

RESEARCH PAPER

Evaluating the Efficacy of Agro-residue Based Culture Media for Growth and Metabolite Production by Endophytic *Fusarium oxysporum*, ZzEF8

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Paper No. 1213

Received: 17-03-2025

Revised: 26-05-2025

Accepted: 03-06-2025

ABSTRACT

Growth media are known to significantly influence secondary metabolite (SM) production with there being a continuing search for cheaper alternatives. Agro-residues constitute potential nutrient sources for growth and metabolite production by different microorganisms. Present study evaluated the potential of rice bran, wheat bran, soyabean chunks and potato peel for umbelliferone (UMB) production by endophytic fungal isolate ZzEF8 (*Fusarium oxysporum*) and compared the data with earlier optimized potato dextrose broth (PDB) medium. Biochemical studies identified higher reducing sugar (975 ± 15 mg/100 ml) and total carbohydrate content (870 ± 1.3 mg/100 ml) in PDB compared to the evaluated agro-residues. These results were supported by higher growth (Fresh weight: 2.08 ± 0.49 g and Dry weight: 0.214 ± 0.06 g) and metabolite production by ZzEF8 mycelia in PDB compared to the other evaluated agro-residue based media. Therefore, despite the environmental benefits associated with use of agro-residues, cost-intensiveness in downstream steps and low SM productivity often pose limitations to their utilization for several microbes. PDB as optimal media for growth and UMB production by endophytic ZzEF8 are discussed in the light of observed variability in composition of agro-residues.

HIGHLIGHTS

- ① Agro-residues showed low and variable umbelliferone (UMB) production by endophytic *Fusarium oxysporum*, ZzEF8 despite being sustainable alternatives.
- ② Maximum growth and UMB production by ZzEF8 were achieved in potato dextrose broth (PDB) due to its high nutrient content.
- ③ Low nutrient content and variability in agro-residue media limit their effectiveness for metabolite production.

Keywords: Agro-residues, *Fusarium oxysporum*, Secondary metabolite production, Endophyte

Endophyte bioprospecting studies have placed incredible attention on endophytic fungi (EF) due to their diversity and exceptional ability to produce structurally novel and complex secondary metabolites (SMs) (Strobel, G. 2018). Endophytes constitute novel sources of cytotoxic metabolites (Uzma, F. *et al.* 2018), antibacterial molecules (Radic, N. and Strukelj, B. 2012), biostimulants (Poveda, J. *et al.* 2021) and biological control agents (BCAs) (Poveda, J. and Baptista, P. 2020) facilitating nutrient solubilization (Strobel, G. 2018), plant growth

promotion (Poveda, J. *et al.* 2021) and induction of plant systemic resistance to biotic (Poveda, J. and Baptista, P. 2020) or abiotic (Cui, R. *et al.* 2021) stress. Studies have reported endophytes to establish either commensalistic or latent pathogenic relationship with the host (Hiruma, K. *et al.* 2016; Alam, B. *et al.*

How to cite this article: Harshitha, K. Anshida, T.H. and Nair, A.R. (2025). Evaluating the Efficacy of Agro-residue Based Culture Media for Growth and Metabolite Production by Endophytic *Fusarium oxysporum*, ZzEF8. *Int. J. Ag. Env. Biotech.*, 18(02): 159-166.

Source of Support: None; **Conflict of Interest:** None





2021) or associate as EF which typically include the majority of *Fusarium* spp, that endemically establish in host tissues without causing disease symptoms (Kumar, K.K. and Dara, S.K. 2021). Due to their ability to colonize diverse hosts (Imazaki, I. and Kadota, I. 2015) together with their outstanding biosynthetic ability (Summerell, B.A. and Leslie, J.F. 2011; Bills, G.F. and Gloer, J.B. 2016; Kaul, S. *et al.* 2016), endophytic *Fusarium* spp. have been isolated from diverse plant taxa (Qin, S. *et al.* 2011; Strobel, G. and Daisy, B. 2003). Though *Fusarium* spp. is generally considered as plant pathogens (Michielse, C.B. and Rep, M. 2009; Fisher, M.C. *et al.* 2012; García-Bastidas, F. *et al.* 2014), several studies have shown the symbiotic association of *Fusarium* spp. with host plants (Jumpponen, A. and Jones, K.L. 2009). Studies have identified *Fusarium* spp. to dominate below ground tissues (Guevara-Araya, M.J. *et al.* 2020). Amongst the diverse *Fusarium* species complexes identified till date from belowground tissues of different plants, *Fusarium oxysporum* (Fo) has been identified as the most abundant EF (Bao, J. *et al.* 2004) such as in *Aristolochia chilensis* (Guevara-Araya, M.J. *et al.* 2020), lawn grass (*Axonopus compressus*) (Zakaria, L. and Ning, C.H. 2013), *Zingiber zerumbet* rhizome (Keerthi, D. *et al.* 2016), tomato (Bao, J. *et al.* 2004), *Oryza granulata* (Naik, B.S. *et al.* 2009), *Oryza sativa* (Yuan, Z.L. *et al.* 2010) and mangroves (Xing, X. and Guo, S. 2011). Being adapted to diverse geographical sites and climatic conditions, *Fusarium* represents a polyphyletic genus documented with global distribution (Stępień, Ł. *et al.* 2018; Qu, B. *et al.* 2008). Due to their ability to produce biomass degrading hydrolases (da Rosa-Garzon, N.G. *et al.* 2019), *Fusarium* spp can colonize diverse plant hosts as endophytes (Bills, G.F. and Gloer, J.B. 2016; Stępień, Ł. *et al.* 2018). Endophytic *Fusarium* spp. from diverse plant sources have been identified to produce more than 100 SMs (Kaul, S. *et al.* 2016) belonging to structural groups like alkaloids, terpenoids, cytochalasins, steroids, anthraquinone derivatives and phenylpropanoids (Ahmed, A.M. *et al.* 2023). With more than 70 species identified so far, *Fusarium* thus represents a cosmopolitan genus and identified as promising resource for bioactive compounds (Toghueo, R.M.K. 2019). Our earlier studies bioprospecting endophytes from rhizomes of medicinal plant, *Zingiber zerumbet* had isolated an EF designated ZzEF8 which was identified as *F.*

oxysporum (Keerthi, D. *et al.* 2016) and characterized as producing the metabolite Umbelliferone (UMB) (Keerthi, D. *et al.* 2023).

SM production by endophytic *Fusarium* spp. have relied on use of potato dextrose (PD) broth or PD agar as commonly used medium (Wongjirathiti, A. *et al.* 2017). Nutritionally rich agro-residues serves as good alternative culture medium for metabolite production that can reduce the expenses by 30-75% of the total production cost (Panesar R *et al.* 2015; Sath, P.K. *et al.* 2018). Several studies have reported the use of various agro residues for production of microbial metabolites (Asagbra, A.E. *et al.* 2005; Ezejiofor, T.I.N. *et al.* 2012; Sukan, A. *et al.* 2014; Saravanan, V. and Vijayakumar, S. 2014). With culture media significantly influencing growth and metabolite production, present study was thus undertaken to determine the effectiveness of agro-residues in enhancing growth and metabolite production by the UMB producing endophyte ZzEF8 (Keerthi, D. *et al.* 2023).

MATERIALS AND METHODS

Biochemical assay of agro-residues

The agro-residues used in present study were procured from local mills and supermarkets. Selected agro-residues (rice bran, wheat bran, soyabean meal and potato peel) were quantitated for various biochemical parameters like reducing sugar by 3,5-dinitrosalicylic acid (DNSA) method, total sugar by phenol sulphuric acid method (Kurzynska-Szklarek, M. *et al.* 2022) and protein content by Lowry's method (Lowry, O.H. *et al.* 1951). For sample analysis, each of the agro-residue (0.2 g) was suspended in 20 ml of 1.5% (w/v) NaOH separately prior to autoclaving at 121°C for 15 min.

Fungal culture and growth conditions

ZzEF8 isolated in earlier studies from *Zingiber zerumbet* (L) Smith (Keerthi, D. *et al.* 2016) and maintained on potato dextrose agar (PDA) slants were used for present study. From the stock cultures, ZzEF8 was sub-cultured on PDA plates (pH 6.5) and incubated at 25 ± 3°C for 7 days in dark. Efficiency of agro-residue to be utilized as growth medium by ZzEF8 was determined by comparing the values with those determined in PDB media that has been optimized in earlier studies for

growth and metabolite production (Saravanan, V. and Vijayakumar, S. 2014).

Effect of various agro-residues on growth and metabolite production

Effect of agro-residues on growth and metabolite production by *ZzEF8* was studied by one-factor-at-a-time method. For this, *ZzEF8* hyphal discs from the actively growing edges of seven-day old culture were inoculated into 50 ml basal media (g/L) [$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6; NH_4SO_4 1.4; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.4; KH_2PO_4 1.6; urea, 0.2; yeast extract, 0.2; peptone 0.25] with agro-residues (rice bran, potato peel powder, soyabean meal, wheat bran (1 g/L) and mineral salt solution [$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 3.34; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ 4.6; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 3 mg/L (pH 7.0)]. Control comprised of PDB inoculated with *ZzEF8* hyphal discs from the actively growing edges of seven-day old culture. Cultures were incubated at $25 \pm 3^\circ\text{C}$ for 11 days. Following completion of incubation period, broth and mycelia were separated using Whatman No.1 filter paper and the mycelial mat was washed with distilled water, moisture removed and fresh weight recorded. Mycelial dry weight was recorded after drying in hot air oven at 50°C for 2 hours.

UMB production in *ZzEF8* culture grown in various agro-residues was determined in both culture broth and mycelia. For the same, the cultures incubated for 11 days were filtrated and centrifuged for 15 min at 4000 rpm at 4°C . Mycelia was cold macerated with dichloromethane (DCM, 5 ml/g) and incubated overnight at 100 rpm at 25°C . Organic phase was collected after centrifugation at 8000 rpm for 15 min and evaporated to dryness at 42°C . Both the supernatant from culture broth and mycelial DCM extract was evaluated for UMB content which was recorded as absorbance units (AU) by spectrophotometric determination over a wavelength range of 190-400 nm (T60 UV-Vis Spectrophotometer, PG Instruments Ltd. UK) as reported in earlier studies (Keerthi, D. *et al.* 2023).

RESULTS AND DISCUSSION

Biochemical parameters of agro-residues: Estimation of reducing sugar, total carbohydrate and protein

Agro-residues serve as natural raw materials that are rich in nutrients like carbohydrates, proteins,

minerals and vitamins thereby constituting potential culture medium for fungal EFs due to their low cost (Lopes, F.C. and Ligabue-Braun, R. 2021). Majority of studies using agro-residues as substrates have been carried out in fungi wherein wheat bran, rice bran and soybean meal have been identified as promising energy sources promoting growth and SM production (Arumugam, G.K. *et al.* 2014). Moreover, present study selected various agro-residues like rice bran, wheat bran, soyabean meal, potato meal based on the fact that *Fusarium oxysporum* isolates display broad host range and have been identified both as EFs from several plant taxa (Ahmed, A.M. *et al.* 2023) as well as phytopathogen infecting diverse crop plants (Michielse, C.B. and Rep, M. 2009). Considering the high variability in nutrient composition of agro-residues, biochemical evaluation was undertaken. Based on the reducing sugar content, the various agro-residues can be arranged in descending order as PDB > Rice bran > Wheat bran > Potato peel > Soya bean meal. Estimation of total carbohydrate content arranged the agro-residues in ascending order as Soya bean meal < Rice bran < Potato peel < Wheat bran < PDB. Total protein present in the agroresidues were determined using BSA as standard. Based on the protein content, the agroresidues were arranged in ascending order as PDB < wheat bran < Potato peel < Rice bran < Soyabean meal. Results thus reveal higher reducing sugar (975 ± 15 mg/ 100 ml media) and total carbohydrate content in PDB (870 ± 1.3 mg/ 100 ml media) compared to the agro-residues tested in present study. However it had the lower protein content (52.04 ± 0.68 mg/ 100 ml media) compared to soybean meal (98.7 mg/ 100 ml media).

ZzEF8 growth and MB production in different culture media

Alterations to the culture media are known to influence not just microbial growth but SM production as well (Miao, L. *et al.* 2006; Antipova, T.V. *et al.* 2015). Despite the classical one-variable at a time (OVAT) process optimization method being time-consuming, the approach is still followed and relevant for optimizing fermentation media of potential endophytes (Singh, V. *et al.* 2017). Earlier studies had optimized fermentation conditions for UMB production by *ZzEF8* using PDA (Keerthi, D. *et al.* 2023). Present experiments compared UMB

production by *ZzEF8* cultures under the earlier optimized liquid state fermentation conditions in PDB with that obtained in agro-residue based media. *ZzEF8* hyphal growth was observed in all of the agro-residue based media as determined based on evaluation of fresh weight and dry weight of the harvested mycelia (Fig. 1). Higher mycelial growth was observed on soya bean meal and PDB (Fig. 1 a and e) compared to other agro-residues (Fig. 1 b-d). Further visual inspections showed higher thickness of mycelial mat and pigmentation in PDB, compared to other media evaluated (Fig. 1 a-e).

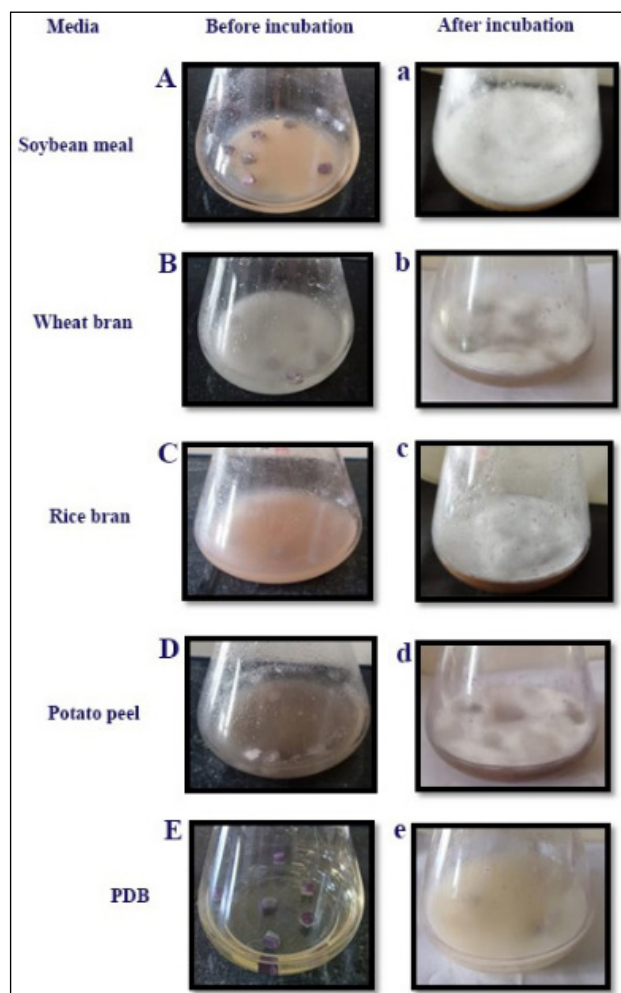


Fig. 1: Growth morphology of *ZzEF8* in different media, A-E: Before incubation. a-e: after incubation period of 11 days

Though in soya bean meal media the mycelial thickness was comparable to PDB, lower pigmentation was observed compared to PDB. Evaluation of *ZzEF8* biomass (wet weight and dry-weight) revealed higher values corresponding to fresh weight (2.08 ± 0.49 g) and dry weight

(0.214 ± 0.06 g) of *ZzEF8* in PDB media followed by soybean meal media (Fig. 2). Low biomass was observed in media based on rice bran, wheat bran and potato peel (Fig. 2). Significant difference in biomass (wet weight) was observed in PDB compared to wheat bran ($p \leq 0.001$) and rice bran ($p \leq 0.01$) (Fig. 2).

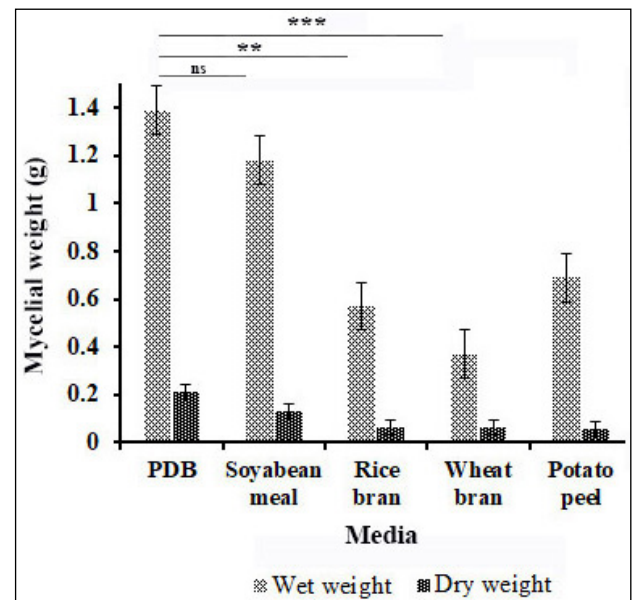


Fig. 2: Growth profile (wet weight and dry weight) of *ZzEF8* in different media. The data represents mean $\pm$ SEM values from triplicate samples for each group, and the significance are shown as $p \leq 0.001$: ***; $p \leq 0.01$: ** and ns: not significant

The differential growth of *ZzEF8* in various agro-residues can be accounted to the inhibitory effect of various agro-residues on microbial growth (Gomes-Araújo, R. *et al.* 2021). Moreover, various studies have cited growth suppression of pathogenic *Fusarium* spp in soil amended with plant residues or agro-residues such as suppression of *Fusarium* wilt in banana by pineapple residue amendment (Yang, J. *et al.* 2023) and inhibition of *F. oxysporum* growth in melon, tomato and cucumber following amendment with plant residue-based compost (Yogev, A. *et al.* 2006).

UMB production was evaluated in *ZzEF8* grown in the selected agro-residue based media. A peak at wavelength ranging from 196 -212 nm was found common to all the samples (Supplementary Fig. 1). In mycelial extract, peak at wavelength ranging from 240 -275 nm was found common to all the samples (Supplementary Fig. 1a-e). Earlier studies had characterized the *ZzEF8* metabolites in PDB

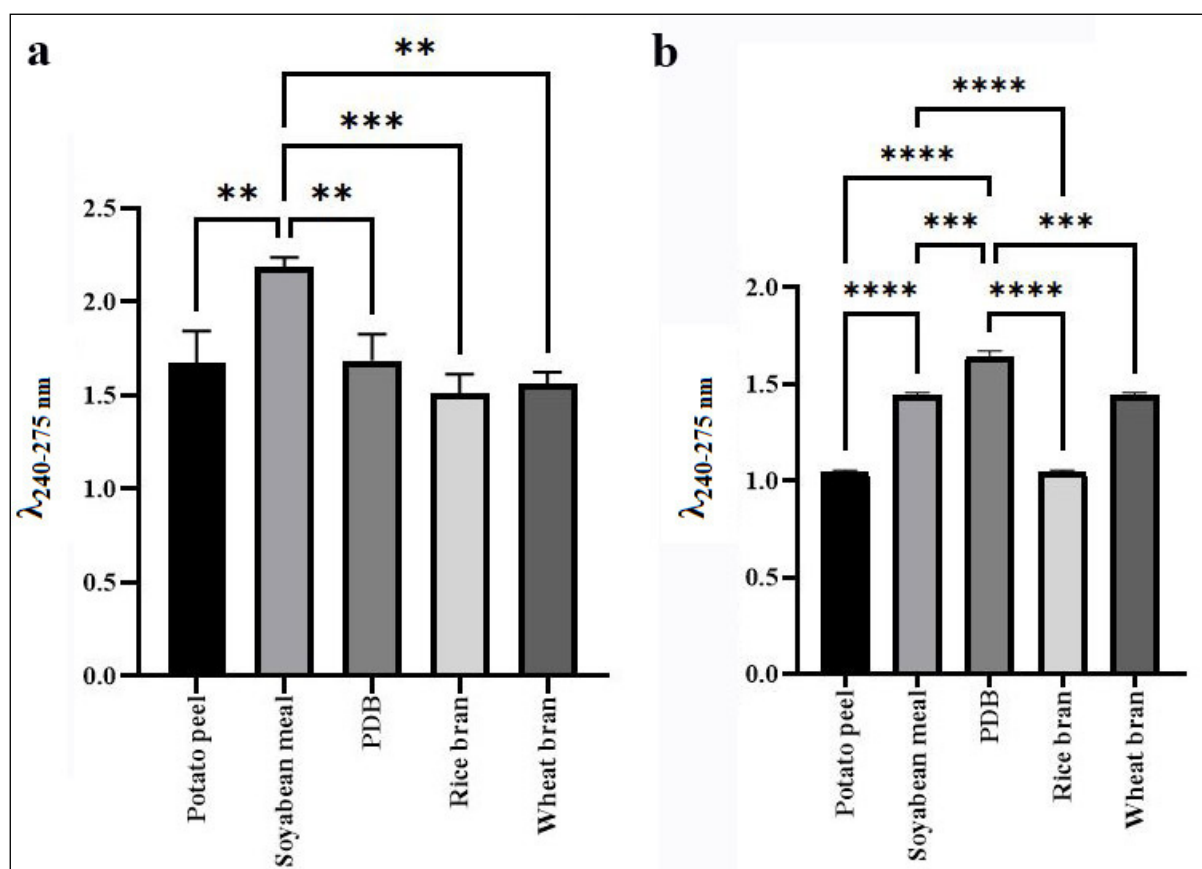
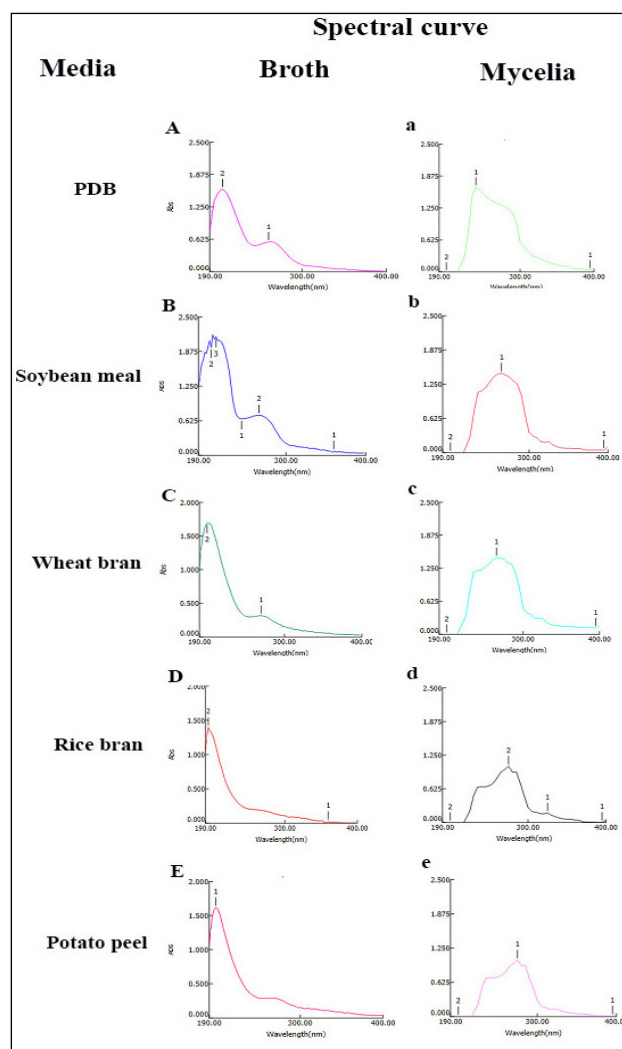


Fig. 3: Metabolite absorbance profile of ZzEF8 (190-400 nm) extract prepared from **a)** broth and **b)** mycelia grown in different media. The data represents mean $\pm$ SEM values from triplicated samples for each group with significance at $p > 0.002$ shown as '**', $p > 0.0002$ as '***' and $p < 0.0001$ shown as '****'.

medium and identified this peak to correspond to UMB (Keerthi, D. *et al.* 2023). Evaluation of metabolite content in broth showed significantly higher UMB content in soyabean meal media (2.18 ± 0.05 AU) compared to PDB (1.68 ± 0.14 AU) and potato peel media (1.67 ± 0.16 AU) ($p > 0.002$) (Fig. 3a). In the mycelial extract, significantly higher UMB content was observed in PDB (1.64 ± 0.02 AU) compared to soya bean meal media (1.44 ± 0.01 AU) and wheat bran (1.44 ± 0.01 AU) ($p > 0.0002$) (Fig. 3b). No additional peaks were detected amongst the media supplemented with various agro-residues but lower peak intensity was observed in rice and wheat bran based media. Higher content of recalcitrant components such as lignocellulose, hemicellulose and lignin in agro-residues like rice and wheat bran could be deterring their utilization for growth by the endophytic ZzEF8. Experiments thus reveal PDB as a better medium for ZzEF8 UMB production compared to agro-residue based media. These observations corroborate earlier reports wherein PDA was identified as best media supporting EF

growth and yielding higher metabolite production such as in *Monascus* spp. for citrinin production (Abdel-Raheam, H.E.F. *et al.* 2022), prodigiosin production by *Serratia marcescens* (Xia, Y. *et al.* 2016) and SM, E2.2 by *Phaleria macrocarpa* (Gasong, B.T. and Tjandrawinata, R.R. 2016). In *Aspergillus fumigatus* strain MF1 (Kalyani, P. *et al.* 2023), *A. terreus* KC 582297 and *Aspergillus* strain TSF 146 (Bhattacharyya, PN and Jha, D.K. 2011; Mathan, S. *et al.* 2013). Present study identified PDB as the best culture medium for yielding higher production of UMB. In *Fusarium* spp., many biosynthetic gene clusters (BGCs) involved in carotenoid pigment production are reported to remain silent under standard laboratory conditions. Such silent BGCs get activated under *in vitro* conditions depending on the nutritional input in media. Present study provides evidence to this aspect as revealed by higher pigment production in PDB medium compared to soybean meal indicative of the requirement of glucose rich sources by ZzEF8 for pigment production. Contrarily soybean meal which

is rich in nitrogen source (amino acids) despite stimulating mycelial growth did not induce pigment production, presumably due to the non-involvement of amino-acid derived intermediary metabolites in pigment production.



Supplementary Fig. 1: Spectral analysis (180- 400 nm) of secondary metabolites observed in extracts from broth (A- E) and mycelia (a- e) of *ZzEF8* cultures grown in different media

CONCLUSION

SM production is known to critically depend on cultivation conditions (Antipova, T.V. *et al.* 2015) such that alterations to the culture medium influences not just microbial growth but metabolism as well (Miao, L. *et al.* 2006). Changing nutrient regimes and medium composition thus constitutes one of the simplest strategies to induce the cryptic biosynthetic gene(s) under *in vitro* conditions (Reen, F.J. *et al.* 2015). Culture media based on agro-residues is considered an efficient and profitable

way to diminish the cost of production (Sadh, P.K. *et al.* 2018). Though several studies have reported agro-residues as alternate energy sources for fungal growth on large scale, these substrates tend to be highly variable due to the differences in traditional processing techniques that fail to guarantee a standardized product (Sadh, P.K. *et al.* 2018). Therefore, despite being considered expensive and relying on a food crop, PDA which contains potato infusion and dextrose as carbohydrate source constitutes a universal fungal media. Study thus demonstrates PDA as ideal medium for growth of endophytic *ZzEF8* which supports luxuriant mycelial growth thereby yielding higher UMB from mycelial extract compared to agro-residue based media.

ACKNOWLEDGEMENTS

HK acknowledges the Grant received from UGC (University Grants Commission, Govt. of India) for the Junior Research Fellowship received (No. 20/12/2015(ii)EU-V). Authors are thankful to Central University of Kerala for the research facilities extended to undertake the work.

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