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Biomass Production of Fungal and Bacterial Bio-control Agents Using Various Agro Wastes as Natural Culture Media

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ABSTRACT

Seven micro-organisms, four fungi (*Trichoderma harzianum*, *T. pseudokoningii*, *Gliocladium roseum* and *Penicillium pinophilum*) and three bacteria (*Bacillus subtilis*, *Leuconostoc mesenteroides* and *Pseudomonas fluorescens*), were acted as potential bio-control agents against a range of phytopathogenic fungi. These bio-control agents were used for biomass production. An attempt was made to multiply these micro-organisms with the objective of evaluating the suitability of locally available organic materials for their production and to compare their growth in different media. Various agro wastes, as inexpensive agricultural co-products, including wheat bran, sawdust, rice straw, rice peels, in addition to date extract (Dibis) were tested as natural substrates. To evaluate these non-synthetic media, Potato Dextrose broth and nutrient broth, as synthetic media were used for biomass production. The growth of all the bio-control agents was better when nitrogen ($NaNO_3$ 1% w/v) was used with all natural substrates. Generally, the growth of the tested bio-control agents was faster in date extract, followed by wheat bran, rice peels, rice straw and sawdust. Results indicated that these micro-organisms can be multiplied well by using locally available materials at a very low cost. According to the available surveys and literature, using of date extract as natural medium for biomass production may provide a new record for this medium.

Key words: Production, Fungal, Bacterial Agents, Agro Wastes Media.

INTRODUCTION

Biological control is an environmentally friendly approach involving the use of specific micro-organisms to control pathogens or diseases (Muriungi *et al.*, 2013 and Lopez *et al.*, 2014). The microbial inoculants as bio-control agents are effective and attractive alternatives to prevent the deficiencies brought about by the exclusive reliance on chemicals (Abd-El-Khair *et al.*, 2011; Nusrat *et al.*, 2013 and Srivastava *et al.*, 2014). The market for bio-control agents has been growing continuously over the last few decades due to the adverse environmental impacts of chemical pesticides (Butt *et al.*, 2001 and Verma *et al.*, 2005). Micro-organisms such as *Trichoderma* spp., *Gliocladium* spp., *Verticillium* spp., *Neumoria* spp. and Actinomycetes *etc.*, are important bio-control agents that have been most extensively studied (Papavizas, 1985; Sarhan and Hassan, 2001; Harman *et al.*, 2004; Sharma *et al.*, 2005; Vargas Gil *et al.*, 2009 and Devakumar *et al.*, 2014). These filamentous fungi are wide spread at high population densities in soils. They are saprophytic, quickly growing and easy to culture and they can produce large amount of conidia with long shelf life (Nusrat *et al.*, 2013). Liquid-state fermentation is an effective method for the mass production of fungal and bacterial biopesicides since it provides micropropagules with higher conidia content (Raimbault *et al.*, 1998; Ooijkaas *et al.*, 2000 and Pandey *et al.*, 2000).

Antagonists for commercial use of micro-organisms are necessary to produce maximum biomass with least economic cost. So, it is important to find a suitable and cheap media for growth of antagonist micro-organisms. Studies carried out by Khandelwal *et al.* (2012), Sargin *et al.* (2013) and Subash, *et al.* (2014) used various culture media for growth *Trichoderma* spp. and micropropagule production in solid-state fermentation.

Considering the above facts, the present study aimed to evaluate locally available and cheaper agricultural residues as natural substrates in addition to date extracts for efficient growth and micropropagule production in seven micro-organisms under liquid-state fermentation conditions.

MATERIALS AND METHODS

Isolation and identification of bio-control agents

The bio-agents were isolated from the representative soil samples by following the serial dilutions and pour plate method. The last 3 dilutions, 10^{-2}, 10^{-4} and 10^{-6}, were used for isolation of bio-agents. One ml of suspension from respective dilution was transferred aseptically into a Petri plates containing the potato dextrose agar (PDA) and nutrient agar (NA) media separately and incubated at 25±2°C for 5-7 days for fungi and 2-3 days for bacteria. The beneficial fungi and bacteria were picked up and purified. They were identified and examined under microscope on the basis of cultural and morphological characters. Individual colonies were picked up and maintained on PDA slants in pure culture for further study.

Cultures of fungi and bacteria

Five natural nutrient substrates (wheat bran,

sawdust, rice straw, rice peels and dates extract) were evaluated for their suitability as growth media for production of seven bio-agents. Seven micro-organisms were determined by the growth and sporulation ability that include four fungi (*Trichoderma harzianum*, *T. pseudokoningii*, *Gliocladium roseum* and *Penicillium pinophilum*) and three bacteria (*Bacillus subtilis*, *Leuconostoc mesenteroides and Pseudomonas fluorescens*). These micro-organisms have previously been proven to have bio-control agents' potential in terms of its mycoparasitic activity and lytic enzyme activities (Sarhan, 2009 and 2013). As a control treatment, potato dextrose broth was used for fungal cultures, while nutrient broth was used for bacterial cultures. The natural nutrient substrates, as carbon sources, were supplemented with nitrogen source ($NaNO_3$ 1% w/v) to support the microbial multiplication. The experiments were conducted with and without nitrogen supplementation.

Biomass production of fungi and bacteria

According to the methods of (Sarhan and Hassan, 2001 and Khandelwal *et al.*, 2012), 200 grams of each natural nutrient substrate was washed well and boiled in 500 ml distilled water for 1 hr. After cooling, the substrates were filtered through a double layered muslin cloth, then from each substrate makeup 1 liter with distilled water. Each natural nutrient substrate was individually transferred to 500 ml conical flask (100 ml each, three replicates for each treatment) and autoclaved at 121°C for 15 minutes. After cooling, the media were aseptically inoculated with 1.0 % (v/v) of spore suspension (1.0×10^8 spores/ml) and incubated at 25±2°C. After the desired cultivation period (7 days for fungal inoculants and 48 h for bacteria), biomass was harvested finally from each replicate. Fresh fungal and bacterial biomass yield was assessed by collecting biomass yield on pre-weighed filter paper. Dry weight was determined after 24 h of oven drying at 60°C. Bacterial count was estimated as dry cell weight (DCW) by measuring the optical density using spectrophotometer at wavelength of 660 nM, and related the optical density to DCW. Otherwise; fungal spores were harvested by washing them thoroughly with sterilized water containing Tween-20 (0.2%) with the help of a small sterile metal spatula. Suspension of spores was made using distilled water with Tween-20 (0.2%) and filtered through a double layered muslin cloth. Spore count was made after necessary serial dilutions (with the aid of haemocytometer) under phase contrast microscope. Colony forming units (cfu) were estimated by plating technique.

RESULTS AND DISCUSSION

Five different naturally available substrates were tested as liquid media for mass production and sporulation of seven micro-organisms, included 4 fungi (*T. harzianum*, *T. pseudokoningii*, *G. roseum* and *P. pinophilum*) and 3 bacteria (*B. subtilis*, *L. mesenteroides* and *P. fluorescens*). All the antagonists utilized the natural media efficiently as compared to the control media (PD Broth and Nut. Broth). Maximum biomass yield was on dates extract medium and minimum on sawdust medium. Also, potential for production of biomass yield among the tested micro-organisms was differed significantly. Performance of natural media revealed that biomass production in both fungi and bacteria was maximum (mg/100ml) in the extract medium of dates, followed by wheat bran, rice peels, rice straw and sawdust medium (Tables 1 and 3). To determine the effect of nitrogen supplementation on micro-organism propagules production, ($NaNO_3$ 1% w/v) as nitrogen source was tested in the natural media, the results are shown in tables (2 and 4). Highest fresh weight of fungi was recorded in *T. harzianum*, 376 mg /100 ml without nitrogen supplementation (Table 1), and 558 mg /100 ml with nitrogen supplementation (Table 2). In addition, highest fresh weight of bacteria was recorded in *B. subtilis*, 141.6 mg /100 ml without nitrogen supplementation (Table 3), and 152.1 mg /100 ml with nitrogen supplementation (Table 4). Abundance of minerals in the extract medium of dates may enhance the growth of fungi and bacteria. Wheat bran and rice peels media also supported the growth of tested micro-organisms (both fungi and bacteria). Moreover, in many of tested media from natural substrates, spore production with fungi was relatively higher when nitrogen source was used as compared to the media without nitrogen supplementation. Similarly for fungal spore production, maximum number of spores per ml of culture media was produced by all the tested fungi on dates extract medium and minimum spore production was observed on sawdust medium. Spore count was the maximum in *T. harzianum* on dates extract medium ($5.9\text{x}10^8$) and the least was obtained in sawdust medium ($3.3\text{x}10^2$), also, *T. pseudokoningii G. roseum* and *P. pinophilum* responded in similar fashion for spore production (Table 2). This is in accordance with the investigations of Khandelwal *et al.* (2012) who found that a high biomass of *Trichoderma* spp. was produced on agro products especially the vegetable wastes. These results indicated that these natural substrates were rich with carbon source but need nitrogen supports which was very important for growth and metabolism. Also, the fungal micropropagules and bacterial counts obtained in nitrogen-supplemented media, prepared with the same conditions for natural substrates, was higher than those obtained in media without nitrogen supplementation. However, potential for production of biomass yield among the antagonist bacteria did

Table (1): Biomass production and spore formation of the fungi as bio-agents on different natural substrates without nitrogen source supplementation

Bio-control agent (Fungi)	Substrates (broth media)	Biomass yield		Spore yield (spores / ml)	Fungal-propagules (cfu / ml)
		Fresh weight (mg / 100 ml)	Dry weight (mg / 100 ml)		
Trichoderma harzianum	Rice straw	142	125	2.1×10^2	2.5×10^2
	Rice peels	190	174	6.6×10^3	2.1×10^3
	Sawdust	101	89	1.8×10^2	1.4×10^2
	Wheat bran	251	232	2.9×10^5	2.2×10^3
	Date extract	376	351	7.1×10^5	3.5×10^4
	Potato Dextrose	436	412	6.8×10^3	5.1×10^3
T. pseudokoningii	Rice straw	135	101	1.7×10^2	1.2×10^2
	Rice peels	184	155	5.3×10^3	4.6×10^3
	Sawdust	91	72	1.4×10^2	1.1×10^2
	Wheat bran	242	215	4.4×10^3	3.7×10^3
	Date extract	358	330	5.1×10^4	4.3×10^4
	Potato Dextrose	416	389	7.9×10^3	5.6×10^3
Gliocladium roseum	Rice straw	102	86	2.3×10^2	1.9×10^2
	Rice peels	184	184	3.6×10^3	2.2×10^3
	Sawdust	99	81	1.8×10^2	1.0×10^2
	Wheat bran	250	231	5.2×10^3	4.3×10^3
	Date extract	348	323	2.5×10^4	7.5×10^3
	Potato Dextrose	406	379	7.7×10^3	6.9×10^3
Penicillium pinophilum	Rice straw	115	89	2.5×10^2	1.5×10^2
	Rice peels	162	135	6.4×10^3	4.6×10^3
	Sawdust	79	64	2.8×10^2	1.7×10^2
	Wheat bran	222	205	5.2×10^3	2.9×10^4
	Date extract	340	319	4.5×10^4	3.1×10^4
	Potato Dextrose	404	387	1.6×10^4	6.0×10^3

Table (2): Biomass production and spore formation of the fungi as bio-agents on different natural substrates with nitrogen source supplementation

Bio-control agent (Fungi)	Substrates (broth media)	Biomass yield		Spore yield (spores / ml)	Fungal-propagules (cfu / ml)
		Fresh weight (g / 100 ml)	Dry weight (mg / 100 ml)		
Trichoderma harzianum	Rice straw	162	141	1.8×10^4	2.6×10^2
	Rice peels	333	315	2.4×10^5	1.9×10^4
	Sawdust	175	147	3.3×10^2	2.5×10^2
	Wheat bran	463	435	4.6×10^6	4.2×10^5
	Date extract	558	530	5.9×10^8	3.5×10^6
	Potato Dextrose	422	399	1.1×10^5	2.7×10^4
T. pseudokoningii	Rice straw	155	132	3.2×10^3	1.2×10^2
	Rice peels	282	255	2.8×10^5	2.3×10^4
	Sawdust	159	134	6.6×10^2	4.1×10^2
	Wheat bran	426	408	5.3×10^5	4.4×10^4
	Date extract	510	489	1.7×10^8	2.1×10^6
	Potato Dextrose	417	391	2.9×10^4	3.5×10^3
Gliocladium roseum	Rice straw	137	116	3.6×10^3	5.0×10^2
	Rice peels	294	271	2.8×10^4	2.1×10^3
	Sawdust	125	106	7.4×10^2	3.3×10^2
	Wheat bran	360	337	3.5×10^5	2.6×10^5
	Date extract	448	423	6.5×10^5	4.2×10^6
	Potato Dextrose	402	377	4.7×10^4	3.7×10^4
Penicillium pinophilum	Rice straw	188	161	8.5×10^4	3.5×10^2
	Rice peels	320	304	2.1×10^5	2.6×10^4
	Sawdust	183	159	4.8×10^3	1.8×10^2
	Wheat bran	491	462	2.9×10^6	2.9×10^4
	Date extract	566	541	1.1×10^7	4.1×10^5
	Potato Dextrose	446	421	3.3×10^5	5.0×10^3

Table (3): Biomass production of bacteria as bio-agents on different natural substrates without nitrogen source supplementation

Bio-control agent (Bacteria)	Substrates (broth media)	Biomass yield		Bacterial counts (No. / ml)
		Fresh weight (mg / 100 ml)	Dry weight (mg / 100 ml)	
Bacillus subtilis	Rice straw	83.4	70.2	2.37×10^4
	Rice peels	102.6	93.5	3.60×10^6
	Sawdust	91.2	76.0	2.65×10^3
	Wheat bran	137.3	128.8	3.25×10^7
	Date extract	141.6	132.2	3.17×10^7
	Nutrient broth	123.7	121.4	6.88×10^6
Leuconostoc mesenteroides	Rice straw	65.5	58.4	4.21×10^3
	Rice peels	84.6	75.3	2.63×10^4
	Sawdust	61.2	55.7	3.65×10^3
	Wheat bran	121.0	109.9	1.15×10^6
	Date extract	127.4	114.1	1.22×10^6
	Nutrient broth	118.7	109.0	7.80×10^5
Pseudomonas fluorescens	Rice straw	70.0	63.6	3.30×10^5
	Rice peels	92.8	83.0	2.45×10^6
	Sawdust	80.3	71.2	1.65×10^3
	Wheat bran	116.0	107.3	1.20×10^7
	Date extract	122.6	110.1	1.27×10^7
	Nutrient broth	125.7	113.4	7.22×10^6

Table (4): Biomass production of bacteria as bio-agents on different natural substrates with nitrogen source supplementation

Bio-control agent (Bacteria)	Substrates (broth media)	Biomass yield		Bacterial counts (No. / ml)
		Fresh weight (mg / 100 ml)	Dry weight (mg / 100 ml)	
Bacillus subtilis	Rice straw	98.5	87.0	4.35×10^6
	Rice peels	115.7	103.4	2.21×10^7
	Sawdust	102.3	90.8	2.88×10^4
	Wheat bran	147.9	138.2	3.92×10^8
	Date extract	152.1	140.6	3.40×10^8
	Nutrient broth	134.6	125.7	5.13×10^7
Leuconostoc mesenteroides	Rice straw	79.0	68.5	6.35×10^5
	Rice peels	98.6	87.0	1.21×10^6
	Sawdust	72.3	61.6	8.88×10^3
	Wheat bran	128.9	119.0	2.92×10^6
	Date extract	132.1	124.4	2.40×10^6
	Nutrient broth	124.0	113.6	3.73×10^5
Pseudomonas fluorescens	Rice straw	88.7	79.5	5.85×10^5
	Rice peels	110.2	100.6	5.21×10^6
	Sawdust	89.0	79.9	2.18×10^4
	Wheat bran	139.3	127.9	6.92×10^7
	Date extract	145.6	134.1	1.40×10^7
	Nutrient broth	130.8	121.6	3.73×10^6

not differ from fungi. Performance of culture media revealed that bacterial count was maximum in *B. subtilis* on dates extract medium ($3.40x10^8$) and least was obtained on sawdust medium ($2.88x10^5$), followed by *P. fluorescens* and *L. mesenteroides* (Table 4). The three tested bacteria gave the same results on wheat bran medium and date extract medium as compared to other natural media and control media (Tables 3 and 4). Subash *et al.* (2014) studied the using of agricultural wastes as a substrate for that the growth and sporulation of *T. harizanum* where they got good results with sugarcane bagasse as natural substrate. Growth of fungi was better in date extract medium, while growth and performance of bacteria was better in date extract medium and wheat bran as compared to other natural media. In date extract medium and wheat bran, available nutrients might have encouraged the growth of *T. harzianum* and *B. subtilis* compared to other bio-control agents. Growth of the fungi was maximum in date extract medium and it might be due to the fact that the date extract medium contained nutrients in readily available form and also in a balanced proportion. Wheat bran supported growth of all the tested bio-control agents and it might be due to the higher content of nutrients and lower pH of wheat bran as compared to other natural media. Similar results were reported by Sargin *et al.* (2013) who reported that high micropropagules count of *T. harzianum* was achieved in wheat bran medium.

In conclusions, the bio-control agents can be multiplied locally with low cost. Mass-production of micro-organisms, used as bio-control agents, was possibly present and locally available in certain organic materials such as, wheat bran, sawdust, rice straw, rice peels and date extract. Performance of bio-control agents (fungi and bacteria) was better in date extract and wheat bran media with nitrogen supplementation. From all the above results, it was found that date extract was more effective for biomass production of fungi and bacteria as compared to the synthetic media, potato dextrose broth and nutrient broth. Date extract as natural substrate for biomass production of micro-organisms, which was evaluated for first time, may provide a new record for this medium.

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