Solid-State Production of Beneficial Fungi on Apple Processing Wastes Using Glucosamine as the Indicator of Growth

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Three *Trichoderma* species, a *Penicillium* species, and a *Rhizopus* species were grown on apple pomace at 25 °C through solid-state fermentation. The effects of $CaCO_3$, water, and nitrogen sources on the growth of selected fungi on apple pomace were investigated. Soluble protein and glucosamine contents of fermented pomace were measured as the parameters of fungal growth. The maximum growth of all fungi was established on apple pomace supplemented with 0.05 g of $CaCO_3$, 2 mL of water, and 0.05 g of NH_4NO_3 or 0.3 mL of fish protein hydrolysate per gram of pomace. The optimal water activity of the medium for fungal growth was 0.96 at 25 °C. This research has provided a clear indication for using glucosamine as a proper indicator of fungal growth in heterogeneous solid-state systems. It also shows potentials for bioconversion of apple processing wastes by beneficial fungi into valuable bioinoculants that are being targeted for agricultural and environmental applications.

Keywords: *Apple pomace; bioinoculant; biomass; fish protein hydrolysate; glucosamine*

INTRODUCTION

Apple pomace is the waste residual remaining after apple juice extraction and represents ~25% of the original fruit. In the United States, >500 food processing plants generate a total of ~1.3 million metric tons of apple pomace as byproduct each year. Apple pomace consists of insoluble carbohydrates with lesser amounts of protein, minerals, and some remaining juice with sugars and other substances (Carson et al., 1994). Traditionally, the apple pomace is mainly used as animal feed or disposed to soil. However, its value as animal feed is relatively limited due to its low protein content. The disposal procedure of apple pomace by trucking to land for application to the soil is costly and also poses serious environmental problems (Jewell and Cummings, 1984; Hang, 1987). More recent research interest has focused on biological conversion of apple processing waste into various value-added products through solid-state fermentation, and such products include ethanol (Hang, 1982; Gupta et al., 1989; Ngadi and Correia, 1992), citric acid (Hang and Woodams, 1986), mushrooms (Worrall and Yang, 1992), and various enzymes (Hang and Woodams, 1994a,b, 1995). In this direction, more practical approaches and more valuable products are to be developed.

Some *Trichoderma* species are able to produce various industrially important enzymes such as proteinases (Manonmani and Joseph, 1993), ribonuclease (Vasileva-Tonkova, 1993), xylanase (Ujiie et al., 1991), and chitinases (Cruz et al., 1992). Certain *Trichoderma* species can also be used in agriculture as biological control agents against pathogenic organisms that usually cause many plant root diseases (Adams, 1990; Andrews, 1992). *Trichoderma harzianum* was also found to be capable of degrading certain organochlorine pesticides such as DDT, dieldrin, endosulfan, pentachloronitrobenzene, and pentachlorophenol and hence has potential applications for environmental bioremediation (Katayama and Matsumura, 1993). In the present study three commonly used *Trichoderma* species were selected for potential *Trichoderma* bioinoculant development.

Rhizopus oligosporus was also used in the present study because it has been used in solid-state fermentation for several centuries, especially in Asia for preparing many fermented foodstuffs such as tempeh (Rehms and Barz, 1995). It not only enhances the digestibility and protein content of foodstuffs but also prevents the formation of toxic substances (Soccol et al., 1994). It could be useful in developing potential protein-enhanced product from apple pomace for use as animal feed. Another fungus used in this study is a *Penicillium* isolate that is capable of decolorizing polymeric dyes in liquid system (Zheng et al., 1997). This isolate has potential applications in bioremediation of water or soil contaminated with polymeric dyes.

In addition to apple processing waste, fish offal, a major fishery byproduct, is another food processing waste the disposal of which causes environmental problems. Due to its high nitrogen content it can be complimentary to apple pomace (Martin and Chintalapati, 1989), and its hydrolysate could be supplemented to apple pomace to enrich the medium for fungal growth. The objective of the present investigation was to determine the feasibility of growing the above beneficial fungi on apple pomace wastes, coupled with utilization of fishery waste, to generate value-added fungal bioinoculant products. A secondary objective of this investigation was to determine if glucosamine content could be a reliable indicator of fungal growth in the solid-state system based on apple pomace waste.

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MATERIALS AND METHODS

Microorganisms. *Trichoderma viride* IF-26, *T. harzianum* ATCC 24274, and *Trichoderma pseudokoningii* ATCC 26801 were obtained from American Type Culture Collection (Rockville, MD); a *Penicillium* sp. ATCC 74414 that decolorized polymeric dyes was isolated in our laboratory (Zheng and Shetty, 1997); and a tempeh fungus, *R. oligosporus*, was obtained from Life-Life Foods Co., Greenfield, MA.

Media and Cultivation Condition. All fungi were maintained on potato dextrose agar (PDA) medium at 4 °C and subcultured monthly. All fungi were cultured at room temperature for 7 days before use. Solid-state fermentation was carried out in 125-mL Erlenmeyer flasks, each containing 10 g of apple pomace, 0.5 g of $CaCO_3$, 20 mL of water, and 0.5 g of NH_4NO_3 or 2 mL of fish protein hydrolysate (FPH) as the supplemental nitrogen source. The apple pomace obtained from Veryfine, Inc., Westford, MA was dried, ground to <2 mm in size, and stored in refrigerator before use. The water content of apple pomace was 10.5% (w/w, wet basis). FPH was obtained from Ocean Crest (Gloucester, MA) as herring waste containing 0.6575 g/mL of soluble solids. The spores from one PDA plate were inoculated into ~20 flasks. The culture flasks were incubated at 25 °C for 4 days.

Protein Assay. One hundred milliliters of distilled water was added into cultured flasks, and the culture was homogenized using a Waring blender and then centrifuged at 1500*g* for 15 min. The supernatant was used for protein assay. Soluble protein was determined using a commercial assay kit (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) with bovine serum albumin as standard, according to the procedure described by Bradford (1976). The soluble protein produced by fungal strains in the apple pomace medium was expressed as milligrams per gram of pomace.

Moisture Content and Water Activity Determination. A known weight of the sample (apple pomace medium) was heated to 105 °C to constant weight; the moisture content (MC) was calculated on wet basis. The water activity (a_w) of apple pomace medium was determined according to the method described by McCune et al. (1981). A reference material (circle of filter paper) of known sorption isotherm was equilibrated for 24 h to the sample, and the water activity of the equilibrated filter paper was calculated using the linear equation of isotherm curve from its moisture content, which was determined by measuring weight gain of the filter paper during equilibration. The water activity of the sample was equal to that of the equilibrated filter paper. The relationship between MC (wet basis) and a_w of the sample was expressed as the following equation

$$\mathrm{MC} = A + B \log(1 - a_w)$$

where, at 25 °C, $A = 0.0033$ and $B = -0.1155$.

Glucosamine Assay. The glucosamine content of fermented pomace was used to estimate the fungal biomass during the solid-state fermentation, which was also the indicator of growth. It was determined according to the modified method of Sakurai et al. (1977). The culture mixture in each flask was mixed with 100 mL of distilled water and homogenized using a Waring blender. One milliliter of suspension of the homogenized sample was mixed with 2 mL of H_2SO_4 (98%) in a test tube. After standing for 24 h at 25 °C, it was diluted with 47 mL of water and autoclaved at 120 °C for 1 h. The hydrolysate was then neutralized with NaOH to pH 7.0 and diluted to 100 mL, from which 0.5 mL was mixed with 0.5 mL of $NaNO_2$ (5%) and 0.5 mL of $KHSO_4$ (5%) in a centrifuge tube. After shaking occasionally for 15 min, it was centrifuged at 1500*g* for 2 min; 0.6 mL of supernatant was mixed with 0.2 mL of $NH_4SO_3NH_2$ (12.5%) and shaken for 3 min. To the mixture was added 0.2 mL of 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH; 0.5%, prepared daily), and then the mixture was boiled for 3 min and immediately cooled to room temperature; 0.2 mL of $FeCl_3$ (0.5%, prepared within 3 days) was added. After standing for 30 min, the absorbance at 650 nm was measured spectrophotometrically. The glucosamine content was calculated as milligrams per gram of pomace according to the standard curve.

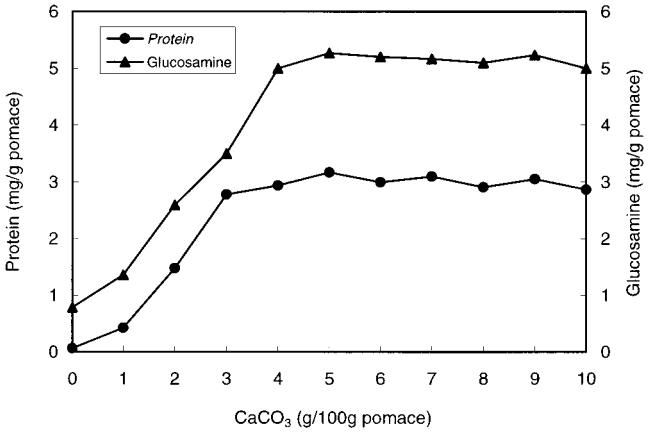

Figure 1. Effect of $CaCO_3$ addition on growth of *T. viride* IF-26 on apple pomace medium.

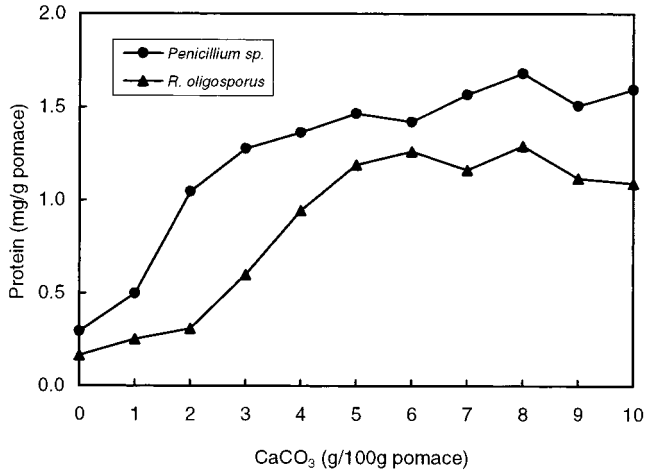

Figure 2. Effect of $CaCO_3$ addition on growth of *Penicillium* and *Rhizopus* strains on apple pomace medium.

RESULTS AND DISCUSSION

Effect of $CaCO_3$ Addition on Fungal Growth. Due to organic acid content, apple pomace has relatively low pH (≈3.1−3.8). To obtain the maximal fungal growth, it is necessary to neutralize the pomace medium before inoculation. Considering the desired pH range for fungal growth, $CaCO_3$ was an ideal neutralizer because it increased the pH of apple pomace medium to 5.8−7.0 and it is inexpensive for large scale use. *T. viride*, *R. oligosporus*, and *Penicillium* sp. were used to examine the effect of $CaCO_3$ addition on fungal growth on apple pomace (10 g) supplemented with 20 mL of water and 0.05 g of NH_4NO_3 in each flask. The growth study indicated that the addition of 0.05 g of $CaCO_3$/g of pomace would be satisfactory for the growth of all three selected fungi (Figures 1 and 2). It was also revealed that the soluble protein measurement was consistent with the glucosamine measurement as the growth indicator of *T. viride* (Figure 1). In our previous studies on the growth of these fungi on cranberry pomace, similar amount of $CaCO_3$ was also required for fungal growth (Zheng and Shetty, 1997).

Effect of Water Addition on Fungal Growth. Inconsistent moisture levels can negatively impact porosity, oxygen diffusion, and gas exchange. A higher

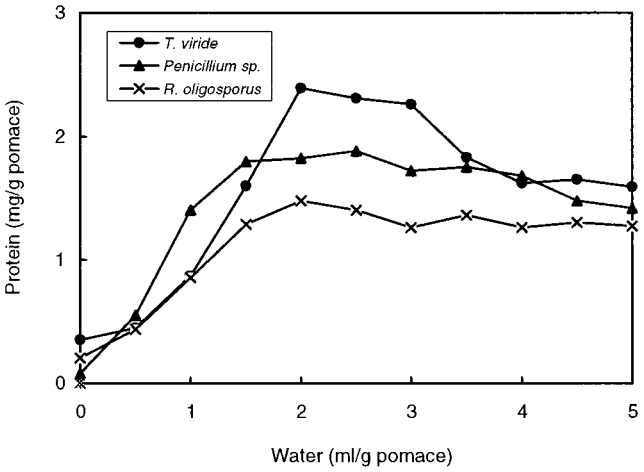

Figure 3. Effect of water addition in apple pomace medium on growth of *T. viride*, *Penicillium*, and *Rhizopus* strains.

Table 1. Effect of Water Addition on the Moisture Content (MC) and Water Activity (a_w) of Apple Pomace Medium at 25 °C

water addition (mL/g of pomace)	MC (%, wet basis)	a_w
0	10.5	0.457
1	68.3	0.960
2	80.7	0.981
3	86.2	0.996
4	89.2	0.997
5	91.1	0.998

moisture content as a result will increase aerial mycelium, and low moisture content will reduce fungal growth. The moisture content of the medium is therefore a critical factor influencing the growth of fungi in solid-state fermentation (Cannel and Moo-Young, 1980; Zheng and Shetty, 1997). To determine the optimal amount of water content for fungal growth, the soluble protein content was measured as the growth parameter of *T. viride*, *Penicillium* sp., and *R. oligosporus* on apple pomace medium with NH_4NO_3 as the additional nitrogen source. All fungi tested in the experiment grew very poorly if no additional water was added to the medium, while they exhibited maximum growth when 2−3 mL of water/g of pomace was added. It was also observed that all three fungi had very similar growth patterns in response to water addition (Figure 3). These results are also consistent with studies on cranberry pomace (Zheng and Shetty, 1997).

Moisture Content and Water Activity. It is important to understand the effect of moisture level of the medium on the fungal growth, and this can be best expressed in a solid-state medium using water activity (a_w), which indicates the availability of water for fungal growth. The moisture content and water activity of the apple pomace medium corresponding to each level of water supplemented to the medium were determined using the methods described under Materials and Methods. Since addition of ∼2 mL of water/g of pomace gave the optimal fungal growth for all selected fungi (Figure 3), the corresponding optimal moisture level was ≈81% (wet basis) and the water activity was ∼0.98 at 25 °C (Table 1). Our previous study showed that these fungi were able to grow optimally on cranberry pomace with a lower moisture content of 67%, while the water activity was 0.99, which is close to our present result (Zheng and Shetty, 1997).

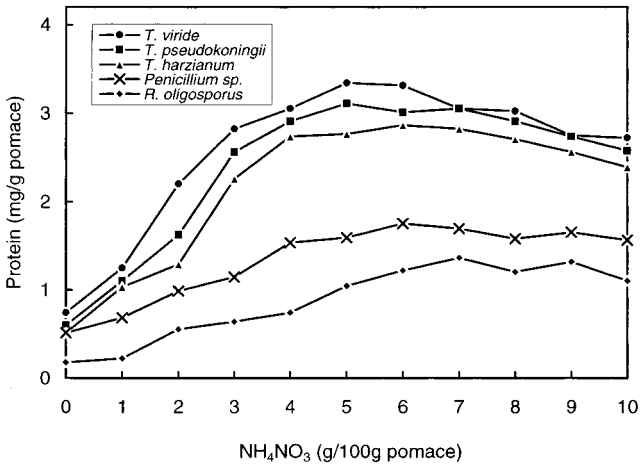

Figure 4. Effect of NH_4NO_3 addition in apple pomace on growth of *Trichoderma* spp., *Penicillium*, and *Rhizopus* strains in terms of soluble protein production.

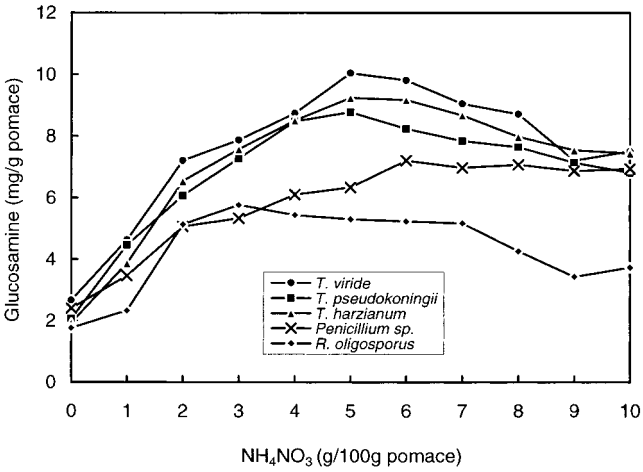

Figure 5. Effect of NH_4NO_3 addition in apple pomace on growth of *Trichoderma* spp., *Penicillium*, and *Rhizopus* strains in terms of glucosamine production.

Effect of NH_4NO_3 Addition on Fungal Growth. One of the critical factors of nutritional regulation of microbial growth in solid-state fermentation is the C/N ratio. In most solid-state fermentation systems, the carbon source comes from the natural soluble and insoluble carbohydrates, while the nitrogen source is added. In this study, apple pomace contains enough fermentable carbohydrates that could be used as carbon source, but a relatively low level of nitrogen source available for fungi becomes the limiting factor for fungal growth. Therefore, to obtain the optimal growth of fungi on apple pomace, additional nitrogen source was needed. Two kinds of nitrogen sources, NH_4NO_3 and FPH, were used for five selected fungi. The fungal growth was monitored by measuring both soluble protein and glucosamine content of the mycelia. The effect of NH_4NO_3 on the growth of all selected fungi is shown in Figure 4 (in terms of soluble protein production) and Figure 5 (in terms of glucosamine content). Both soluble protein and glucosamine measurements indicated that addition of ∼0.05 g of NH_4NO_3/g of pomace resulted in the maximal growth of all selected fungal strains (Figures 4 and 5). Growth studies indicated that the soluble protein content coincided with the glucosamine content in all of the selected fungi, which is consistent with previous observations (Figure 1). Similar results were

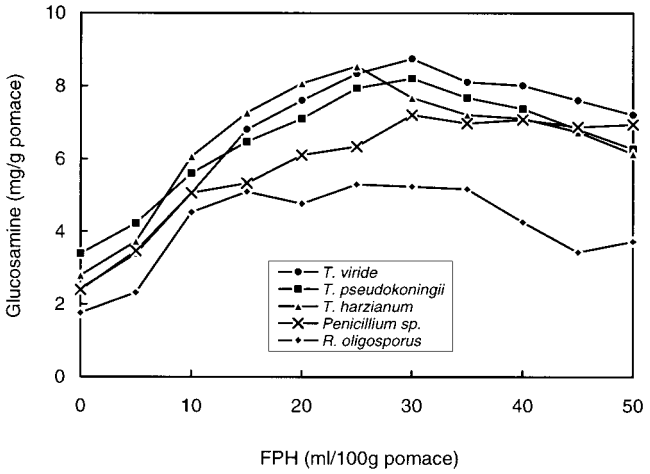

Figure 6. Effect of fish protein hydrolysate (FPH) addition on growth of selected fungi on apple pomace in terms of glucosamine content.

also observed for fungal growth studies in cranberry pomace (Zheng and Shetty, 1997).

Effect of FPH on Fungal Growth. Besides apple pomace, fish offal is another important food processing waste, and its disposal problems need new solutions (Martin and Chintalapati, 1989). By enzyme and acid hydrolysis, the fishery byproduct was converted into FPH in which high concentrations of nutrients such as nitrogen were expected to enhance the fungal growth in apple pomace medium. It has been reported that the growth of *Scytalidium acidophilum* on acid peat hydrolysate was enhanced when it grew in fish offal−peat compost (Martin and Chintalapati, 1989). Here we proposed to replace NH_4NO_3 with FPH as an alternative nitrogen source for apple pomace medium.

The effects of addition of herring FPH in apple pomace on the growth of *T. viride, R. oligosporus,* and *Penicillium* sp. were investigated. The fungal growth was expressed in terms of both soluble protein and glucosamine content. However, because the partially hydrolyzed fish protein product FPH contained polypeptides that interfered with the protein assay, its addition to the medium resulted in a significant increase in total soluble protein content of the fermented pomace. Therefore, using soluble protein as growth indicator resulted in misleading growth curves having the soluble protein constantly increasing as the level of FPH increased (data not shown). Soluble protein content therefore is a poor indicator of growth and can only be used as fungal growth indicator when the medium contains a negligible amount of soluble proteins. It is more reliable to use the glucosamine content as the growth indicator in this case. Figure 6 shows that the addition of ∼0.2−0.3 mL of FPH/g of pomace resulted in the maximal growth of all selected fungi. These results also coincided with that observed for fungal growth in cranberry pomace (Zheng and Shetty, 1997).

Biomass Estimation. One of the most important problems encountered in solid-state fermentations is the biomass determination. In solid-state fermentation, fungal mycelia are intimately bound to the solid matrix and cannot be quantitatively separated from it, so direct measurement of fungal biomass is impossible. Previous studies have described different methods of indirect biomass estimation including measuring fungal cell constituents, such as ergosterol, nucleic acids, protein, nitrogen, and chitin, or primary metabolites, such as CO_2, ATP, and enzymatic activity, or nutrient consumption (Desgranges et al., 1991; Roche et al., 1993). Our previous studies in cranberry indicated glucosamine is a good indicator of fungal growth in heterogeneous solid-state systems (Zheng and Shetty, 1997). Glucosamine is an essential and stable component in chitin of mycelial cell wall; the glucosamine content is a useful parameter for the estimation of the total sum of the growing mycelium, and its changes may correspond to the development of the mycelium, although the values cannot be converted to mycelial weight quantitatively (Sakurai et al., 1977). Furthermore, it was also suggested that the glucosamine measurement gave a good indication of fungal biomass development, but the biomass indicator could only be used to compare different media having the same constituents even if the C/N ratios were different (Desgranges et al., 1991). This correlation between biomass and glucosamine content of fungi during solid-state fermentation was also suggested by other laboratories (Roche et al., 1993).

In the present study, two methods for biomass estimation were compared when additional $CaCO_3$ or nitrogen source (NH_4NO_3 or FPH) was added to the medium. In the case when NH_4NO_3 was used as nitrogen source, as shown in Figures 1 and 2 or Figures 4 and 5, it seemed that a consistent relationship between the measurement of soluble protein and the measurement of glucosamine content existed. Under such conditions, therefore, either soluble protein or glucosamine content could be used as the fungal biomass indicator in solid-state fermentation. However, when FPH was added to the medium, only glucosamine content was a suitable indicator of fungal growth (Figure 6). This study on apple pomace clearly confirmed our previous observations in cranberry pomace (Zheng and Shetty, 1997) that glucosamine content is a better indicator of fungal growth in such heterogeneous solid-state systems. On the basis of glucosamine content, the biomass was estimated for *T. harzianum*, and this is translated into ∼30% of substrate loss during solid-state fermentation. Specific biomass estimation and viability test for the fungal inoculants and their individual applications are also being undertaken.

CONCLUSIONS AND IMPLICATIONS

Apple processing waste can be used as a carbohydrate source for fungal growth. To obtain the maximum growth of the selected *Trichoderma* spp., *R. oligosporus,* and *Penicillium* sp., the apple pomace waste needs to be supplemented with 0.05 g of $CaCO_3$, 2 mL of water, 0.05 g of NH_4NO_3, or 0.3 mL of FPH per gram of pomace. The optimal moisture content for fungal growth was ∼0.81, while the water activity was ∼0.98 at 25 °C.

The soluble protein and glucosamine content of the culture could be used for the estimation of fungal biomass in solid-state fermentation. However, if the medium contains significant amounts of soluble proteins or peptides, only glucosamine content can be used as the growth indicator.

Apple pomace could serve not only as an excellent carbon source but also as an organic carrier for fungal inoculants for food, agricultural, and environmental applications. Solid-state fermentation provides a potential way to convert the apple processing waste into various potential value-added products such as fungal bioinoculants. The results of this investigation revealed

some possibilities of utilization of apple pomace by *Trichoderma*, *Rhizopus*, and *Penicillium* species. For instance, many organochlorine pesticides are persistent in the soil environment; thus, it is feasible to make the *T. harzianum* bioinoculant grown on apple pomace and to apply it into the soil environment for the purpose of enhancing pesticide degradation, or in the case of polymeric dye pollution, a novel *Penicillium* sp. bioinoculant could be used. On the other hand, food grade *R. oligosporus* grown on apple and fish wastes could be targeted as an animal feed.

FPH, a high-nitrogen-containing fishery byproduct, can serve as a good supplemental nitrogen source for apple pomace. The utilization of apple processing waste can be coupled with the utilization of fishery waste, therefore integrating two different natural resource sectors in a beneficial manner. When compared to cranberry pomace (Zheng and Shetty, 1997), apple pomace is a better substrate for growing *Trichoderma* spp. and *Penicillium* sp. but a relatively poorer substrate for growing *R. oligosporus*. These differences in substrate utilization will be probed further to maximize growth for production of diverse bioinoculant formulations required for agro-environmental and animal feed industries.

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