (UGC) in the qualifying criteria of the UGC-NET (National Eligibility Test). According to a statement issued by UGC earlier qualifying criteria for UGC-NET Exam involved qualifying top 15% of those candidates in each subject and category, who obtained the minimum required marks in paper-I, paper-II and paper-III according to the category of the candidates. Subsequent to the orders of the High Court of Kerala, University Grants Commission had revised the procedure and criteria of qualifying candidates and as such it has been decided that 6% of the total candidates who appear in the UGC-NET examination will be declared qualified.

New UGC regulations for PhD admissions in Universities: According to the new sets of draft regulations uploaded by University Grants Commission (UGC) in its website, getting admission to PhD will be difficult. The draft regulation has suggested that the institutions which come under 'Category III Institution', would enroll candidates only who have qualified the NET or SLET or SET examinations for their PhD course.

NOBLE PRIZES 2017 ANNOUNCED

Medicine Nobel Prize for work on Biological Clocks: Jeffrey Hall, Michael Rosbash and Michael Young (all are Americans) awarded for their discoveries of molecular mechanisms controlling our biological clocks and raising awareness about the importance of getting proper sleep.

Physics Nobel Prize for work on Gravitational waves: The 2017 Nobel Physics Prize was divided, one half awarded to Rainer Weiss, the other half jointly to Barry C. Barish and Kip S. Thorne for decisive contributions to the LIGO detector and the observation of gravitational waves.

Chemistry Nobel Prize for work on cryo-electron microscopy: The Nobel Prize in Chemistry 2017 is awarded to Jacques Dubochet, Joachim Frank and Richard Henderson for the development of cryo-electron microscopy, which both simplifies and improves the imaging of biomolecules. This method has moved biochemistry into a new era.

OPPORTUNITIES

Post-Doctoral Fellow opportunity at Indian Institute of Science Education and Research Thiruvananthapuram: IISER, Thiruvanantha-puram invites applications Postdoctoral fellow

opportunity for the Project on 'Role of Periostin-Integrin-áv in adult and fetal hematopoiesis'. For further details, Refer website http://www.iisertvm.ac.in/ openings/read_opening/224.phpx.

Institute Nano Science And Technology, Mohali Post-Doctoral Research Fellows: INST, Mohali invites applications from various science and engineering aspirants for Post doctoral fellow opportunity. Refer http://inst.ac.in/careers.php.

Postdoctoral Opportunities at NCBS/inStem: Applications are invited for Post doctoral fellow opportunities at NCBS/in STEM. Refer website https://www.ncbs.res.in/academic/postdoc

SERB-National Post Doctoral Fellowship (N-PDF): Applications are invited for post doctoral opportunities for SERB- N-PDF. Refer web site http://www.serb.gov.in/npdf.php

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