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Screening of Organic Acids by Fungi Grown on Extracts from Various Leaves after Protein Isolation *In Vitro* : The Novel Industrial Approach

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ABSTRACT

Green crop fractionation (GCF) consists of its by products viz., pulp, pressed crop (PC), juice and deproteinised juice (DPJ). The main fraction of GCF is Leaf protein concentrate (LPC). Experiments proved DPJ induces fungal growth including yeast and their metabolites like enzymes. In this experiment, to grow the fungi, DPJ from different foliages used as a medium for experimental basis and Potato Dextrose broth (PDB) medium as control, for comparison. The liquid medium of grown cultures of fungi were filtered and collected to study the production of organic acids by Sunstornsuk method. The mycelia growth created the zones of yellow halo. *Raphanus* DPJ was found to induce more production of organic acids as compared with PDB medium. As compared with cabbage and cauliflower DPJ, Raphanus DPJ was found more significant. *Trichoderma* was found more significant as compared with *Aspergillus niger* and *Curvularia*. The organic acid values were calculated by Acid Unitages (AU). The separated yellow coloured zones of organic acids caused regrowth of the mycelium of few fungi while some DPJ reduced fungal growth after the yellow halo formation.

INTRODUCTION

Background of DPJ Research

During the preparation of Leaf protein concentrate (LPC) suggested by Prof. N. W. Pirie by the process of green crop fractionation, juice is formed by squeezing pulp of fractionated leafy crop. This juice is heated at 90°C to coagulate the proteins to form the curd and it becomes precipitated. The supernatant formed is isolated and mostly disposed randomly called as deproteinised juice (DPJ). This DPJ is used in research because it consists of carbohydrates, minerals, vitamins, non protein nitrogen *etc* (Jadhav 2018 b). In previous research, it was used to grow economically conventional industrial fungi and can also be as used as the source of metabolites (Gogle *et al*, 2001; Doiphode and Mungikar, 2004 ;Sayyed, 2015; Shende and Gogle, 2016) and can also be used for single cell protein production by yeast fermentation and its secondary metabolites (Mungikar and Jadhav, 2005 ; Jadhav 2018 a). Doiphode *et al*., 2011 investigated the production of organic citric acid by culture filtrate of *Aspergillus niger* cultivated on DPJ from Lucerne (Alfalfa). There was decrease in invertase enzymes by yeast fermentation using DPJ. It was found that DPJ enhances productivity of alcohol by yeast fermentation (Jadhav and Deshmukh, 2018). When different flours were added in DPJ for fungi growth, there was enhancement in the mycelial growth as compared with control as well as productivity of enzyme amylase (Sayyed, 2014). In earlier findings DPJ used to study the activity of fungal enzymes grown on it viz., proteases, cellulases and lipases. This is the novel industrial approach. Activity of the enzyme nitrate reductase varies when the DPJ employed from various plants and treated as soil conditioner for growth of various plants (Jadhav and Gare, 2018). Raddish DPJ also enhanced the rate of seed germination and retarded the dormancy (Jadhav, 2018 c).

During present investigation, the experiment is conducted to use this DPJ as a medium to grow fungi *Trichoderma, Aspergillus niger* and *Curvularia lunata* and to observe its effect on the production of organic acids by sunstornsuks method (Shaikh and Qureshi, 2013). These fungi were also grown on PDB as control to compare DPJ to find out its productivity. DPJ also used from foliages of three different crops viz., cabbage cauliflower and radish for comparative observations.

Organic acids from fungi

Production of lactic acid and succinic acid was studied by HPLC after the effect of phosphate solubilisation for rubber tree growth promotion (Promwee et *al*., 2014). An organic acid is an organic compound with acidic properties. The most common organic acids are the carboxylic acids whose acidity is associated with their carboxyl group -COOH. Organic acids have long history of being utilized as food additives and preservatives for preventing food deterioration and extending the shelf life of perishable food ingredient (Cherrington *et al*., 1991). The organic acids produced by various microbes are citric, gluconic, itaconic, lactic, oxalic, fumaric, malic acid. *Aspergillus niger* produces gluconic acid (Milson and Meers, 1985). Plant pathogens like *Trichoderma* produce oxalic acid, citric acid, succinic acid and lactic acid. Mostly *Curvularia lunata* found as a biotransforming agent of organic compounds and also forms chemicals of hydrocarbons. *Aspergillus niger* can also produce single cell protein. All the cultures of three fungi were further checked for their potential to produce organic acid and their acid unitage value was calculated on modified mineral salt agar incorporated bromocresol green. Unsaturated (cinnamic, sorbic), hydroxylic (citric, lactic), phenolic (benzoic, cinnamic, salicylic) and multicarboxylic (azelaic, citric, succinic) all organic acids are distinguished from other acids by the functional group COOH to which an organic group or a hydrogen atom may be attached. Common names used to describe this group of organic compounds include fatty, volatile fatty, lipophilic, weak, or carboxylic acids.

MATERIALS AND METHODS

Fungi inoculation on PDB and DPJ and their culture filtrates

Fresh DPJ from Cabbage *(Brassica oleracia. Var capitataL)*, Cauliflower *(Brassica oleracia. Var botrytis L)* and radish *(Raphanus sativus.L)* were prepared by green crop fractionation. Each 50 ml DPJ were taken in conical flasks in triplicates and under aseptic conditions, the three fungi *Trichoderma viride, Aspergillus niger* and *Curvularia lunata* inoculated on it. As a control a liquid Potato dextrose broth (PDB) was used to grow these fungi in triplicates.

After 7 days, the liquid medium were filtered and dry biomass was measured obtained on whatman filter paper. The culture filtrate obtained, were collected to use for the study of organic acid detection by zones of yellow halo formation by sunstornsuk method *in vitro*. These culture filtrates were poured in cavities created by cork borer in petri dishes of the solid media of sunstornsuk in aseptic conditions. After the zones of organic acids yellow halo

formation, whether again the initiation of fungal growth on yellow halo takes place was recorded.

Screening for Organic acid production

Isolated cultures were subjected for screening of organic acid production by determining the acid unitage (AU) values. A loopful of fungal spore solution was inoculated on petriplates containing mineral agar acid indicator medium as described (Sunstornsuk *et al.*, 1994) with slight modifications and incubate for the formation of yellow zone around the mycelial growth. The medium used was contained (g/l): Glucose 120g, $(NH_4)2SO_4$ 3.02 g, $MgSO_47H_2O$ 0.25 g, $ZnSO_4.7H_2O$ 0.04 g, KH_2PO_4 0.15 g, Agar 20g, Bromocresol green 0.2, Triton X 100 1.5 ml/l in distilled water (pH 5.5).

Acid unitage (AU) value of the colonies were determined by dividing the diameter of the yellow zone by the diameter of the colonies**.** The colonies having notable acid unitage values were picked up and stored at 4°C. The fungi grown on PDB and DPJ, filtered and the culture filtrates with significant levels of organic acid production was filled by pouring in cavity wells aseptically on sunstornsuk solid media in petri dishes by cork borer (Mukadum and Gangawane, 1982) and the zones of fungi colony along with yellow halo was measured in mm. Yellow halo indicates presence of different organic acids.

RESULTS AND DISCUSSION

Fungal mycelia biomass grown on PDB and DPJ was compared. It was found more on various DPJ culture medium as like earlier investigations. Table 1 indicates the enhanced zone *i.e.* the more productivity of organic acid unitage because of the use of radish foliage DPJ as compared withPotato dextrose broth used *i.e* 2.4 by *Trichoderma*, 2.00 by *Aspergillus niger* and 2.5 by *Curvularia* fungi. The other two DPJ made up of Cabbage and cauliflower foliage induced less acid unitage (AU) of the zone of organic acids as compared with PDB. Table 2 indicates the mean value.

Table 1. Effect of three foliage DPJ on organic acids production by three fungi.

Liquid Medium for Fungal Growth.	Fungi culture filtrates (organic acids zone diameter)								
	Trichoderma viride			*Aspergillus niger*			*Curvularia sp*		
	Zone with fungi (mm)	Fungi Zone with Yellow hallo (mm)	Acid unitage (AU)	Zone with fungi (mm)	Fungi Zone with Yellow hallo (mm)	Acid unitage (AU)	Zone with fungi (mm)	Fungi Zone with Yellow hallo (mm)	Acid unitage (AU)
PDB (Control)	20	32	1.6	25	42	1.68	18	30	1.66
Cabbage DPJ	10	20	2.0	30	40	1.33	25	32	1.28
Cauliflo-wer DPJ	28	35	1.25	18	26	1.44	20	30	1.5
Radish DPJ	15	36	2.4	20	40	2.00	10	25	2.5

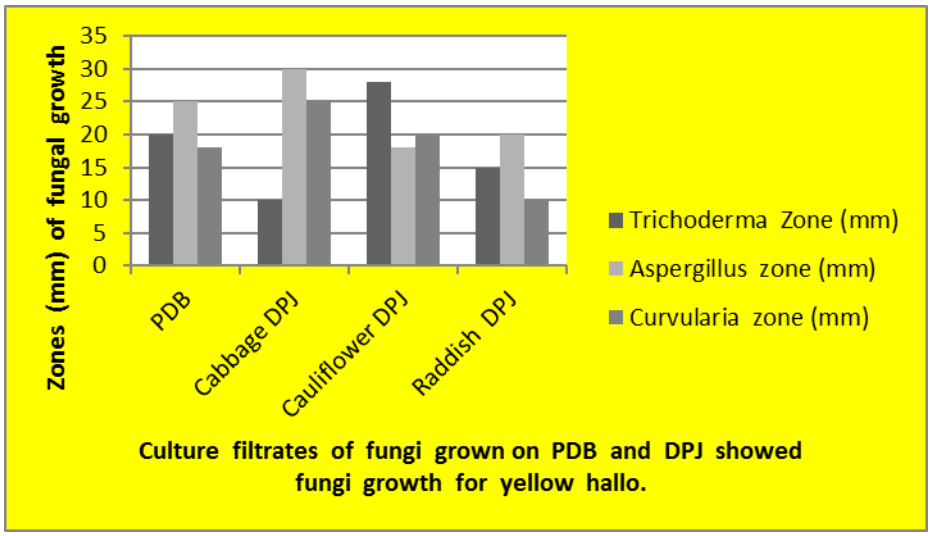


Figure 1. Illustration of various fungi growth after the cavity made by cork borer by pouring culture filtrates of PDB and various DPJ.

Figure 1 illustrates graphically only the growth of fungi in the form of zonal colony *in vitro* on sunstornsuk solid media. These fungal zones form the surrounding zones of organic acids of yellow colour to the mycelial colonies illustrated graphically in figure 2. Figure 2 shows both fungi as well as organic acids combined zone. The formation of zones found started after 4 days.The complete conspicuous zones appearance found after 7 days. There were variation in the effects on fungal colonies and zonal areas by DPJ made from different leaves as compared with PDB grown fungi.

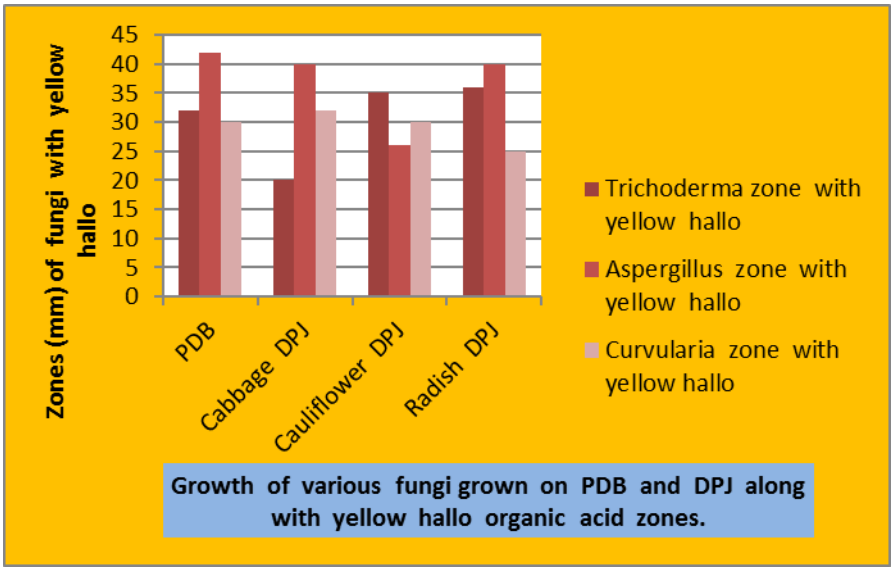


Figure 2. Illustration of Yellow hallo zone of organic acids formation by mycelial surroundings of various fungi from the culture filtrates of PDB and DPJ.

of acid unitage is more by the raddish DPJ i.e. 2.3 A.U. as compared with PDB as control shows 1.64 acid unitage. All the three fungi viz. *Trichoderma viride, Aspergillus niger* and *Curvularia lunata* produced best acid unitage by raddish DPJ. Therefore it proves clearly that if any favourable DPJ is taken into consideration, it can also enhance the production of organic acid. The DPJ from foliages of cabbage and cauliflower showed decreased organic acid productivity as compared to PDB used for fungal growth. During previous investigations it was already proved that DPJ media induces more metabolites by fungi like enzyme productivity *etc* than that of artificial medium. Table 2 indicates cabbage and cauliflower leaf DPJ show comparatively least production of organic acids. Figure 1 shows the graphical presentation of acid unitages (AU) of zones formed by various fungi grown on different deproteinised leaf extracts.

Table 2. Mean Acid Unitage (AU) values by diameter of the zones under the influence of various DPJ used for productivity of organic acids by fungi.

Liquid Medium used for Fungal Growth	Fungi culture filtrates Acid Unitage (AU)			
	Trichoderma viride	*Aspergillus niger*	*Curvularia sp*	Mean
PDB (Control)	1.6	1.68	1.66	1.64
Cabbage DPJ	2.0	1.33	1.28	1.53
Cauliflower DPJ	1.25	1.44	1.5	1.39
Radish DPJ	2.4	2.00	2.5	2.3
Mean	1.81	1.61	1.73	

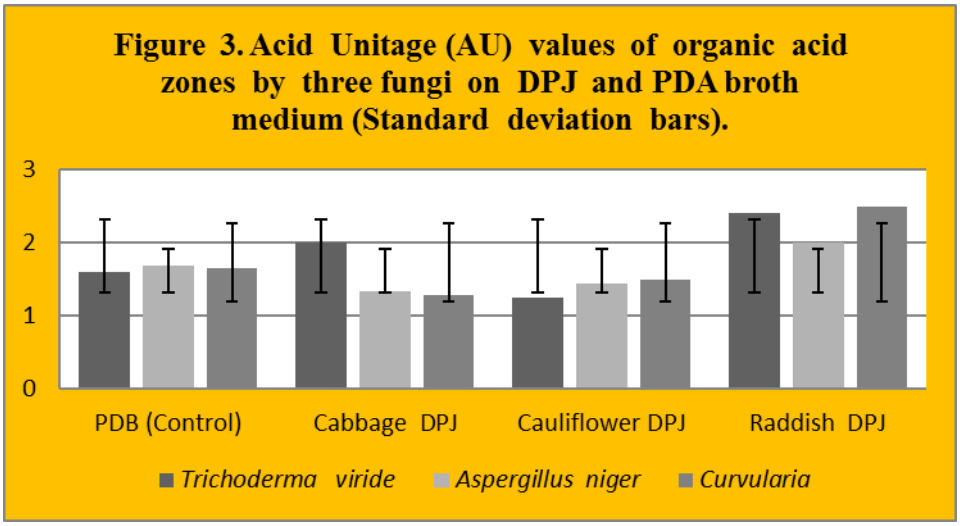
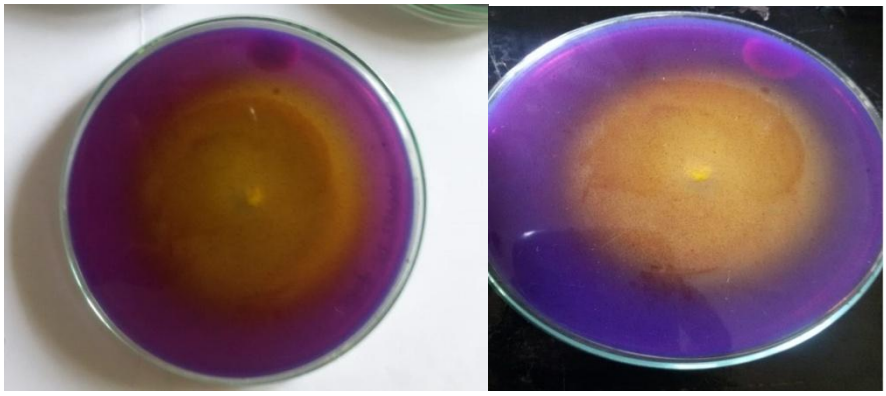


Figure 3. Acid Unitage (AU) values of organic acid zones by three fungi on DPJ and Potato dextrose broth medium (Standard deviation bars)

Figure 3 illustrates the calculated Organic Acid Unitage values of the yellow zones formed surrounding the mycelial biomass of various fungi. It illustrates the standard deviation error bars among the comparative values of PDB as control and DPJ as treated.



(a) (b)

Figure 4. Yellow halo of organic acids from culture filtrates of *Curvularia* grown on (a) PDB (control) and by (b) raddish DPJ.

The figure 4 (a, b) and 5 illustrates the production of organic acid on solid medium *in vitro*, a yellow halo is produced around the colonies which produce organic acid. Figure 4 b petridish showed complete yellow coloured zone of organic acid. Figure 5 shows yellow halo by *Aspergillus niger* grown on cabbage DPJ inducing rapid growth of fungi by the organic acids. Acid unitage values have been determined by measuring the diameter (mm) of colony. Table 1 represents the zone diameters along with yellow halo and acid unitage values. Out of three fungal isolates, *Trichoderma* culture filtrate showed significant acid production. Culture filtrate of *Aspergillus niger* found to be average, *Curvularia* was more than average, and *Trichoderma* were the best.

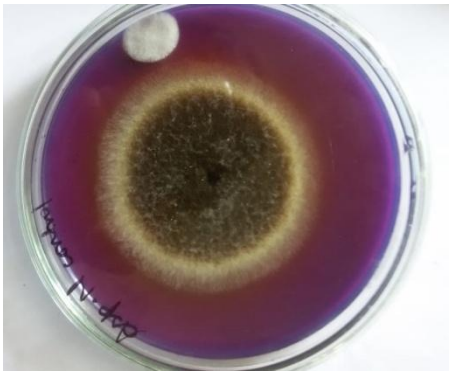


Figure 5. Yellow halo of organic acids of *Aspergillus niger* on Sunstornsuk media in petridish after pouring culture filtrate of DPJ in cavity by cork borer, it also shows the fungi growth on organic acid.

Ramadan *et al* (2015) founded the inhibitory influence on fungal growth by organic acids and formation of toxins at lowest percentages. Table 1 illustrates that as compared with PDB (zone 20 mm), cabbage DPJ (zone 10 mm) reduced the *Trichoderma* growth after formation of organic acids as indicated in table 1. In case of cauliflower DPJ, it reduced *Aspergillus* fungal colony as compared with PDB culture filtrate. While in case of radish DPJ it also reduced the *Curvularia* colony after organic acid zone formation from 18 mm (PDB) to10 mm. Therefore all the DPJ had the efficacy in having the reduction of fungal colonies by their organic acids, especially more by radish DPJ.

Table 1 illustrates the zones of fungi grown in petridishes which forms the yellow halo of organic acids. *Trichoderma* fungi zone on sunstornsuk media was higher because of Cauliflower DPJ, while *Aspergillus* and *Curvularia* zones of fungi was higher by cabbage DPJ as compared with PDB. Therefore this result indicates that DPJ induced growth of fungi after separating the organic acids by sunstornsuk media. Figure 3 clearly indicates the *Aspergillus* growth on yellow halo of organic acid by culture filtrate of cabbag DPJ. Therefore it clearly proves that organic acids can induce fungal growth at specific concentrations by cabbage and cauliflower DPJ. Radish DPJ seems inhibitory to fungal growth after the organic acid formation.

CONCLUSION

Therefore it is concluded that the artificial medium (PDB) when used to grow the fungi, it produces the organic acids. When leafy deproteinised juice is used as a medium to grow the fungi, it enhances mycelial dry weights and also increase the organic acids productivity. It was found that radish DPJ enhanced the organic acids, but reducing regrowth of fungi after yellow halo. Therefore it is predicted that if suitable DPJ is used for fungal growth, it can produce the appreciable amount of organic acids by the suitable fungi. The enhanced yield of organic acids by DPJ can be of industrial or pharmaceutical approach. After the formation of organic acid yellow halo, both cabbage and cauliflower DPJ was responsible to induce the growth of *Aspergillus* and *Curvularia* mycelia. Therefore organic acids can cause the growth of fungi. Few plant species DPJ may reduce the fungal growth after organic acid ormation. Radish DPJ enhanced organic acid unitage values among all fungi as compared with cabbage and cauliflower DPJ. There were variations in expression by fungi to form organic acids by the influence of DPJ and vice versa. Further investigations on possibilities of organic acids

production on commercial scale are needed to exploit full potential of the DPJ left after leaf protein production.

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