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ALTERNATIVE CULTURE MEDIA FOR FUNGAL GROWTH USING DIFFERENT FORMULATION OF PLANT MATERIAL

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ABSTRACT

Keeping in view the cost of the yeast extract as well as difficulty in its procurement in remote places, suitable alternative to such conventional media components needs to be explored in order to facilitate the applicability of the treatment process in remote small- scale units. Habitat conditions such as nutrient availability, temperature, humidity, etc greatly affect the growth of microorganisms, decreasing their activity and field viability. Due to several practical difficulties, such a biological system may not be feasible for effluent treatment in remote rural industries. Hence, for evolving a viable biological system, a suitable formulation of efficient strain would form an important input for the effluent treatment plant. This study describes the development of three *A.lentulus* formulations using organic bases as rice based fungal formulation (RBFF), wheat based fungal formulation (WBFF) and maize based fungal formulation (MBFF)which could function both as nutrition as well as a carrier. All three formulations were evaluated in terms of shelf life and metal removal efficiency at definite intervals. Based on the findings of this study, it is observed that the development and evaluation of the shelf life of three formulations formulations i.e rice based fungal formulation, maize based fungal formulation and wheat based fungal formulation performed well in controlled conditions i.e 30^{0}C & 40C upto seven months.

KEYWORDS: Alternative media, lyophilized, rural industries, fungal formulation organic bases

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INTRODUCTION

For ensuring the utility of efficient strains in remote small- scale industries/ units, it is essential that suitable locally available alternates to the commercial expensive growth media be worked out.[1] Surprisingly, such studies on media formulations using locally available nutrients are very scanty.[2]Therefore, a need is felt to explore widely available plant- derived components as alternatives to animal – based microbiologically media.[3-4] Alternative substance opted for media substitution should be abundantly available and cost effective as far as possible. Therefore, as a source of alternative media, two widely grown plants namely *Leucaena leucocephala* and *Sesbania* sp have been selected.*L. leucocephala* is an evergreen, fast growing; multipurpose leguminous tree species which provides highly palatable foliage and can also helps in afforestation of ever increasing wastelands.[5] It is being used as an option for agroforestry in dryland. A three year study conducted in sandy loamy soil resulted in increased total herbage productivity using intercropping of cereal forages with *Leucaena.*[6] *Sesbania* sp. (commonly referred to as *Dhaincha* in India and sometimes named as *S. aculeate*) is a crop generally cultivated for providing nutrient to soil. It is cultivated in monsoon season almost throughout India and grows well in loamy, clayey, black and sandy soils. It is an ideal green maturing crop as it is fast growing, succulent, easily decomposable with low moisture content and produces maximum amount of organic matter and nitrogen in the soil [7,8,9]. Majority of the studies have tried to explore alternative carbon substrate. Molasses is the residual syrup derived from sugar production/ sugar beet pulp, which strongly stimulates microbial growth. [10-11] Therefore, molasses has been used as the carbon source in the media. Donmez showed that in the molasses media high concentrations of copper were bioaccumulated by mixed culture. Sharma tested and compared several carbon and nitrogen source for growth and Cu removal by fungal strains. Corn, sorghum, millet meal extract have been used as alternate culture supplements (for potato) in potato dextrose agar for *Aspergillusr niger, Fusarium moniliforme, Penicillium sp., Cercospora* sp. Fortification of the media containing mannitol or glucose as a carbon source with amaranthus seed meal (as a supplement providing growth factor) resulting in high cell number culture of *Sinorhizobium fredii* has also been reported. Solaimane valuated soy molasses as combined nitrogen and carbon source for production of sophorolipids by *Candida bombicola*. *Guar gum*, non-toxic colloidal polysaccharides and Gum Kataria have been used as cheap alternates to agar for gelling agent in microbial culture media. Majority of such on alternative media have been reported for solidified growth medium, hence there is a large scope of research for alternative liquid growth medium or both. Further, most of the existing investigations could lead to only functional supplements but not the complete substitute. Another very important and interesting aspect, which needs to be investigated, is that whether the alternative media can solely support the growth and metal removal under stress conditions [12-13]. Microbial formulation development is an important aspect which ensures easy and regular availability of the particular microbial cultures for specific applications. In most of the studies researchers have developed microbial formulations for use as biocontrol agents and also as biofertilizer.These studies include production of solid formulations, liquid oil- based formulations. Researchers have been adopting low cost preferably waste byproducts as the materials for developing formulations [14]. Vidyarthi, studied the production of *Bacillus thuringiensis* (BT) based bio pesticides using wastewater sludge as the raw material [15]. Starch wastewater has been used for liquid bio pesticide formulation based on *B. thruingesis*.Aqueous flowable (suspension) formulations for *B. thruingesis*. However, no study describes a product / microbial formulations for wastewater treatment which will act as a seed for replication in effluent treatment systems. [16] For evolving a viable biological system, a suitable product or formulation of efficient strain (for use as the seed culture in remote small scale industries) would form an important input for the effluent treatment plant. [17-18] In this context a study on microbial formulation of oil degrading bacterial consortia named oilzapper has been reported. Degradable organic matrix was used to make this oil degrading bacterial formulation, which is being found very effective for remediation of the oil- spilled area. Bennet reported the use of fungal compositions, *Marasmiellus troyanus* in combustion with an alginate as a carrier, for degradation of benzopyrene.[19] However, extensive review of the literature revealed that efforts have not been made to formulate microbial strains capable of metal detoxifications. It has been observed that the storage and drying conditions of the formulations affect their shelf- life. Since difficulty in handling the biological strains (requirement of refrigeration and clean sterile environment) hampers their usage, a strong need for development of suitable product which can be stored under ambient conditions without the loss of viability is perceived. [20-21].

MATERIALS AND METHODS

Collection of Plant material

The plants parts pods in case of *L. leucocephala* and seeds in case of *Sesbania* sp.were collected from sector -15, Noida, Ghaziabad, Uttar Pradesh, India and identity was confirmed using a taxonomist in the University**.**

Test fungi

In this study, fungi namely; *A.lentulus* were tested. These fungal cultures were obtained from the culture collection of Department of Microbiology, Jiwaji University Gwalior, India. Prior to the study above fungi were sub-cultured on fresh PDA medium.

Preparation of test powder

The plant parts chosen for media substitution were pods in case of *L. leucocephala* and seeds in case of *Sesbania* sp. After collection, the plant materials were washed, dried (40^{o}C), grinded and sieved to obtained uniform sized powder (>150 mesh) for sample analysis.

Comparison of fungal growth on alternative growth medium

A comparison of two plant based media composition for the growth of *A.lentulus* was designed.

On the basis of carbon and nitrogen content in *L. leucocephala* and *Sesbania* sp. these materials were presumed to be suitable substitutes for carbon and nitrogen sources, in place of glucose and yeast extract. To prepare uniform growth medium using these materials, the powdered materials (at designated doses) were dissolved in water, heat treated and filtered. The final broth that was obtained was termed as Water Extractable *Lucenia* Pods (WELP) and Water Extractable *Sesbenia* Seeds (WESS). To investigate whether the WELPor WESS will suffice alone or would have to be supplemented with other nutrients, several combinations including addition of glucose and yeast extract were evaluated and compared with the conventional composite media.

Selection of organic bases for fungal formulation

In order to facilitate storage and transportation of A. lentulus for field application, initially suitability of different organic bases namely rice, maize, wheat flours, rice husk and wheat bran was tested for effective preparation of formulation. The materials were grinded and dried at 40 ^{0}C for 1h.After drying fungal spores were inoculated (5% v/v) on homogenized and dried bases while maintaining the moisture level up to 60-70% using sterile distilled water. These fungal inoculated bases were incubated at 30 ^{0}C for 120 h.

Preparation of fungal formulation

To make the formulation bases, flours of all three grains (rice, wheat and maize) were collected from the market. Wheat and maize were obtained as flour, whereas rice was grinded to fine powder. To make these materials moisture free, these flours were dried at 40^{o}C ± 2^{o}C for 30 to 60 min. After drying, fungal spores were inoculated (5% v/v) on homogenized and dried bases while maintaining the moisture level up to 60- 70% using sterile distilled water. These fungal inoculated bases were incubated at 30^{o}C for 120 h.

Evaluation of stored fungal formulations

All three were formulations were evaluated in terms of shelf life and Cu (II) removal efficiency at definite intervals during the period of storage these formulations filled in sterilized plastic containers were kept in different conditions (ambient temperature, at 4^{o}C, at 30^{o}C) for ~ 1 year (Sep 2010- Sep 2011). At controlled temperature of 30^{o}C, formulations were kept in incubator and for 4^{o}C, refrigerator was used. To provide ambient conditions containers were kept under open atmosphere. Sample was withdrawn after every month. Weighed amount (0.1g) of formulation was added into the composite media with or without Cu (II) amendment. The flask was kept at 180 rpm and 30^{o}C for 120 h. For measuring the shelf life, biomass was measured at the end of exponential phase in both the conditions i.e. in absence and in presence of Cu (II).

RESULTS

Characterization of selected plants

The results of the proximate and ultimate analysis of these materials are given in Fig 1 &Table 1, which shows that both the plant materials have comparable moisture, crude fiber and crude fat content. The carbon content in *L. leucocephala* and *Sesbania* sp. was 40% and 30%, respectively. The carbon content of *L. leucocephala* is equivalent to the carbon content present in glucose. On the other hand, the nitrogen content in *Sesbania* sp (6.4%) was found to yeast extract (9.4%) as compared to lower content (1.76%) in *L. leucocephala.*

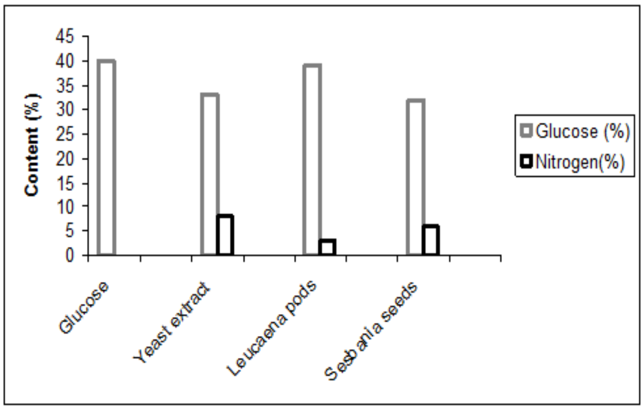

Figure 1
Comparison of the carbon and nitrogen content in composite media components (glucose and yeast extract) with alternative media components (Leucaena pods and Sesbania seeds)

Evaluation of alternative growth medium for A. lentulus

(a) Growth of A.lentulus using WELP/ WESS supplemented with glucose and yeast extract in absence of Cu (II).

Results are shown in Fig 2. Looking at the performance of plant materials alone, WESS was found better than WELP. Further, using *L.leucocephala* pods (15g/l); maximum fungal growth (biomass concentration 4.6 g/l) was obtained when WELP was supplemented with yeast extract. On the other hand, using *Sesbania* Seeds (7.5 g/l), maximum growth (4.5 g/l) was recorded when WESS was supplemented with glucose. In comparison to composite media, there was only 2.1% reduction in fungal growth when WELP supplemented with yeast extract was used while 38.3% relative reduction was observed when WEPL alone was used. Similarly, in comparison to composite media, there was only 8.1% reduction in fungal growth when WESS supplemented with glucose was used while 30.6 relative reductions were observed when WESS alone was used.These results further corroborated that due to its high nitrogen content *Sesbania* seeds could replace the nitrogen source (yeast extract) while *L. leucocephala* pods with higher C/N ratio could replace the carbon source.

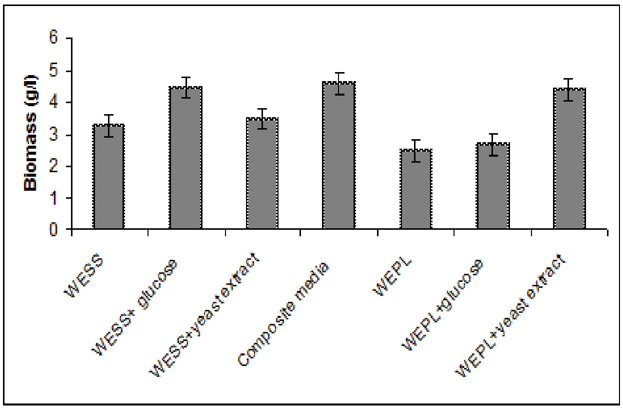

Figure 2
Biomass production using WESS (7.5 G/L) and WELP (15 g/l) supplemented with glucose (10 g/l) and yeast extract (5 g/l) in absence of Cu (II)

(b) Growth of A. lentulus using alternative media alone (WELP+ WESS) in absence of Cu (II)
To investigate the optimum dose of *L.leucocephala* pods and *Sesbania* seeds, three combinations of different doses were used. It was found that as the dose of L. *leucocephala* pods and *Sesbania* seeds was increased from a minimum of 10 g/l (WELP) and 5 g/l (WESS), respectively to a maximum tested 15 g/l (WELP) and 7.5 g/l (WESS), there was a substantial change in fungal biomass production from 3.1 g/l to 4.4 g/l (Fig 3)

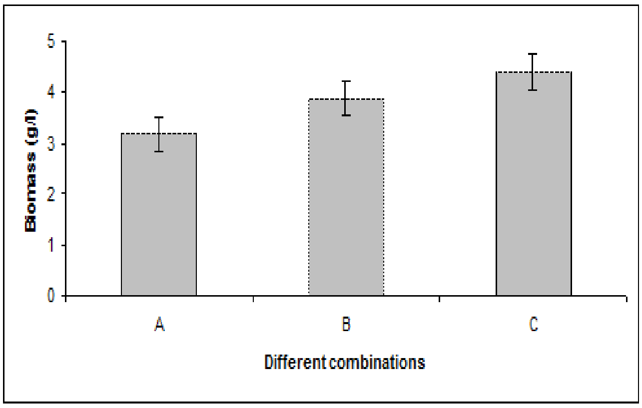

Figure 3
Effect of various doses of alternative media on fungal biomass production
(A) 5 g/l of WESS + 10 g/l WELP (B) 6 g/l of WESS + 12.5 g/l of WELP (C) 7.5 g/l of WESS + 15 g/l of WELP

Effect of Cu (II) on the growth of A.lentulus using plant based media
(a)Growth of A.lentulus using WELP/ WESS supplemented with glucose and yeast extract in presence of Cu (II)
When the conventional composite media was employed, there was insignificant relative reduction (2.0%) in biomass yield in presence of Cu (II) as compared to that in the absence of Cu (II) Fig 4.

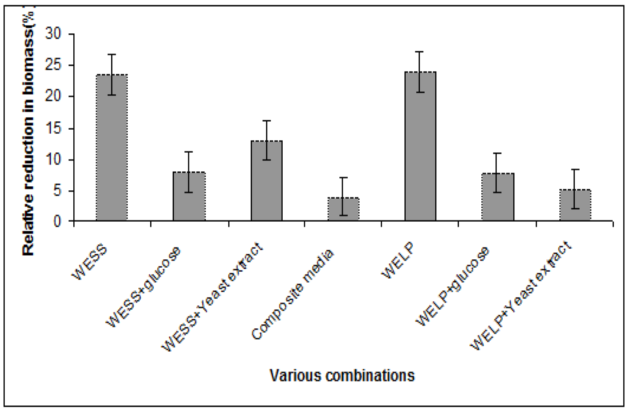

Figure 4
Relative reduction in biomass production using WESS (7.5 g/l) WELP(15 g/l) supplemented with glucose (10 g/l) and yeast extract (5 g/l) in presence of Cu (II).

(b)Growth of A.lentulus using alternative media aloneWELP/ WESSin presence of Cu(II)
The relative reduction in biomass production due to the presence of Cu (II)in case of alternative media alone was 6.6%. This was slightly higher than 2% relative reduction observed in case of composite media.

Fungal formulation for field application
All the three bases, which performed well, were characterized to know their C: N ration. The C: N ratio of these bases was 21.4 (rice), 25.2 (maize) and 22.2 (wheat). In present study, rice flour having low C: N ratio was found to be most effective for fungal formulation in comparison to maize and wheat flour previously.

Table 1
Characterization of Plant materials selected as alternative media components (values in percentages)

Plant	Moisture content	Crude fiber	Crude fat	Protein	Total sugar	Ash content	Carbon	Hydrogen	Nitrogen
Leucaena leucocephala	9.0	4.0	12.0	11.0	49.0	4.2	40.0	6.1	1.7
Sesbania sp.	7.4	3.0	10.0	40.0	40.0	5.8	29.4	4.1	6.4

Shelf life of fungal formulations
Since the main aim of these formulations was achieved the Cu removal from industrial effluent, the viability in presence of Cu (II) was also tested. The Shelf life was evaluated over a period of one year i.e from October, 2010 to September 2012.

Formulation stored at 30 ^{0}C

All the three formulations performed well for six months. After 7th months there was slight reduction in biomass in RBFF (8.5%) and MBFF (12.4%) in absence of Cu (II) (Fig. 5 & Fig.6). In the case of RBFF after 9th month relative reduction in biomass production increased to 16% and eventually reached to 24.4% at the end of 12th month. Similar trend was observed in presence of Cu (II) whereby 11.8% and 30% relative reduction was recorded in 7th and 12th month, respectively.

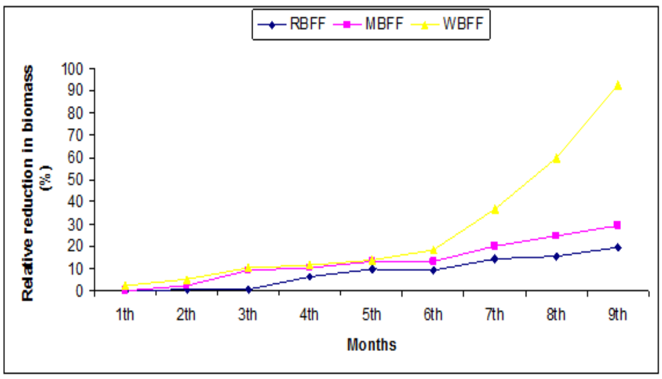

Figure 5
Relative reduction (%) in biomass production using RBFF, MBFF and WBFF stored at 30°C for one year in absence of Cu (II).

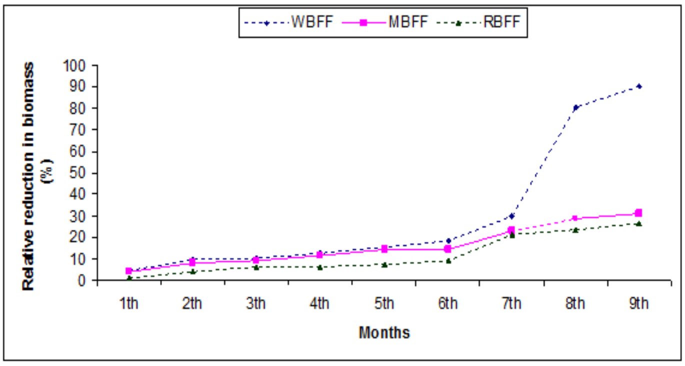

Figure 6
Relative reduction (%) in biomass production using RBFF, MBFF and WBFF stored at 30°C for one year in presence of Cu (II)

Formulation stored at 4^0C

While testing the shelf life of formulations stored at 4^0C, it was observed that the trend of deterioration was quite similar for WBFF stored at 30^0C, while in case of MBFF and RBFF, there is lesser reduction in fungal biomass as compared to that observed with formulations stored at 30^0C.In case of RBFF there was no reduction in biomass production up to 7^{th} month while 11.2% 9^{th} month and 14.3% reduction was observed after 1 year (Fig 7). Formulations stored at 4^0C were relatively more stable than those stored at 30^0C.One of the study it was observed that storage temperature had pronounced effect on the stability of the formulated Fusarium oxysporum in Pesta granules.[23]They found that fungal pellets stored at 4^0C were more stable and remained viable for up to 16 weeks of storage.

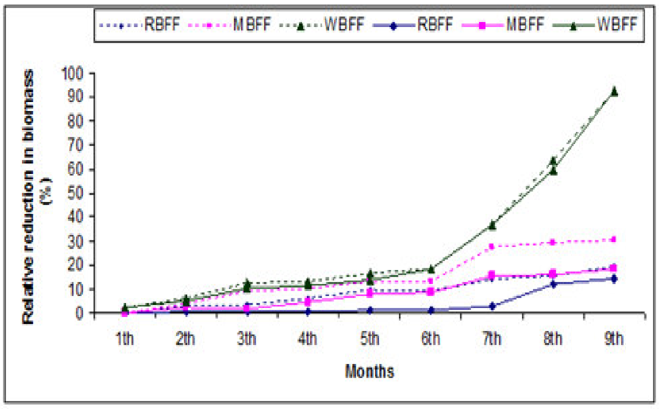

Figure 7
Relative reduction (%) in biomass production using RBFF, MBFF and WBFF stored at 4°C for one year in absence of Cu (II). Solid lines (absence of Cu (II), dotted lines (presence of Cu (II).

DISCUSSION

Since yeast extract is very expensive and difficult to procure, suitable alternatives to such conventional media components were explored as a source of alternative media, two widely available plant materials namely *Leucaena leucocephala* pods and *Sesbania* sp. seeds were used By chemical analysis of *L. leucocephala* pods and *Sesbania* sp. seeds it was found to have carbon and nitrogen % equivalent to yeast extract and glucose. Therefore, it was envisaged that a combination of the two plant parts designated further as alternative medium would be an ideal substitute to yeast extract and glucose. The results point out that refrigeration may not be required to ensure the viability and activity of above fungal formulations. The present investigation provides a biological system based on a newly isolated efficient and versatile fungal strain along with low cost media substitutes based on wasteland growing plants and a storable formulation (for as a seed culture) to facilitate Cu detoxification in remote small-scale industrial units.[22] One of the study soy molasses as combined nitrogen and carbon source for production of sophorolipids by *Candida bombicola*. Guar gum, non toxic colloidal polysaccharides and *Gum Kataria* have been used as cheap alternates to agar for gelling agent in microbial culture media. Majority of such on alternative media have been reported for solidified growth medium, hence there is a large scope of research for alternative liquid growth medium or both.[24-25] For ensuring the utility of efficient strains in remote small- scale industries/ units, it is essential that suitable locally available alternates to the commercial expensive growth media be worked out. Surprisingly, such studies on media formulations using locally available nutrients are very scanty [25]. Therefore, a need is felt to explore widely available plant- derived components as alternatives to animal – based microbiologically media.Corn, sorghum, millet meal extract have been used as alternate culture supplements (for potato) in potato dextrose agar for

Aspergillusr niger, Fusarium moniliforme, Penicillium sp., Cercospora sp. Fortification of the media containing mannitol or glucose as a carbon source with amaranthus seed meal (as a supplement providing growth factor) resulting in high cell number culture of *Sinorhizobium fredii* has also been reported.[24] In most of the studies researchers have developed microbial formulations for use as biocontrol agents and also as biofertilizer. These studies include production of solid formulations, liquid oil- based formulations. However, no study describes a product / microbial formulations for wastewater treatment which will act as a seed for replication in effluent treatment systems. For evolving a viable biological system, a suitable product or formulation of efficient strain (for use as the seed culture in remote small scale industries) would form an important input for the effluent treatment plant.[27-28] It has been observed that the storage and drying conditions of the formulations affect their shelf- life. Since difficulty in handling the biological strains hampers their usage, a strong need for development of suitable product which can be stored under ambient conditions without the loss of viability is perceived.

CONCLUSIONS

In order to enhance the applicability of biological strains in remote rural industries, strong need to develop suitable formulations, which can be produced, handled and stored conveniently in simple set up, was perceived. Based on the findings of this study, it is concluded thatthe development and evaluation of the shelf life of three A. lentulus formulations using organic base, all the three formulations i.e rice based fungal formulation (RBFF), maize based fungal formulation (MBFF) and wheat based fungal formulation (WBFF) performed well in controlled conditions i.e 30^0C & 4^0C upto seven months. Hence, among all the three, RBFF came out as potential fungal formulation. Overall, the formulations could be stored for 3 months under ambient conditions and 6-12 months under controlled conditions (30ºC).Further work on a suitable rector design using the data and resources (media substitute and formulations) generated in the present investigation would help translating these results into decentralized effluent treatment system for small scale industries located in remote/ rural section.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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