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RESEARCH ARTICLE

Dynamics of acid phosphatase production by cell suspension system and its further characterization

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ABSTRACT

The current study compares the influence of media engineered media on production of acid phophatase (APase) with its characterization in suspension cell cultures of *Atropa acuminata*, authenticated by rbcL and ITS. Various parameters like pH, inoculum size, temperature, nutrient sources in the medium were optimized for APase hyperproduction in *in vitro* conditions. APase production was accomplished to be evidently amplified at 5% inoculum size (94.90 U/gFW), pH 5.0(99.75 U/gFW) and incubation temperature of 30°C (91.5 U/gFW). Sucrose as carbon and ammonium nitrate as nitrogen sources were eminently suited for maximum enzyme harvest (91.20 U/gFW and 94.56 U/gFW). The APase activity was observed to be distinctly increased in phosphate (Pi) starved suspension cells (197.84 U/gFW). Overall, 6.5 fold increase in APase activity was achieved as compared to the activity before optimization studies. Hence the present Apase production and its activity from *Atropa acuminata* suspension culture may have apparent use for future industrial, agricultural and biotechnological application.

Keywords: Acuminata; Acid phosphatase; p-nitrophenylphosphate; Suspension cells; Pi-starvation

INTRODUCTION

Acid phosphatases (APases; E.C. 3.1.3.2) catalyse the hydrolytic reaction of phosphate (Pi) in phosphate monoesters and anhydrides under an acidic environment (Vincent et al., 1992). Acid Phosphatases are responsible to bring about the production, transport and reprocessing of Pi, a critical macronutrient in cellular metabolism and bioenergetic system (Dotaniya et al., 2019). These enzymes arise as ubiquitous and abundant in plants, animals, fungi, and bacterial systems and in addition take a crucial task in food manufacturing industries to decline phosphate content of food (Yenigun and Guvenilir, 2003; Tagad and Sabharwal, 2018). Eukaryotic APases endure as tissue/cellular specific isozymes that displaying diversity in subunit molecular weight, their localization, substrate specificity and sensitivity to inhibition by various divalent cations and metabolites (Vincent, 1992; Duff et al., 1994). APases obtained from snake venom and various other microbial sources have been used commercially as food additives, (Andrews, 1991) biochemical reagents (wen Su, 1995) and potential therapeutic targets (Bull et al., 2002). Research has shown that plant APases are genetically expressed under varied environmental and developmental conditions that include water or salt-deficit stress, flowering, fruit ripening, seed germination, senescence, pathogen infection and nutritional Pi-deficiency (Vincent et al., 1992; Bozzo et al., 2002; Deng et al., 2022). Several researches have proven an induction of APase in various plants tissues or cultures under phosphate deprived nutritive conditions. In many species, APase induction by Pi deficient plants has been associated with de nova APase systems (Bozzo and Plaxton, 2008). APases are of various types and can be classified on relative substrate specificities as nonspecific APases, with no clear specificity or APases with defined but no absolute substrate specificity.

Purple acid phosphatases (PAPs) catagorised by a distinct class of non-specific APases with a clear a binuclear metal-ion complex at their active site (Vincent et al., 1992; Bozzo et al., 2002). They are what distinguish phosphatases from others in their inherent pink or purple colour, due to the presence of characteristic phenolate to metal charge transfer complex, and insensitivity to L-tartrate inhibition**.** PAPs

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play an important role in plants and animals carrying iron, resorption of bone, redox reactions and antigen presentation in animals (Buhi et al., 1982; Hayman et al., 1996). Plant PAPs can be distinguished from mammalian PAPs in having either Fe(III)– Zn(II) or Fe(III)–Mn(II) binuclear metal centre and having homodimers (110 kDa) (LeBansky et al., 1992; Del Pozo et al., 1999), whereas mammalian PAPs possess only Fe(III)–Fe(II) entity in their active site mostly occuring as 35 kDa monomers (Schenk et al., 2000). However, they own certain similarities also like some structures with conserved sequences possessing metal ligating residues (Schenk et al., 1999). Moreover plant APases appear to have been isolated pertinent and then characterized from varied and considerable sources like roots (Panara et al., 1990), seeds (Park and Van Etten, 1986), fruits (Turner and Plaxton, 2001) and leaves (Staswick et al., 1994). Some of which have been crystallized as well (Strater et al., 1992).

In recent years, plants are being exploited as safe and alternative source for production of commercially important enzymes (Streimikyte et al., 2022). However, plant derived enzymes show poor activities due to presence of polyphenols and thus is not preferred for large scale industrial production (Kunwar and Priyadarsin, 2011). The problem posed by polyphenols can be circumvented by employing plant tissue culture technique. *In vitro* regenerated plant has provided a better option for the accomplishment of many commercially important enzymes. Lately, broadening of plant cell culture system to the production of heterologous proteins (Xu and Zhang, 2014) is being considered now as new enzymatic source in the fermentation industry. *Nicotiana tabacum* cell cultures successfully exploited by Bethesda Research Laboratories, USA has been considered as credible source for production of phosphohydrolases (Buitelaar and Tramper, 1992). Similarly, *Catharanthus roseus* suspension cultures have been utilized for phosphohydrolases production as well (Tanaka et al., 1985). Several studies have deduced greater impact of external factors on the acid and alkaline phosphatases production and their activity both in microbial cultures (Antibus et al., 1986; Manna et al., 2007). However, in accordance with information we have, there are almost no research on the factors optimising enhanced APase production in plant suspension cultures and therefore, immense need is required to explore them for APase production in detail. Keeping this in view, we therefore, attempted to optimise different physical factors such as inoculum density, pH, temperature of culture medium, nutrient factors like nitrogen, carbon, phosphate sources to maximize the enzyme production by the suspension culture cells derived from *Atropa acuminata*. We also tried to study the phosphate starved response of *Atropa acuminata* suspension cultures on the production of APase, in order to check enzyme dynamics in response to these factors. This study thus could provide valuable information for extensive future research on APase in plant cell culture systems.

MATERIAL AND METHODS

Atropa acuminata seed germination

The surface sterilization of seeds of *Atropa acuminata* with 1% sodium hypochlorite were treated with 0.5 M Gibberellic acid. Seed germination was conducted on sterile petriplates using moistened filter paper with distilled water for 15-20 days (at 25°C ±3; relative humidity of 85%). The experiment was done in three set of replicas (Nikolaeva, 1982)

Maintenance of *in vitro* regenerated *Atropa acuminata* cultures

Seedlings of *A. acuminata* were transferred aseptically into MS media and different morphogenetic lines were regenerated as reported earlier (Ahuja et al., 2002). Non-differentiated callus culture with higher enzyme activity (Khan et al., 2016) was exploited for further studies.

Phylogenetic analysis of *Atropa acuminata*

Before embarking on the study, two barcode loci, ITS and rbcL of *in vitro* regenerated plant, were amplified, sequenced and subjected to NCBI database analysis as described by (Ghareb et al., 2020)

Preparation of suspension culture

Heterogeneous cell suspension cultures of *A. Acuminata* were grown as previously described by (Thomas and Street, 1970), till the culture exhibited continual and steady biomass for the inoculum preparation for future studies of different experimental setups of media engineering.

Media engineering for hyperproduction of APase from *A. acuminata*

In order to augment the APase production in *A. acuminata* suspension cultures, optimizing experimental set ups were conducted in triplicates by culturing suspension cells for 16 days before harvesting, under different physical and nutrient conditions: inoculum density (1-10%); incubation temperature (10-40 °C); pH of medium (4.0-8.0); carbon sources (oxalic acid, glucose, dextrose, lactose, fructose, maltose and mannose); sucrose concentration (1-10%); nitrogen sources (urea, ammonium nitrate, casein, yeast, peptone ammonium phosphate, potassium nitrate and ammonium chloride,); phosphate sources (ammonium phosphate, sodium di-hydrogen phosphate, potassium mono-hydrogen phosphate and sodium mono-hydrogen phosphate). To manifest evident, the effect of phosphate starvation on the APase production, phosphate source of the culture medium was depleted in one of the flasks as stated by (Bozzo et al., 2002, 2004a). In all experimental

procedures, production of Apase was analysed following the Tabatabai and Bremner method (1969).

Preparation for assessing activity of crude enzyme extract from optimized *A. acuminata* suspension culture

Cell suspension cultures of *A. acuminata* cultured on normal MS medium were kept under continuous shaking in dark for 8 days, at 28 °C. Subsequently, cells were harvested by following (Bozzo et al., 2002). The centrifuged cells followed by washing with medium deficient in Pi (- Pi) were utilised to inoculate 100 ml fresh optimized (inoculum density of 5 %, initial pH of 5.0, a growth temperature below 40°C, sucrose as a carbon source and ammonium nitrate as a nitrogen source), and Pi starved MS media (-Pi) in culture flasks and then allowed to grow for 16 days on shaker in the dark. The cells were obtained by harvesting and filtering through a Buchner funnel and then filtrate was concentrated employing the method of Bozzo and co-workers (Bozzo et al., 2002, 2004a).

RESULTS AND DISCUSSION

Seed germination and maintenance of *in vitro* raised cultures of *A. acuminata*

Giberallic acid treated *A. acuminata* seeds germinated within 10-12 days at an ambient temperature of 25±2 °C. These young plantlets were used for generating different morphogenic lines including roots, proliferative shoots and non-differentiated callus in different media as reported by Ahuja (Ahuja et al., 2002). The cultures were precisely established and sub cultured after every four weeks as shown in Fig. 1.

Phylogenetic identification of *A. acuminata*

Molecular taxonomical approach employing barcoding with rbcL and ITS primers were used to authenticate and confirm the plant species. rbcL and ITS specific primers amplified 500 bp and 600 bp products respectively and the results obtained after employing sequencing shared revealed that the source plant shared with 96 % similarity *Solanum lycopersicum* thereby confirming it to *A. acuminata* (Fig. 2).

Influence of growth and nutritive factors of culture medium on APase production and its activity from suspension culture of *A. acuminata*

Since static callus cultures exhibited the highest APase activity during early stages of culture growth (Khan et al., 2016), thus it was developed as a progressively proliferating suspension culture by culturing the cells in MS media containing NAA and BAP (1 mgL^{-1}) for further experimentations to scale up the production of APase.

We investigated the effect of growth conditions and nutritive factors on APase production from suspension

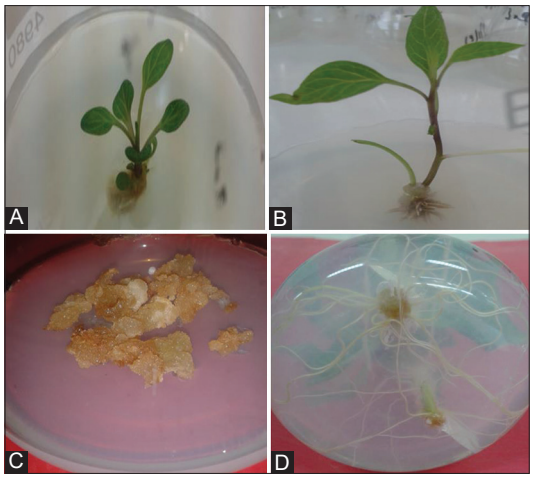

Fig 1. *In vitro* cultured tissues of *Atropa acuminata* regenerated from seedlings following Ahuja et al., 2002 protocol**.** A seedling; B *In vitro* regenerated shoot; C Undifferentiated callus; D *In vitro* regenerated root.

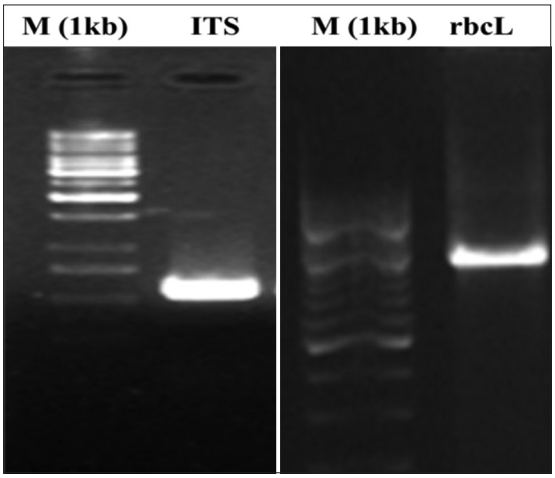

Fig 2. PCR amplification of two regions rbcL and ITS from genomic DNA of *A. acuminata.*

culture of *A. acuminata*. The plant cell culture production scaffolds has distinctly been broaden to the manufacturing of imperative bioactive metabolites and novel enzymes (Buitelaar and Tramper, 1992). The dynamics of APase from varied *in vitro* raised cell cultures including suspension cultures regenerated from *Atropa acuminata* seedlings, were evaluated to recognize optimum producing cell line. The present investigation was aimed to optimize and understand the role of various culture conditions like inoculum size, incubation temperature, pH and various nutritive factors like phosphate carbon and nitrogen sources for enhanced making of APase as such conditions are known to be the fundamental factors affecting microbial APase (Liu et al., 2013). Hence, studies on each part were employed to acquire the suitable environment for hyperproduction of APase. Among the various percentage of inoculum studied (1-10 %), 5 % density exhibited maximum enzyme activity (94.90 U/g FW) as shown in Fig. 3A. Consequently, inoculum

of 5 % size was utilised for further optimization studies. The inoculum density is of prime importance in plant cell cultures, which not only can impact on the growth of cell, but also the production of biomolecules. In our study, APase production was found to be low at lesser inoculum density (1%), while it increased with increasing density, and reached the maximum at an inoculum density of 5%. However, further increase in inoculum density displayed a drop in the enzyme production. Similar results have been discovered in numeral plant cell cultures systems (Wang et al., 1997).

Another fundamental parameter studied was incubation temperature, which determines the dynamics of enzyme. The culture was grown at seven different temperatures ranging from 10 to 40°C. However, aside from 40°C showing eminent decline in APase activity, no prominent effect of incubation temperature was seen (Fig. 3B). Abiotic factors like temperature has multitude of effects as in the metabolic regulation mechanisms, permeability processes, nutritional requirements, and the progression of intracellular systems in plant cell cultures (Zhang et al., 1997). Probably, change in temperature of growth medium would alter the physiology and metabolism thereby sequentially affecting enzyme production. These changes in APase activity may be attributed to the high temperature interference with the cell growth and metabolism.

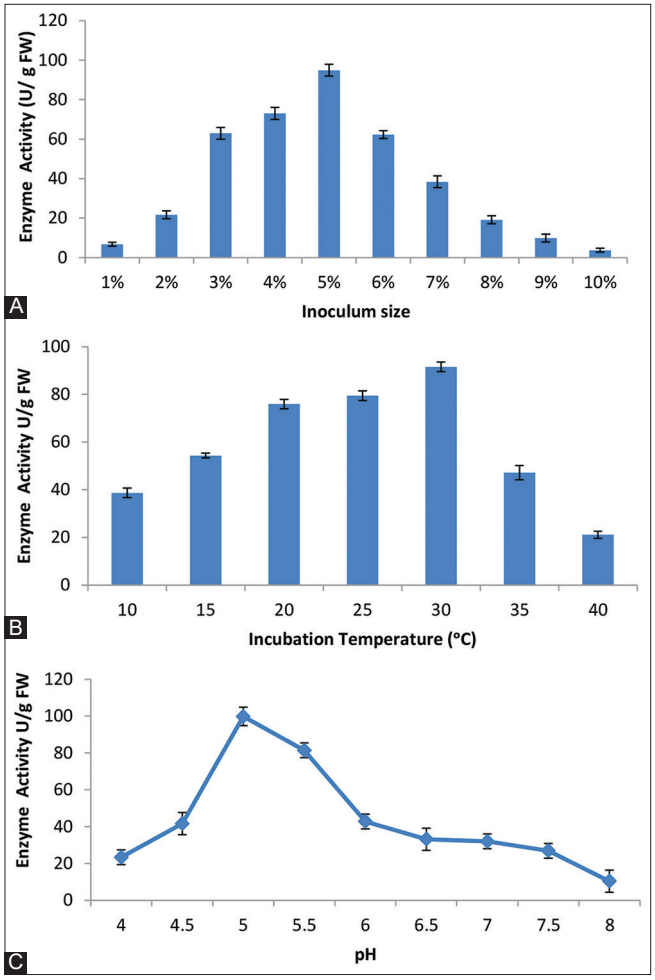

Fig 3. Effect of various factors on Acid phosphatase production. A Inoculum size; B Temperature; C pH. Values of enzyme activity of APase are the mean of three replicates ± standard error.

In another set of experiment, the production medium pH was varied from pH 4.0 to 8.0 and growing the culture at constant experimental conditions. As shown in Fig. 3C, results ranged between 23.33 and 10.35 U/gFW. Among the range of pH investigated, pH 5 was observed to be the best for obtaining optimum APase activity of (99.75 U/gFW). Low pH has been reported to be optimum for varied production of biomolecules in suspension cultures systems (Teixeira da Silva et al., 2016).

Optimization of nutritive factors of culture medium was achieved by various experimental set ups. Nutrition is required to keep cells growing. It was observed that sucrose among various carbon sources was best to achieve maximum enzyme activity. The suspension culture of *A. acuminata* was cultured using eight different carbon sources (sucrose, oxalic acid, glucose, dextrose, lactose, fructose, maltose and mannose) wiliest keeping the rest of media constituents and growth conditions constant. Among the various sources used the maximum production was observed in sucrose (91.20 U/gFW) and minimum was seen in oxalic acid as displayed in Fig. 4A. The sugars oxalic acid and lactose, although they initiated growth of the green callus, did not improved APase production. Fructose (58.20 U/g FW), mannose (61.90 U/g FW), and maltose (70.08 U/g FW) produced less APase activity than sucrose and glucose (79.47 U/g FW). Besides offering the energy required for growth and maintenance, sucrose is also utilised to provide the extensive carbon scaffold of key cell structural components. Further, the concentration of sucrose for the maximum activity was also optimized. Optimization results (Fig. 4B) indicated that sucrose concentrations from 1 to 5 % had an increasing positive effect on APase production in the cell cultures with a maximum activity of102.20 U/g FW and minimum of 3.31 U/g FW at 5 and 10 %, respectively. Literature reports suspension cultures of *Perilla frutescens* accumulates enhanced anthocyanin pigments at high (45 gL^{-1}) sucrose concentration (Zhong and Yoshida, 1995). Although being an osmotic pressure regulator, sucrose might carry a crucial role concerning the osmotic pressure of the medium. The advantageous results of osmotic potential on the accumulation of anthocyanins incited by the high medium sucrose concentrations have been well studied in *Daucus carota* cell cultures (Rajendran et al., 1992). Consequently, it can be figured out that a comparative increased sucrose concentration resulting in a certain amount of osmotic stress, could be quite encouraging for product formation.

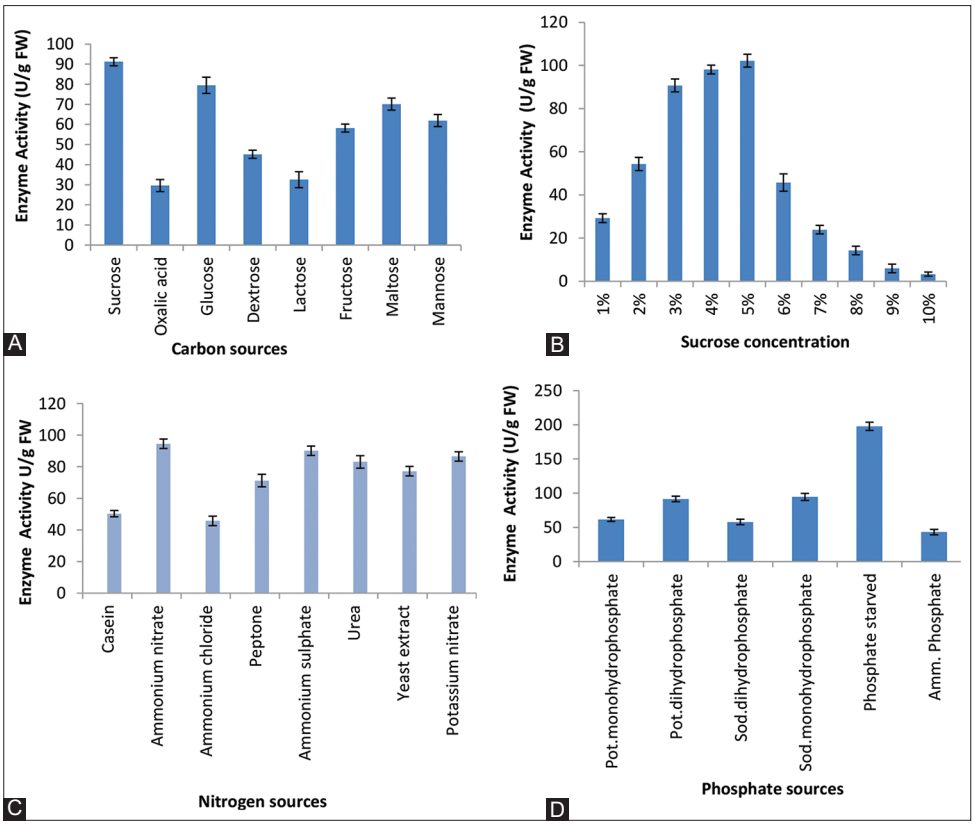

Fig 4. Effect of various substrates on Acid phosphatase production. A Carbon source; B Sucrose concentration, C Nitrogen source; D Phosphate source. Values of enzyme activity of APase are the mean of three replicates ± standard error.

Similarly, while assessing diverse nitrogen sources, ammonium nitrate was found to be best source bearing highest APase activity of 94.56 U/g FW (Fig. 4C). However, ammonium chloride on contrary, lead to the lesser production (45.73 U/g FW) of APase. Besides being the indespensible component of discrete biomolecules, nitrogen plays an key role in making up of nucleic acids and proteins.

Interestingly, in consistent with earlier reports demonstrated that phosphate starved media was best in providing highest APase activity of 197.84 U/g FW (Fig. 4D). It has been reported that APases induction is a distinct and pervasive plant response to Pi starvation. Secreatory APs induced by phosphate starvation have been confirmed in suspension cell cultures and roots of tomato (Goldstein et al., 1988), white-lupin proteoid roots (Miller et al., 2001) and *Arabidopsis thaliana* seedling (Haran et al., 2000).

Therefore, under the above set of optimized conditions as inoculum density of 5 %, growth temperature 30 °C, culture medium pH 5, 5 % sucrose as a carbon source, ammonium nitrate as a nitrogen source with phosphate depleted medium, APase activity in suspension cultures of *A. acuminata* significantly increased to 432.2 U/g FW (Fig. 5). The present investigation could enhance APase activity to 6.5 fold by carrying out engineering of culture

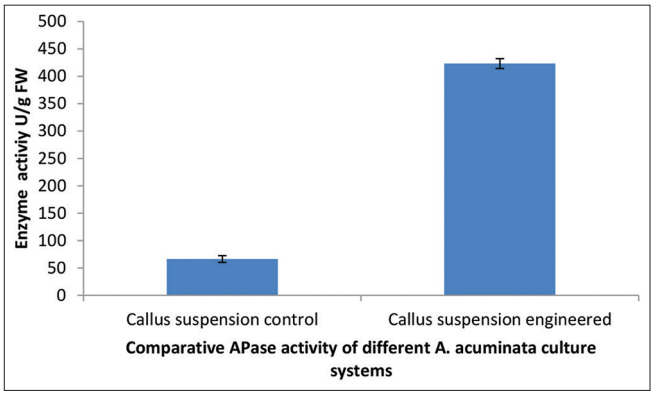

Fig 5. Acid phosphatase activity in optimized suspension culture of *A. acuminata*. Comparative APase activity in various culture systems of *A. acuminata*. Values of enzyme activity of Acid phosphatase are the mean of three replicates ± standard error

media suggesting APase production by *A. acuminata* cell cultures is potentially pragmatic method.

CONCLUSION

The present study implements the existence of cell culture of plant system a viable and expedient approach for APase production at commercial levels. The genetic relationship of source plant was determined by amplifying universal

plastid barcode (rbcL) and nuclear (ITS) barcode loci regions and the results so obtained indicated that the sequence shared 96% similarity thereby confirming it to be *A. accuminata*. In continuation with our previous studies, our media engineering studies involving optimization of abiotic and nutritive factors of production medium particularly while allowing a progression from phosphate sufficiency to its scarcity, have established the possibility for enrichment of production of APase in *A. accuminata* cell cultures. The present investigation of media engineering enhanced APase activity to 6.5 fold that APase production by *A. accuminata* cell cultures is potentially a pragmatic method. The parameters investigated further provided the basis for understanding the molecular regulation of the enzyme. Therefore, the present work is contemplated to be conducive for competent substantial bioprocessing of plant cell cultures performed in bioreactors for future up scaling and bulk production of commercial enzymes.

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Authors' contributions

SK: Experimental methods, data curation, preparation of rough draft. SM: Designing experiments, Manuscript editing. MM and SS: Experimental methods. AA: Supervision of experimental work and resources.

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