

# African Journal of Biotechnology

Volume 13 Number 45, 5 November, 2014

ISSN 1684-5315



*Academic  
Journals*

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**References:** In the text, a reference identified by means of an author's name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author's name should be mentioned, followed by 'et al'. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like 'a' and 'b' after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998;

1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)  
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. *Afr. J. Biotechnol.* 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant *Staphylococcus aureus* in community-acquired skin infections. *Emerg. Infect. Dis.* 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing *Escherichia coli* in the Calgary Health Region: emergence of CTX-M-15-producing isolates. *Antimicrob. Agents Chemother.* 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). *Microbiology: Concepts and Applications.* McGraw-Hill Inc., New York, pp. 591-603.

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Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

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## Full Length Research Paper

# Cellulase activity of filamentous fungi induced by rice husk

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Received 9 February, 2014; Accepted 16 October, 2014

**The objective of this study was to determine the potential of different filamentous fungi to degrade cellulose in rice husk pre-treated with steam explosion or alkaline hydrolysis. A preliminary test performed with carboxymethyl cellulose and nine fungi (*Trichoderma* 1, 2, 3, 4, 5; *Trichoderma reesei*; *Aspergillus niger*; *Rhizopus oryzae* and an isolated fungus from rice husk) allowed the selection of the fungi that can degrade cellulose the most. Subsequently, the fastest growing fungi on the substrate (carboxymethyl cellulose) were subjected to a fermentation bioreactor (18 mL of the fungus with 2 mL of conidial suspension at a concentration of  $5 \times 10^6$  conidia mL<sup>-1</sup>). Their potential to degrade cellulose was determined. This was done by measuring the amount of total carbohydrate and reducing sugars using the anthrone method and 3,5 dinitrosalicylic acid respectively. On the other hand, the endoglucanase, exoglucanase and  $\beta$ -glucosidase activity of the two most promising fungus (*Trichoderma* sp. 1 and *Aspergillus* sp.) was evaluated. Statistical analysis revealed no significant differences; however, the rice husk pre-treated with steam explosion before the fungal strains had the greatest amount of total carbohydrates; it produces 2.9 and 1.4 times more than those not treated with alkaline hydrolysis. Moreover, fungi *Trichoderma* sp. 1 and *Aspergillus* sp. had higher number of total released carbohydrate and reducing respectively, which demonstrated the difference in the enzyme system of the two microorganisms. Endoglucanase and exoglucanase activities had similar performance for *Aspergillus* sp., and *Trichoderma* sp. 1, during the 288 h of the test. Likewise,  $\beta$ -glucosidase activity was similar. After 192 h, values of 0.150 and 0.140 IU mL<sup>-1</sup> were obtained for *Aspergillus* sp. and *Trichoderma* sp. 1, respectively. Finally, the applicability of rice husk in agribusiness as a raw material for subsequent fermentation and for obtaining added-value compounds is shown.**

**Key words:** Enzymatic activity, rice husk, fermentable sugar, agroindustrial wastes, filamentous fungi.

## INTRODUCTION

The use of agro-industrial byproducts as raw materials for the production of high added-value products such as bio-fuels, compost, xylitol, enzymes and compounds for human and animal consumption, among others, has become increasingly important (Sánchez, 2009). In Colombia, rice

farming is a major component of the agricultural sector, with a semiannual output of 1,376,385 t (Dane-Fedearroz, 2013), from which about 50% of rice husk can be obtained (Ahumada and Rodríguez-Páez, 2006). Due to its recalcitrant structure (Yu et al., 2009), abrasive nature, low nutri-



tional value and high ash content (Jurado et al., 2003), this residue has limited use. Additionally, its incineration is questioned, given the high environmental costs of its combustion (Camassola and Dillon, 2009).

Different physical and chemical treatments are used to transform cellulosic wastes (Sun and Cheng, 2002). As a clean alternative, the industry uses enzymes that convert the constituent polymers of the plant cell wall (lignin, cellulose and hemicellulose) into simple sugars (Pérez et al., 2002), but the high cost of these processes is an obstacle for their usage (Biswas et al., 2006). Consequently, the use of microorganisms is gaining relevance because of their ability to degrade polymers such as cellulose and starch which are the major constituents of plant biomass (Ramírez and Cocha, 2003). Moreover, it is important to highlight the role of microorganisms in the degradation of agro-products, for two main reasons: 1) the cost of producing the enzymes for the process is 50% (Galbe and Zacchi, 2002), and 2) the decrease in the inhibitory effect on fermentation processes caused by the preservatives and stabilizers that accompany the use of commercial enzymes (Fujita et al., 2004; Golias et al., 2000).

Different strains of fungi are used in agro-industrial waste degradation, especially those that have exhibited activity on cellulosic substrates. The *Trichoderma* genus was analyzed because of its ability to produce high cellulolytic enzymes activity (Miettinen-Oinonen and Suominen, 2002), that allows the transformation of plant cell-wall constituents or wastes, such as husk, into simple sugars that may become alcohols after the fermentation process. This leads to the conservation of non-renewable resources (Valverde et al., 2007). Therefore, ethanol production becomes relevant, given the possibility of producing 0.25 L of 96°GL alcohol per Kg of husk, which, according to the per liter price Colombia (USD 0.91), could represent an additional income source for producers (Rojas and Cabanillas, 2008). The use of *Penicillium echinulatum* on sugarcane bagasse yields 1.60, 0.21 and 1.49 U mL<sup>-1</sup> for endoglucanase, β-glucosidase and xylanase, respectively; for control cellulose, values of 1.20, 0.20 and 1.46 U mL<sup>-1</sup> were obtained (Camassola and Dillon, 2009). Also, *Aspergillus niger* cellulases, cross linked by glutaraldehyde, maintain their degrading activity during a longer period of time, and hence, further degradation of rice husk at lower cost can be obtained (Sohail et al., 2009).

Therefore, the search for native microorganisms from substrates could be an alternative for obtaining fungal strains with high potential for a cleaner conversion of lignocellulosic materials, and the use of physical and chemical pretreatments will generate cleaner, cheaper processes and without demanding specialized infrastructure

(Llacza and Castellanos, 2012; Martínez-Anaya et al., 2008). In this regard, the objective of this study is to compare the cellulolytic activity of fungal reference strains against those isolated from rice husk, identifying the potential of converting this residue into fermentable sugars.

## MATERIALS AND METHODS

### Plant material

Rice husk was obtained in rice mills located in El Espinal - Tolima Department, Colombia, during the second half of 2011 and was subsequently treated in an electric mill to obtain a size of 1-2 mm. Then, a bromatologica was performed to determine humidity, crude fiber, ether extract, cinder, protein, nitrogen, potassium, phosphorus, copper, zinc, iron, manganese, boron, sulfur, sodium, calcium, and magnesium was done using the methods of AOAC (2012). Analysis was performed in order to determine the percentages of cellulose, hemicellulose, lignin and some oligoelements that could influence fungal growth and cellulase activity.

### Biological material

#### *Fungi isolation and identification*

Untreated samples (rice husk) were introduced into sterile Petri dishes with potato dextrose agar (PDA, Oxoid) and incubated 8 days at 25°C to allow the growth of microorganisms. Later, subcultures were made in order to separate and individualize each fungus. Preliminary identification was performed on a microscope (Advanced Optical, Model XS-402) after staining the fungi with blue-lactophenol; and through taxonomic keys, genera identification was possible.

#### *Preliminary evaluation of cellulolytic capacity*

With some modifications, the methodology proposed by Mikán and Castellanos-Suárez (2004) was used. Strains of *Rhizopus oryzae*, *Aspergillus niger*, *Trichoderma reesei* and *Trichoderma* sp. (five strains) were obtained from the microbiology laboratory of the Research Group of Natural Products of University of Tolima – Colombia. They were identified as follows: T.1, T.2, T.3, T.4, and T.5 and determined for their cellulolytic potential. Also, a strain isolated from rice husk was used. These fungi were placed into a solid culture medium that contained agar-agar and CMC (1 and 2% w/v). Inoculation was performed by placing in the CMC agar center a 5 mm diameter disk of potato dextrose agar (PDA, Oxoid) that was previously inoculated with fungal mycelium. Growth kinetics measurement was performed by triplicate, incubating the microorganisms at 25°C, until the growth of the control samples was observed in the entire 9 mm Petri dish. The degradative activity was manifested through the presence of yellow or unstained areas after the application of Congo red solution (Merck).

### Pre-treatment

#### *Steam explosion (SE)*

The methodology proposed by Sun and Cheng (2002) was used,

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**Table 1.** Experimental design and treatments.

Pre-treatment	Fungi		
	<i>Trichoderma</i> sp.	<i>Aspergillus</i> sp.	<i>Rhizopus oryzae</i>
SE	T.SE	A.SE	R.SE
LIME	T.LIME	A.LIME	R.LIME
BLANCK	T.WT	A.WT	R.WT

SE: Steam explosion; LIME: Alkaline hydrolysis; Blank: rise husk without pre-treatment.

with some modifications. The lignocellulosic material (rice husk) was treated with high-pressure saturated steam and then the pressure was swiftly reduced. The process was performed under autoclave conditions (120°C, 15 psi) during an interval of 45 and 60 min.

#### Alkaline hydrolysis (LIME)

For this assay, the methodology described by Sun and Cheng (2002) was used. 100 g of the rice husk was treated with saturated solution of calcium hydroxide diluted (2 L) in 1:20 ratio, at 60°C for 24 h. Finally, rice husk was washed with distilled water three times.

#### Fermentation

The material was exposed to a fermentation that included a pre-treatment (SE or LIME) coupled with the subsequent degradation of one of the fungal strains used. The total number of treatments was 6, with 3 replicates for each one, wherein blank was included (rise husk without pre-treatment).

The fermentation process of 3 fungi [*Trichoderma* sp., *Aspergillus* sp. (isolated from rice husk) and *Rhizopus oryzae*] with the best performance from the CMC assay was developed in bioreactors of 500 mL, containing 10 g of husk, 18 mL of sterile water and 2 mL of spore suspension ( $5 \times 10^6$  conidia mL<sup>-1</sup>). Finally, the pH was adjusted to 6.5 with 0.1 N HCl and 0.1 N NaOH, and the solution was incubated at room temperature (25°C) with constant stirring (150 rpm) for 30 days. Finally, the leachate samples from the bioreactors were taken every 7 days for a month, whereupon they were vacuum filtered in order to quantify total and reducing sugars.

#### Quantification of carbohydrates

Total carbohydrates were quantified by a spectrophotometer (UV-V Thermo Scientific Helios Gamma UVG154501 model), using the anthrone method described by Witham et al. (1971). Moreover, reducing sugars were quantified by the 3,5-dinitrosalicylic method, described by Miller (1959). Calibration curves were made from 10 to 100 µg mL<sup>-1</sup> for DNS method and 120-2000 µg mL<sup>-1</sup> for anthrone method, and validated according to Quattrocchi et al. (1992).

#### Cellulose activity

##### Endoglucanase activity

The methodology used for this purpose was the one proposed by Gunjekar et al. (2001) and Berghem and Pettersson (1973). A CMC solution (1%) was prepared in sodium acetate buffer (0.05 M, pH 5) and one (1) mL of this solution was incubated with 0.28 mL of the enzyme solution (leachate filter) and assayed at 50°C for 30 min. After reaction completion, DNS reagent (1%) was added. The reducing

sugars concentration produced by the enzyme reaction was measured according to the equation proposed by Eveleigh et al. (2009) and Gunjekar et al. (2001): Endoglucanase activity (U mL<sup>-1</sup>) = reducing sugars released (mg) x 0.66.

##### Exoglucanase activity

In this assay, the methodology used was the one proposed by Gunjekar et al. (2001) and Berghem and Pettersson (1973). One (1) mL of tested enzyme solution (leachate filter) was added to 50 mg of filter paper previously dipped in Buffer sodium acetate (0.05 M, pH 5). After 30 min of incubation at 40°C, DNS reagent (1%) was added and the reducing sugar concentration was measured. Exoglucanase activity was calculated according to the equation proposed by Afolabi (1997): Exoglucanase activity (U mL<sup>-1</sup>) = reducing sugars released (mg) x 0.185.

##### β-Glucosidase activity (cellobiose)

The methodology used was the one proposed by Klesov (1981). Three test tubes were used: the first blank tube contained 1 mL of each solution (cellobiose 15 mM, citrate buffer at pH 4.8 and water), the second blank tube contained 1 mL of the sample (filter leachate) and 2 mL of water, and the third tube contained 1 mL of cellobiose solution, buffer and test sample. All tubes were mixed and incubated at 50°C for 30 min. DNS reagent (1%) was added and the reducing sugars concentration (glucose) was measured by the DNS method. The concentration measurement was obtained by subtracting the absorbance sample from that of the sample blank and cellobiose blank. The β-glucosidase activity was determined according to the equation of Afolabi (1997): β-glucosidase activity (U mL<sup>-1</sup>) = Glucose liberation (mg) x 0.0926.

All tests were made with leachates extracted from a submerged culture assay as described above. But in this case only *Trichoderma* sp.1 and *Aspergillus* sp. were used; moreover, a kinetics analysis was performed every 48 h reaching 196 h.

#### Statistical analysis

All variables were subjected to a Kolmogorov-Smirnov test, in order to obtain a normal data distribution. Then a one-way variance analysis (ANOVA) and a LSD test ( $p \leq 0.05$ ) were made using the Info Stat program (free version) (Di Rienzo et al., 2011). Treatments abbreviations are described in Table 1, which were employed in subsequent graphs.

## RESULTS AND DISCUSSION

As a result of the bromatological test applied, percentages of cellulose, hemicellulose and lignin were determined

**Table 2.** Bromatological test results from rice husk.

Parameter	Value
Cellulose (%)	37.63
Hemicellulose (%)	10.23
Lignin (%)	12.5
Humidity (%)	11
Cinder (%)	19
Crude Protein (%)	1.7
Ether extract (%)	2.6
Brute protein (%)	34
Nitrogen (%)	0.27
Potassium (%K)	0.31
Phosphorus (%P)	0.56
Cooper (mg Kg <sup>-1</sup> Cu)	1.2
Zinc (mg Kg <sup>-1</sup> Zn)	18
Iron (mg Kg <sup>-1</sup> Fe)	12
Manganese (mg Kg <sup>-1</sup> Mn)	39
Bore	ND
Sulfur (%S)	0.20
Sodium (mg Kg <sup>-1</sup> NA)	46

(Table 2). These results were used in the calculation of the material conversion into total carbohydrates and reducing sugars. These findings were compared with reports from other authors regarding the same waste (rice husk), and similar results to those reported were obtained by Sánchez (2009) and Valverde et al. (2007).

The ash (19%) indicated the presence of minerals, such as manganese (39 mg kg<sup>-1</sup>), iron (12 mg kg<sup>-1</sup>) and zinc (18 mg kg<sup>-1</sup>). Likewise, other minerals were found, but in smaller proportions. It is noteworthy that some of the minerals (manganese, iron and zinc) are part of the most widely culture media used in cellulose degradation studies.

### Growth kinetics

Some of the fungal strains (*R. oryzae*, *T. reesei* and *Trichoderma* 1, 2, 3, 4, 5) were present in the microbiology laboratory and *Aspergillus* sp. was recovered from waste. Growth assay on one material cellulosic like CMC allowed the identification of the cellulolytic activity from the strains used as shown in Figure 1. This allowed the identification of *R. oryzae*, *Aspergillus* sp. and *Trichoderma* sp.1 as the ones with the highest speed growth. Husk degradation tests were done with those strains. The fungus *R. oryzae* filled Petri dish in just 48 h, probably for its capacity to grow in different substrates.

### Quantification of carbohydrates

The statistical analysis showed that there is no significant difference between the applied pretreatments; however, the best performance was the one showed by steam

explosion. This treatment released 878.26 µg of total carbohydrates, generating 2.9 (304.44 µg) and 1.4 (643.44 µg) more than those released from the treated (LIME) and untreated husk, respectively. Regarding reducing sugars, the untreated material was the top performer: it released 509.56 µg, generating 1.5 (343.15 µg) and 1.3 (387.49 µg) more than those released with the LIME and steam explosion pre-treatments respectively (Figure 2).

As shown in Figures 2 and 3, the steam explosion pre-treatment favored carbohydrate release. Probably this effect is due to the physical and chemical changes that may occur in this process, such as depolymerisation and breakage of fiber and links with the subsequent release of oligosaccharides; processes that have been previously described by Sun and Cheng (2002). Nonetheless, the performance of reducing sugar release was significantly lower, probably due to other factors such as substrate fungal colonization and their enzymatic efficiency.

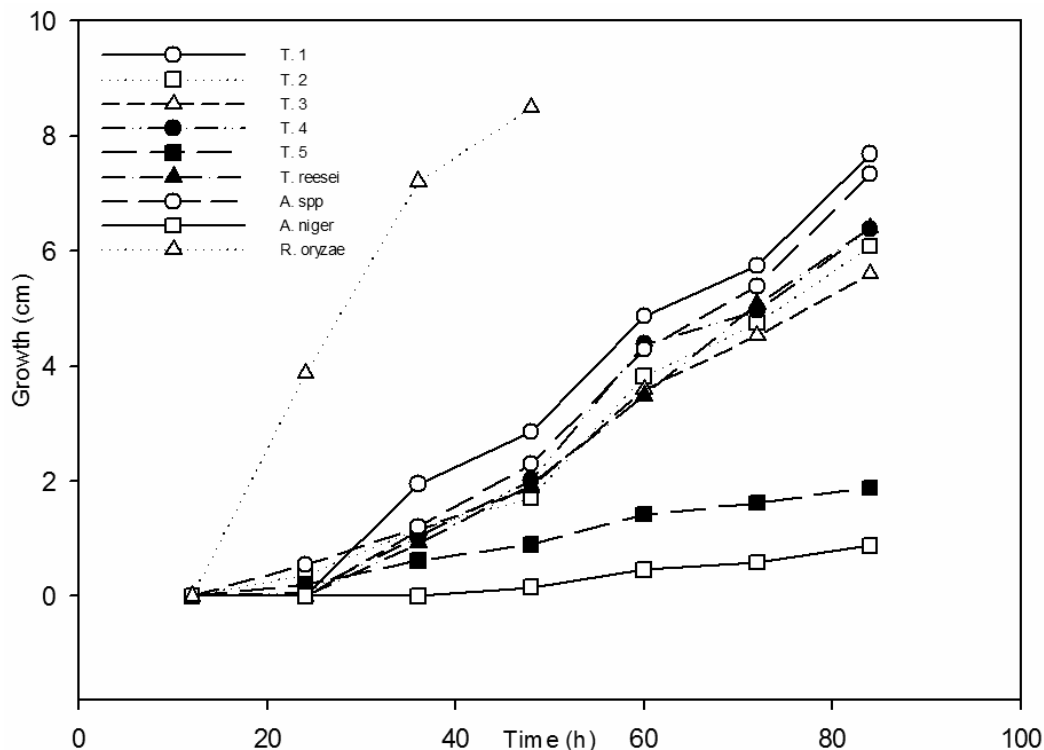
Likewise, between the two most efficient fungi (*Aspergillus* sp., and *Trichoderma* sp.1) statistically significant differences were observed. *Aspergillus* sp. released more reducing sugars and *Trichoderma* sp. 1 produced the largest amount of total carbohydrates (probably related to the β-glucosidases production, responsible for monomeric sugars release). This performance was also observed in *Trichoderma reesei* strains as previously reported by Saloheimo et al. (2007) and Lynd et al. (2002). This will be clarified later in the enzymatic activity discussion.

Figures 4 and 5 show the system performance during each week. In Figure 4, high total carbohydrates release can be observed (1489.41 µg mL<sup>-1</sup> of total carbohydrates from which 610.83 µg mL<sup>-1</sup> correspond to reducing sugars). However, that release decreased with time. This phenomenon has also been observed by other authors, who have highlighted that it is due to several factors, such as fungal demand for taking some of the produced sugars to continue their metabolism (Taniguchi et al., 2005), the absorption of enzymes by cellulose and lignin (Garibello and Melissa, 2013), or the enzymatic activity inhibition due to glucose and cellobiose presence (produced by cellulases) in the medium (Qing et al., 2010).

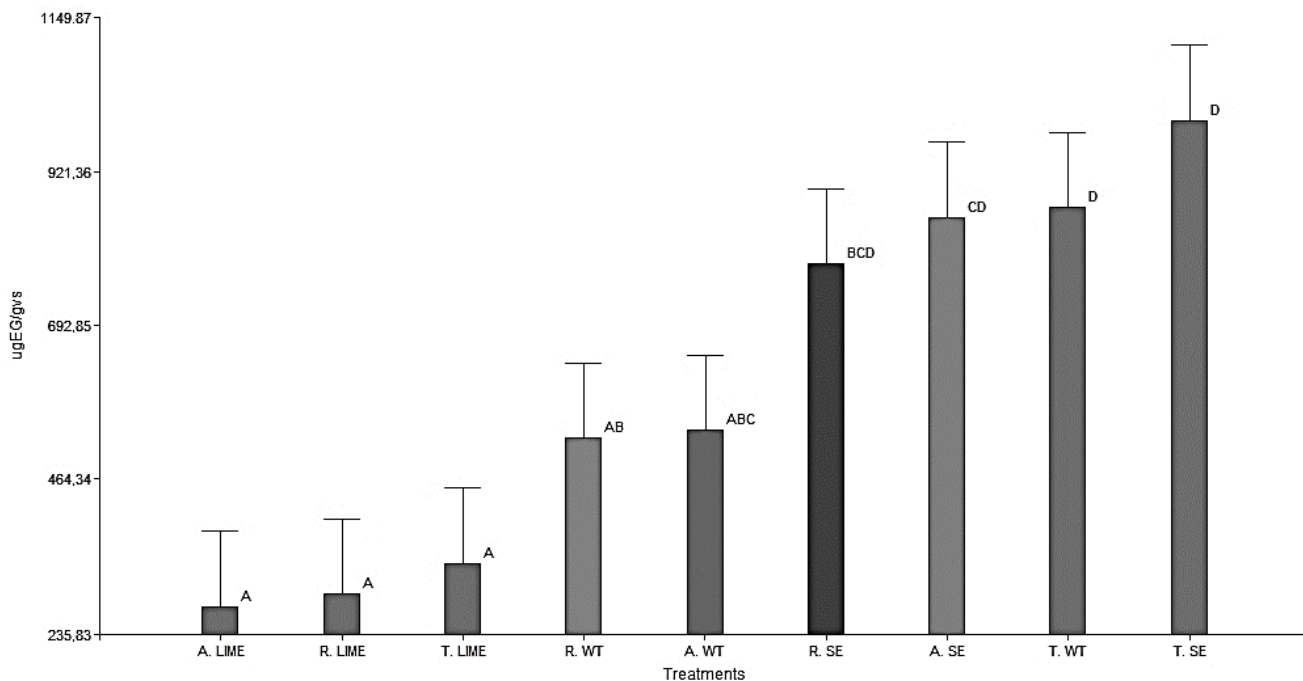
Finally, at week 4 of the treatment, the best conversion ratio, starting with 10 g of husk, was that *Aspergillus* sp. had a transformation percentage of 21.06%. There was a sharp difference in the production of total carbohydrates and reducing sugars, which allowed the choosing of *Trichoderma* sp. 1, and *Aspergillus* sp., as the two microorganisms with the best performance. The cellulase activity was evaluated in order to differentiate their ability to degrade the material.

### Cellulase activity

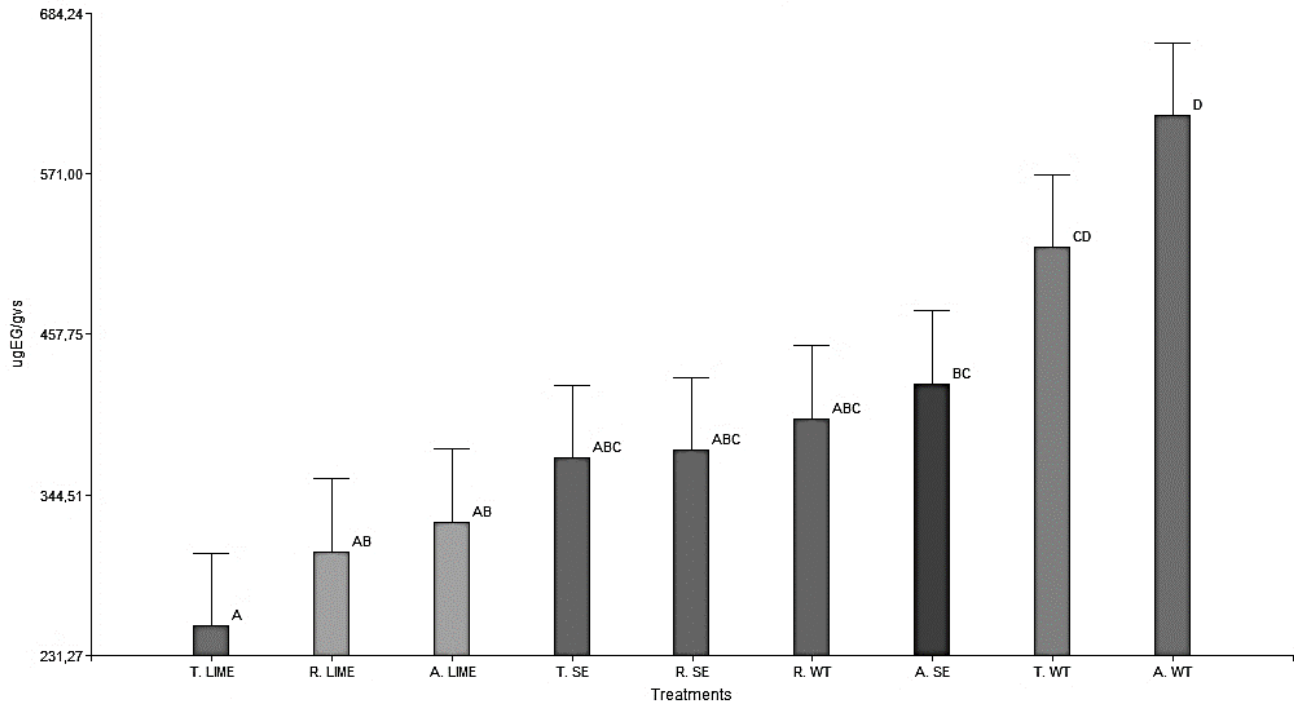
Endoglucanase activity Strains of *Aspergillus* sp. and *Trichoderma* sp. 1 showed similar performance during



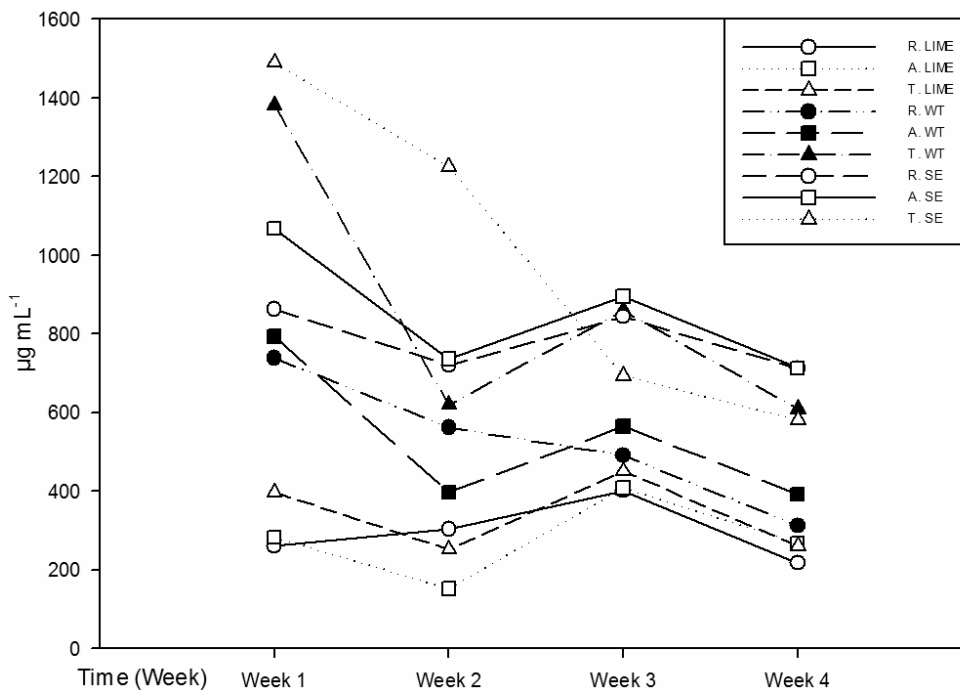
**Figure 1.** Growth kinetics from strains used in agar CMC (2%). T. 1, *Trichoderma* sp 1; T. 2, *Trichoderma* sp 2; T. 3, *Trichoderma* sp 3; T. 4, *Trichoderma* sp 4; T. 5, *Trichoderma* sp 5; T. reesei, *Trichoderma reesei*; A. sp (Asl), *Aspergillus* sp; A. niger, *Aspergillus niger*; R. oryzae, *Rhizopus oryzae*.



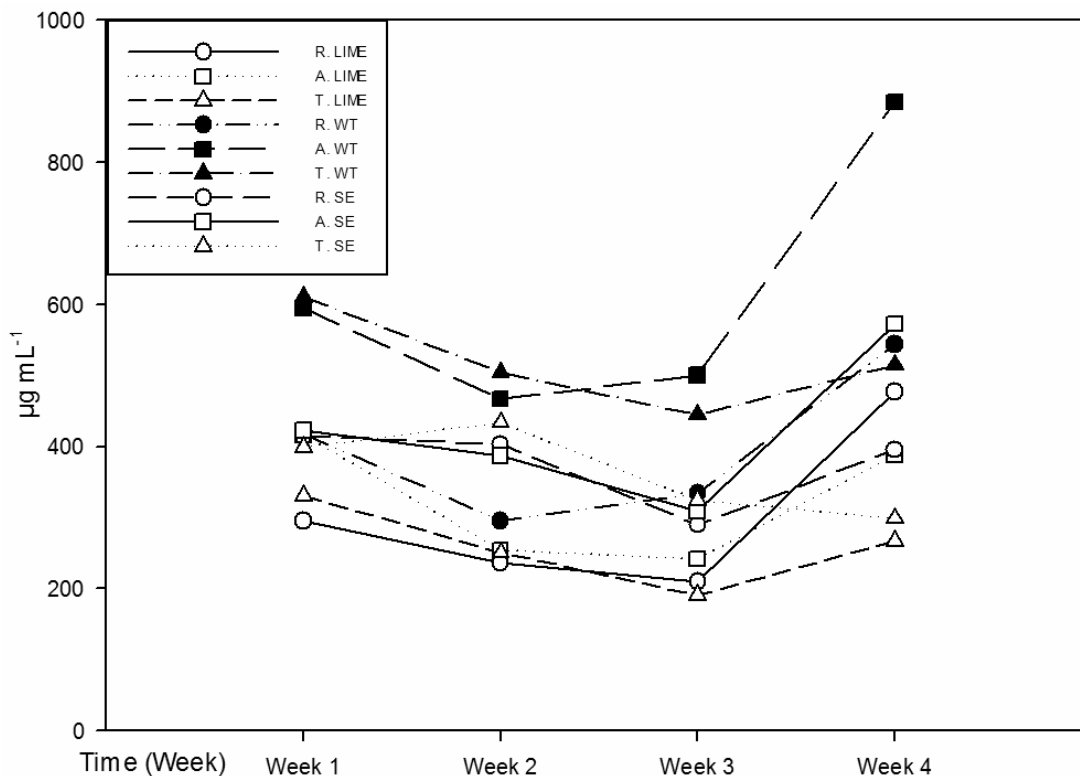
**Figure 2.** Least significant difference (LSD) of treatments. .LIME, *Rhizopus*-LIME; A.LIME, *Aspergillus*-LIME; T.LIME, *Trichoderma*-LIME; R.WT, *Rhizopus*-Without treatment; A.WT, *Aspergillus*-Without treatment; T.WT, *Trichoderma*-Without treatment; R.SE, *Rhizopus*-steam explosion; A.SE, *Aspergillus* steam-explosion; T.SE, *Trichoderma* steam-explosion.



**Figure 3.** Least significant difference (LSD) treatments. T.LIME (Trichoderma-LIME), R.LIME (Rhizopus-LIME), A.LIME (Aspergillus-LIME), T.SE (Trichoderma steam-explosion), R.SE (Rhizopus-steam explosion), R.WT (Rhizopus-Without treatment), A.SE (Aspergillus steam-explosion), T.WT (Trichoderma-Without treatment), A.WT (Aspergillus-Without treatment) in the release of reducing sugars.



**Figure 4.** Kinetics of total carbohydrates released by the treatment used. R.LIME, Rhizopus-LIME; A.LIME, Aspergillus-LIME; T.LIME, Trichoderma-LIME; R.WT, Rhizopus-Without treatment; A.WT, Aspergillus-Without treatment; T.WT, Trichoderma-Without treatment; R.SE, Rhizopus-steam explosion; A.SE, Aspergillus steam-explosion; T.SE, Trichoderma steam-explosion.



**Figure 5.** Kinetics of reducing sugars released by the treatment used. R.LIME, Rhizopus-LIME; A.LIME, Aspergillus-LIME; T.LIME, Trichoderma-LIME; R.WT, Rhizopus-Without treatment; A.WT, Aspergillus-Without treatment; T.WT, Trichoderma-Without treatment; R.SE, Rhizopus-steam explosion; A.SE, Aspergillus steam-explosion; T.SE, Trichoderma steam-explosion.

monitoring, with a unique difference at 240 h wherein *Trichoderma* sp.1 showed higher endoglucanase activity. This result contrasts with previous reports, which indicate that the activity of *Aspergillus* genus has been greater than that of *Trichoderma*. In the present study, at day 10, *Trichoderma* sp.1 showed the highest activity, with 0.350 IU mL<sup>-1</sup> followed by *Aspergillus* sp. with a production of 0.225 IU mL<sup>-1</sup>, as shown in Figure 6. Furthermore, the fungal endoglucanases from the strains assessed, proved to have a production comparable with others enzymes from different investigations performed on different substrates (Ahamed and Vermette, 2010).

### Exoglucanase activity

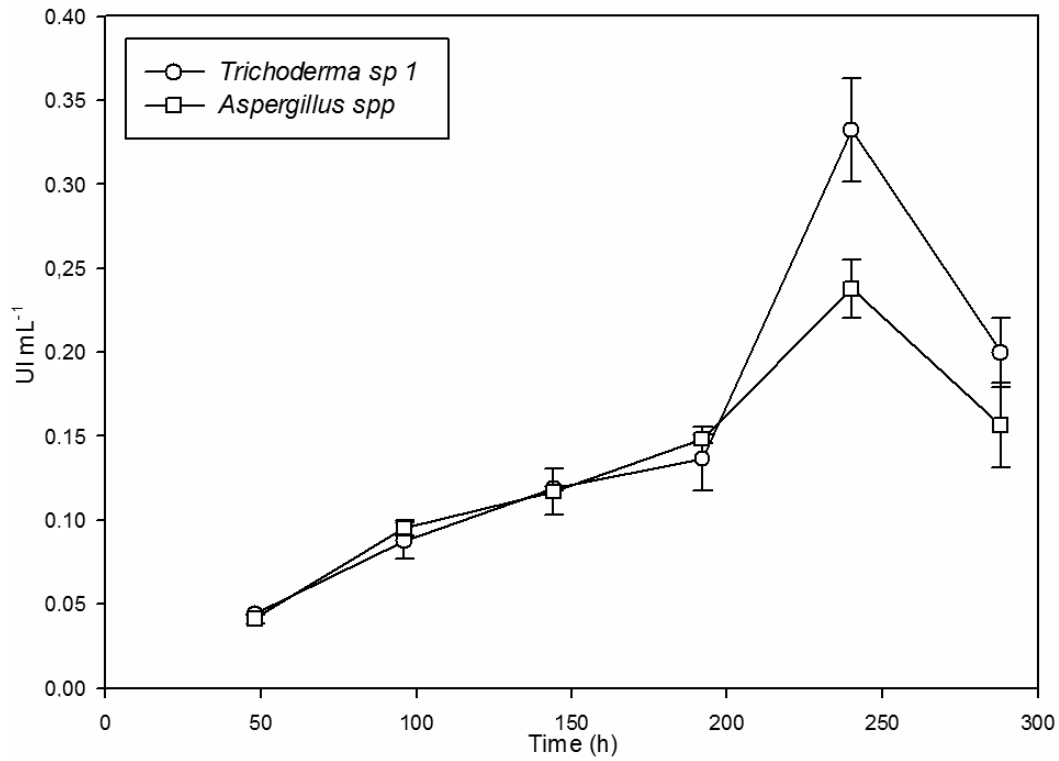
The exoglucanase activity showed no significant differences among the used fungi (Figure 7), emphasizing that the activity is the same on this substrate. However, different performances are observed in the literature when compared strains from the same genus are placed on filter paper substrates (Fang et al., 2010). This indicates the importance of studying the performance of several strains on different substrates and under different culture conditions.

### β-Glucosidase activity

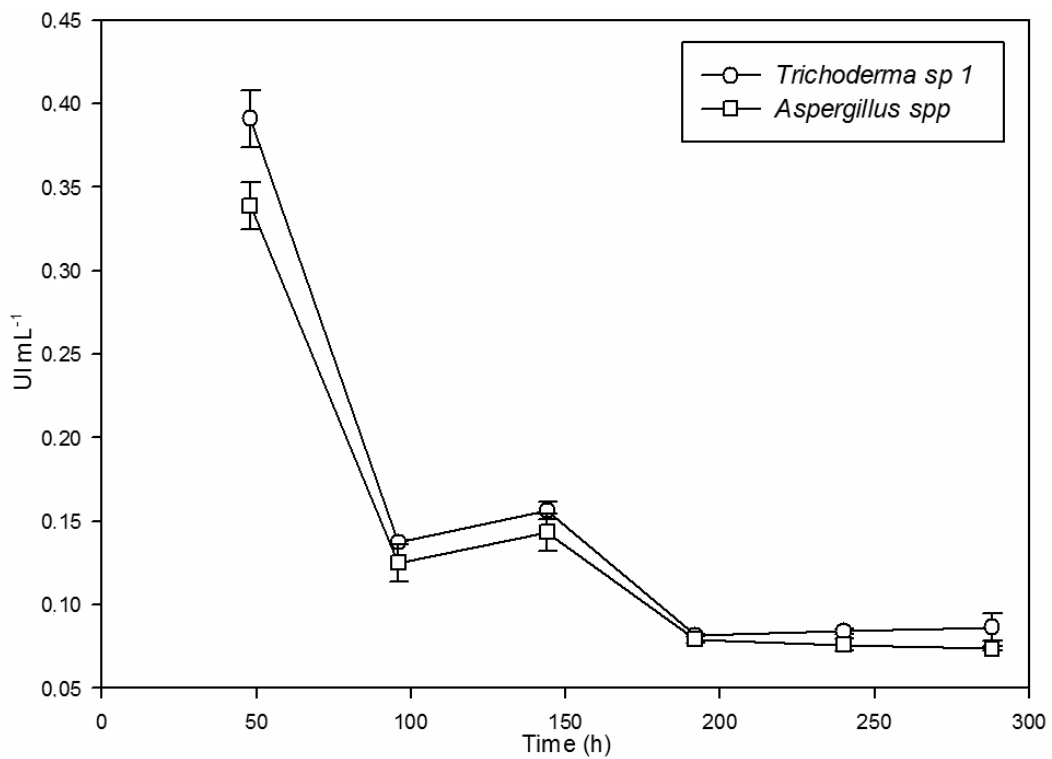
Regarding this activity, there was a similarity between *Aspergillus* sp. and *Trichoderma* sp.1 with maximum values of 0.150 and 0.140 IU mL<sup>-1</sup> at 192 and 288 h, respectively (Figure 8). The β-glucosidase activity in *Trichoderma* sp.1 was lower than the one of *Aspergillus* sp.. This is contrary to the reports of Manjarrés et al. (2011), Fang et al. (2010) and Flachner et al. (1999), wherein an inverse performance is pointed, compared with the one found in the present study. Finally, the enzymatic assay allowed the relating of the endoglucanases production, the greater release of total carbohydrates in *Trichoderma* sp. 1 as well as the greater production of reducing sugars and β-glucosidase in *Aspergillus* sp.

### Conclusions

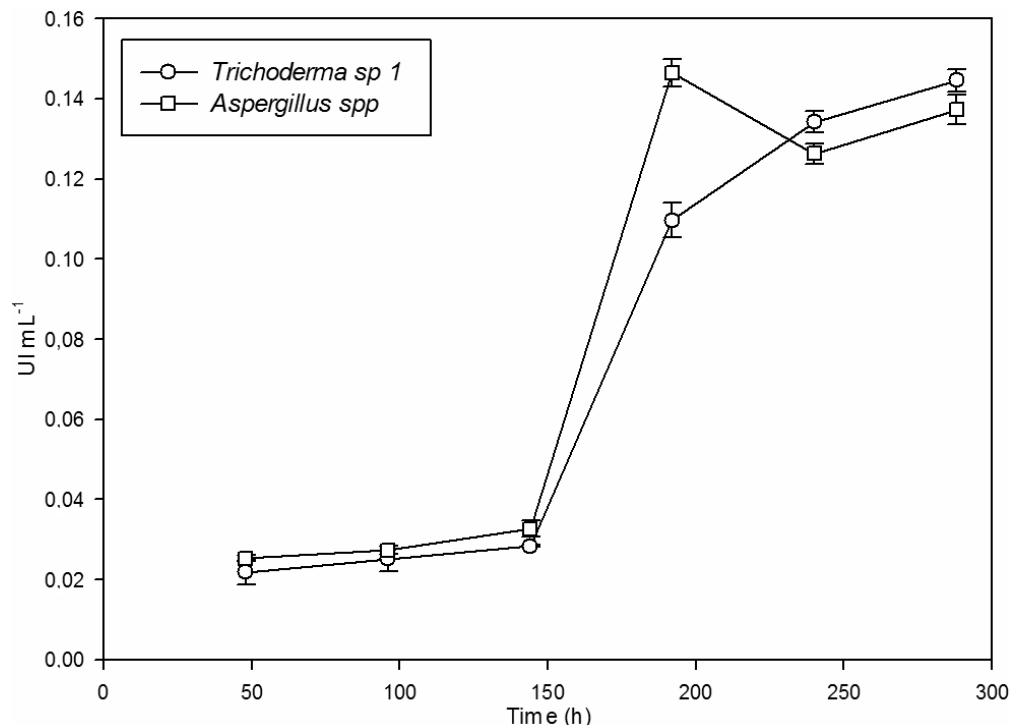
The study presented here showed the efficiency of using filamentous fungi for splitting rice husk. It allows the production of significant amounts of fermentable sugars, which can be subsequently used to produce various added-value compounds, including ethanol. Native fungal



**Figure 6.** Enzyme kinetics of two fungal endoglucanase activity with increased release of total carbohydrate and reducing sugars (*Trichoderma sp 1* and *Aspergillus spp.*).



**Figure 7.** Enzyme kinetics of two fungal exoglucanase activity with increased release of total carbohydrate and reducing sugars (*Trichoderma sp 1* and *Aspergillus spp.*).



**Figure 8.** Enzyme kinetics of two fungal  $\beta$ -glucosidase activity with increased release of total carbohydrate and reducing sugars (*Trichoderma sp 1* and *Aspergillus spp*).

strains from husk, such as *Aspergillus sp.*, offer a potential comparable with that of fungi widely used for similar purposes, and hence, may be used in the cellulosic materials degradation processes.

### Conflict of Interests

The author(s) have not declared any conflict of interest.

### ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Research and Development office of University of Tolima for funding the study presented here.

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## Full Length Research Paper

# Chitinolytic assay for *Trichoderma* species isolated from different geographical locations of Uttar Pradesh

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Received 15 August, 2014; Accepted 13 October, 2014

Chitin is the most commonly available polymer on the earth. Cell walls of most of the fungi are made up of chitin. As we all know that *Trichoderma* produces a wide variety of cell wall degrading enzymes (CWDEs) such as chitinase, xylanase, glucanase and cellulase. Out of these CWDEs chitinase is of prime importance as it is the building block of fungal cell walls. For the detection of chitinase activity we used a simple and sensitive method. We supplemented the chitinase detection media with colloidal chitin as a carbon source and bromocresol purple as pH indicator dye. This method is easy, sensitive, reproducible and economical. Colloidal chitin derived from sea-shells and commercial chitin is supplemented as carbon source in chitinase broth and solid media for the detection of chitinolytic and exochitinase activity. The chitinolytic activities were ranged from 6.2 to 3.9 and 4.8 to 1.8 mg/ml and exochitinase activities ranged from  $0.0133 \times 10^{-3}$  to  $0.0076 \times 10^{-3}$  and  $0.00609$  to  $0.0055 \times 10^{-3}$  U/ml, respectively, with colloidal chitin derived from commercial chitin and sea-shells.

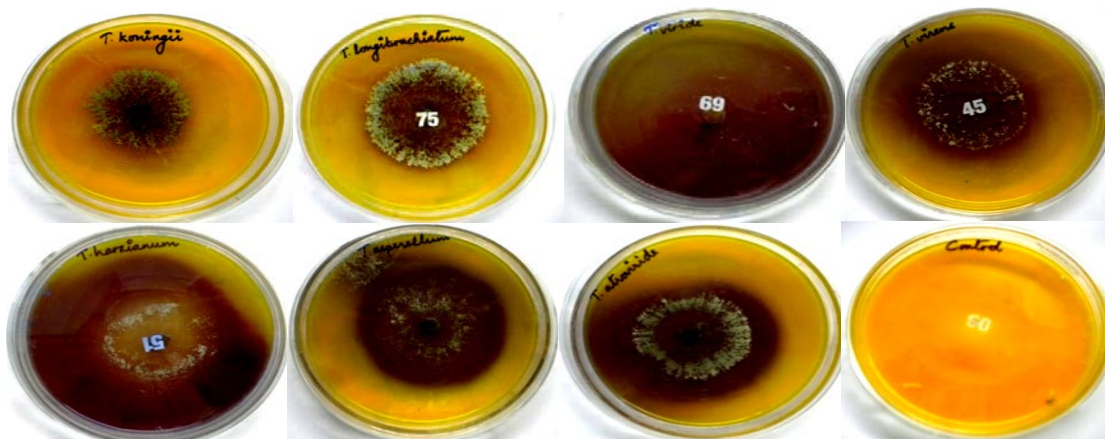
**Key words:** Bromocresol purple, chitin, N-acetyl- $\beta$ -D-glucosamine, p-nitrophenol, *trichoderma*, volume activity.

## INTRODUCTION

Chitin is an unbranched polymer of 1,4- $\beta$ - linked N-acetyl-D-glucosamine (NAGA). Chitin is the building block of fungal cell walls. Chitinase are enzyme that degrade the chitin by breaking the  $\beta$ -1,4 linkages. Chitinases occur in a wide variety of microorganisms including bacteria, fungi, insects etc. In fungi, chitinases are believed to have autolytic, nutritional and morphogenetic roles. In mycoparasitic fungi, chitinases are associated with the degradation of cell walls. *Trichoderma* spp. is the most commonly used biocontrol agents against several soil-borne fungal plant pathogens such as *Sclerotium rolfsii*,

*Rhizoctonia solani* and *Pythium* spp. Etc Bhattacharya et al., 2007). Members of the fungal genus *Trichoderma* spp. produce cell wall degrading enzymes such as glucanase, chitinase xylanase etc., that are involved in the mycoparasitic action. Chitinase enzymes are of great importance as compared to other CWDEs, as fungal cell wall is made up of chitin that is why chitinolytic enzyme degrade phytopathogenic fungi easily (González et al., 2012). *Trichoderma* spp. employ different strategies of defense against phytopathogens such

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**Figure 1.** Chitinase activity observed after 3 days of inoculation in chitinase detection media supplemented with colloidal chitin (set-1).

as: competition for space and nutrients, secretion of cell wall degrading enzymes, induction of resistance etc. (Rifat et al 2013). *Trichoderma* inhibit the hyphal growth of phytopathogens by coiling, it uses hooks to penetrate the fungal cell walls with the help of cell wall degrading enzymes such as xylanase, chitinase, cellulase etc., among the different mechanisms used by *Trichoderma* spp. parasitism, competition and antibiosis are the main mechanisms which are involved in mycoparasitic action. Cell wall degrading enzymes are the key factors which involved in the cell wall destruction of pathogen (Kowsari et al., 2014).

## MATERIALS AND METHODS

### Isolation and maintenance of fungal isolates

*Trichoderma* strains were isolated from soil samples collected from the different geographical locations of U.P. and were maintained on potato dextrose agar (PDA) plates. Colloidal chitin used as a carbon source was derived from sea-shell and commercial chitin (CDH). For colloidal chitin preparation, acid hydrolysis was done by conc. HCl during acid hydrolysis of which the flasks were kept in constant stirring using magnetic stirrer for 24 h. After 24 h, this chitin and acid mixture was kept at 4°C and left for overnight. After incubation period the acid mixture is treated with 2000 ml of ice cold 95% ethanol and kept at 26°C for overnight. After incubation period, it was centrifuged at 3000 rpm for 20 min at 4°C. After centrifugation the supernatant was discarded while pellet is washed with distilled water by centrifugation at 3000 rpm for 5 min, till the smell of alcohol is removed (Saraswathi et al. 2013). The colloidal chitin thus obtained has a soft and white consistency with 90 to 95% moisture and stored at 4°C till use (Roberts and Selitrennikoff, 1988).

### Agar medium for detection of chitinase-positive microorganisms

Chitinase detection medium supplemented with colloidal chitin as carbon source was used for the chitinase plate assay (Shahidi et al., 2005). Lukewarm media was poured into the Petri plates and

allowed to solidify; after solidification, fresh culture plugs of the *Trichoderma* spp. tested for chitinase activity were placed in the middle of the plate. Plates were incubated at 25±2°C and were observed for purple colour zone formation. Chitinase activity exhibited by seven *Trichoderma* spp. was determined by measuring the diameter of purple color zone after three to seven days of incubation (Agrawal and Kotasthane, 2012) (Figures 1 to 4).

### Total chitinolytic activity

Total chitinolytic activity was calculated by measuring the release of reducing saccharised from carbon source (colloidal chitin). A reaction mixture was prepared containing 1 ml culture supernatant and 0.3 ml of sodium acetate buffer (pH 4.6); to this mixture, 0.2 ml of colloidal chitin was added and incubated for 20 h at 40°C. After incubation, the contents of the tube were centrifuged at 13000 rpm for 5 min at 5°C.

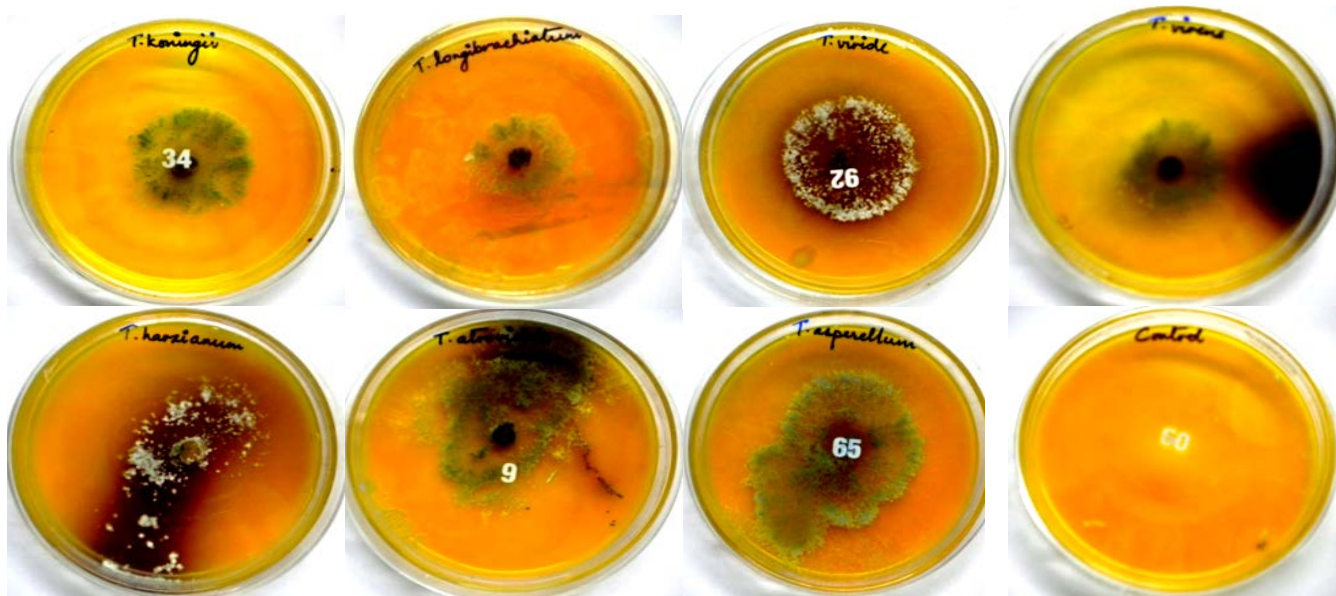
After centrifugation 0.75 ml of supernatant was taken and 0.25 ml of 1% salicylic acid was added to the mixture 1 ml of 0.7 M NaOH and 10 M NaOH were added and heated at 100°C for 5 min. OD of the reaction mixture was taken at 582 nm. Reference curve was made with N-acetyl-β-D-glucosamine (NAGA). Chitinolytic activity was expressed in terms of mg/ml (Muzzarelli et al 1997).

### Exochitinase activity

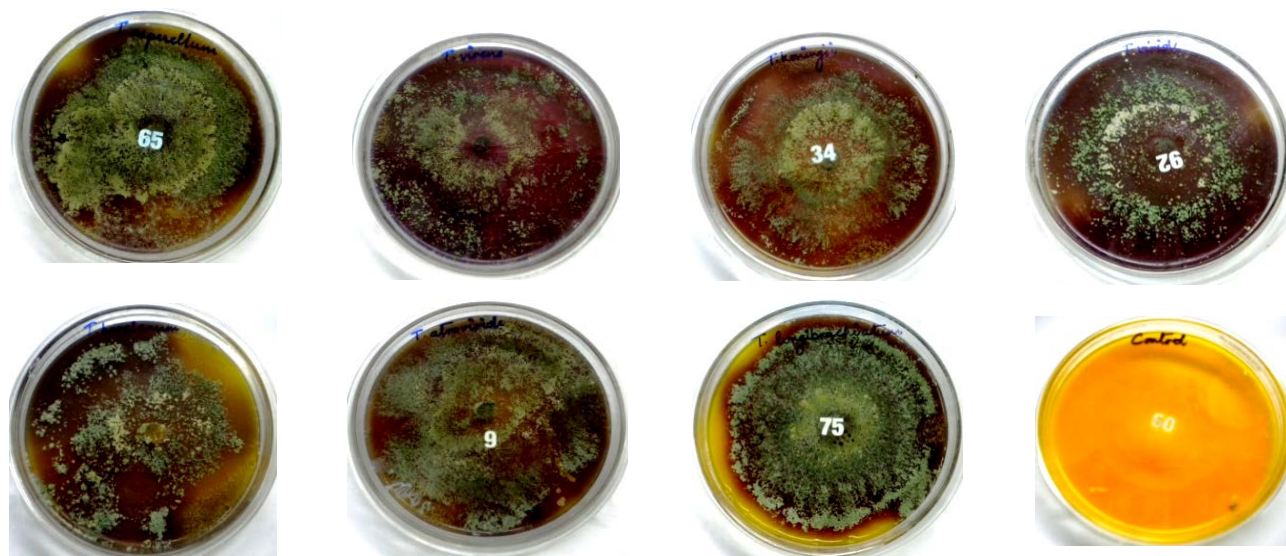
Exochitinase activity was measured by the release of p-nitrophenol (pNP) from p-nitrophenyl-Nacetyl-β-D-glucosaminide (pNPg). A reaction mixture was prepared containing 25 ul of culture filtrate and 0.2 ml of p-nitrophenol solution, to this reaction mixture 1 ml of 0.1 M sodium acetate buffer was added. This reaction mixture was incubated at 40°C for 20 h. After incubation period, contents of the tube were centrifuged at 13000 rpm. After centrifugation, 0.125 M sodium tetraborate- sodium hydroxide buffer was added to the 0.6 ml of supernatant. OD was taken at 400 nm.

## RESULTS AND DISCUSSION

When *Trichoderma* strains were inoculated on chitinase media containing colloidal chitin (carbon source) and bromocresol purple (pH indicator dye) breakdown of



**Figure 2.** Chitinase activity observed after 3 days of inoculation in chitinase detection media supplemented with sea- shell chitin (set-1).



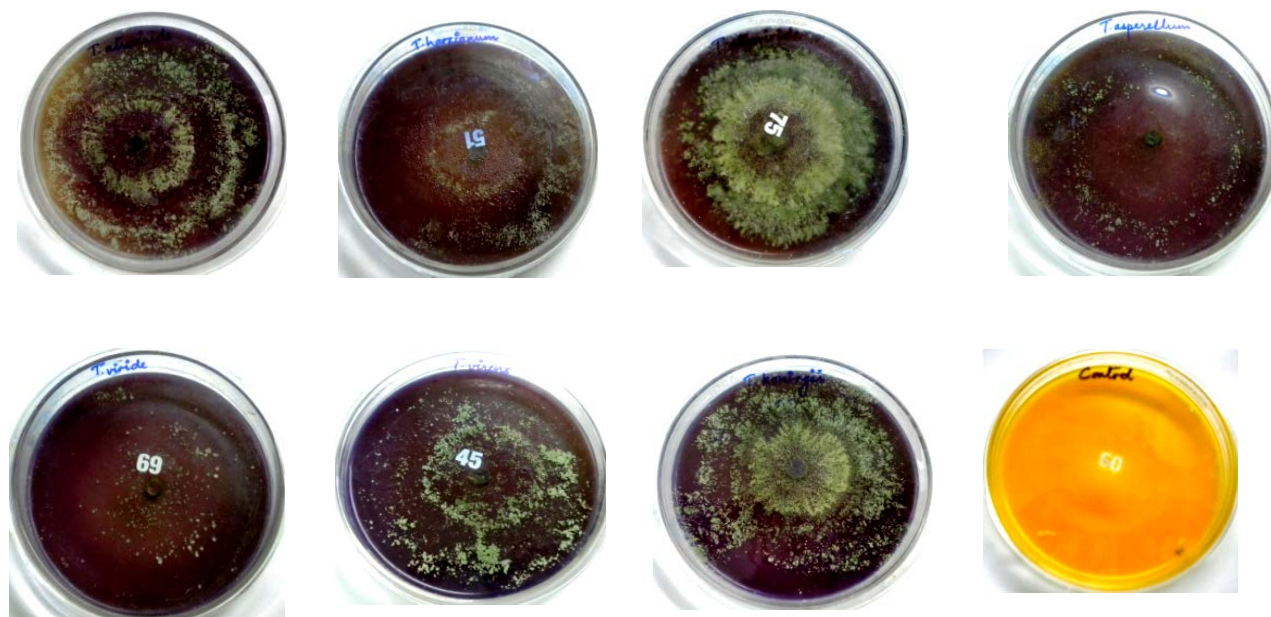
**Figure 3.** Chitinase activity observed after 7 days of inoculation in chitinase detection media supplemented with seashell chitin (Set-2).

chitin occurs into N-acetyl glucosamine which causes a change in pH (acidic to alkaline). This change in pH is indicated by the change in colour of media from yellow to purple zone surrounding the inoculated culture plug area.

Chitinase activity exhibited by the seven strains of *Trichoderma* was evaluated through the formation of purple coloured zone after three and seven days of incubation. No complicated protocols were adopted for the evaluation of chitinase activity (Gomez et al., 2004). Total chitinolytic activity was assayed by measuring the

release of reducing saccharides from colloidal chitin (Table 1).

Standard curve generated by the use of NAGA is used to evaluate the reducing saccharide conc. The observations were in close resemblance with those De la Cruz et al. (1992) and Lorito et al. (1994). Production of hydrolytic enzymes is greatly affected by the cultural conditions. For exochitinase activity, release of p-nitrophenol (pNP) from p-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminide (pNPg) was measured. The volume activity



**Figure 4.** Chitinase activity observed after 7 days of inoculation in chitinase detection media supplemented with colloidal chitin (Set-1).

**Table 1.** Chitinolytic and exochitinase activity of *Trichoderma* species.

Isolate name	Total chitinolytic activity (mg/ml)		Exochitinase activity (U/ml X 10 <sup>-3</sup> )	
	Colloidal chitin	Seashell chitin	Colloidal chitin	Seashell chitin
<i>T. viride</i>	6.0	4.3	0.0125	0.00609
<i>T. harzianum</i>	6.2	4.8	0.0133	0.00607
<i>T. asperellum</i>	5.4	3.3	0.0110	0.00604
<i>T. koningii</i>	5.5	2	0.0097	0.0060
<i>T. atroviride</i>	3.9	3.3	0.0116	0.0061
<i>T. longibrachiatum</i>	5.6	3.1	0.0084	0.0055
<i>T. virens</i>	5.0	1.8	0.0076	0.0056

of pNP ranged from 0.0125 to 0.0076 × 10<sup>-3</sup> U/ml and 0.0069 to 0.0055 × 10<sup>-3</sup> U/ml in commercial chitin and sea shell derived colloidal chitins, respectively.

**Conclusion**

Based on the results of above observations, it is clear that for choosing an effective biocontrol agent, it is essential to provide the optimum cultural conditions. The medium used here for chitinase assay was very effective and economical. The medium used here is very friendly and sensitive. Formation of the purple color zone was found to be the easier alternative method for the selection of chitinolytic strains of *Trichoderma* species.

**Conflict of Interests**

The author(s) have not declared any conflict of interest.

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Shahidi F, Abozaytoun R (2005). Chitin, chitosan and co-products: Chemistry, by production, applications and health effects. Adv. Food Nutr. Res. 49:93-135.

Full Length Research Paper

## Comparative acute toxicity and oxidative stress responses in tadpoles of *Amietophrynus regularis* exposed to refined petroleum products, unused and spent engine oils

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Received 9 August, 2014; Accepted 13 October, 2014

The relative acute toxicity of refined petroleum (diesel, kerosene and petrol), unused and spent engine oils as well as their abilities to alter the activities of superoxide dismutase (SOD) and cause lipid peroxidation in tadpoles of the common African toad, *Amietophrynus regularis* were evaluated. After 48 h of exposures, kerosene was found to be the most toxic ( $LC_{50} = 4930$  mg/L) while the least toxic was unused engine oil ( $LC_{50} = 7777$  mg/L). However, by 96 h of exposure, spent engine oil was found to be the most toxic ( $LC_{50} = 2915$  mg/L) while unused engine oil remained the least toxic ( $LC_{50} = 7353$  mg/L). Further, assessment of oxidative stress markers was conducted using sub lethal concentrations of the test compounds (1/100th 96 h  $LC_{50}$ ). There was significant inhibition of SOD in exposed tadpoles compared to the control ( $P < 0.05$ ) with the least activity recorded in tadpoles exposed to petrol, while unused engine oil recorded the highest. The results of the lipid peroxidation assay, determined by measuring the levels of malondialdehyde (MDA) indicated significantly higher levels in the exposed individuals compared to the control. Unused engine oil caused the highest level of MDA production while diesel induced the least level. Tadpoles exposed to diesel, kerosene, petrol and spent engine oil exhibited consistent responses among the three test parameters, however inconsistent responses were observed in tadpoles exposed to unused engine oils. The relevance of the comparisons in biomarker selection and ecotoxicology were discussed.

**Key words:** Petroleum products, toxicity indices, tadpoles, oxidative stress.

### INTRODUCTION

Crude oil refining, transportation and use according to Pacheco and Santos (2001), is associated with spillage

of petroleum products which is one of the most important pollutant of concern in aquatic ecotoxicology. Their

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toxicological effects have been evaluated in a number of fish species (Sunmonu and Oloyede, 2007; Simonato et al., 2008; Jahanbakhshi and Hedayati, 2012) given the inevitability of contact and possible uptake once they enter aquatic ecosystems.

In Nigeria, petroleum product spills apart from those occurring during refining of crude oil may also result during transfers at the jetty, accidents involving tankers, dispensing of products to vehicles as well as vehicular and generator repairs. Since, the advent of crude oil exploration in the country in 1956 (Akpofure et al., 2000) spillages of crude and petroleum products have been commonplace, raising concerns regarding their polluting effects in aquatic ecosystems (Kadafa, 2012). Petroleum products are a mixture of hydrocarbons and additives which could produce free radicals (Achuba and Osakwe, 2003). Free radicals are one of the major precursors to oxidative stress, binding with the unsaturated fatty acids of the phospholipids of cell membranes, resulting in lipid peroxidation damage (Timbrell, 2000).

Exposure of animals to pollutants such as hydrocarbons in their natural environment and laboratory conditions has been reported to result in oxidative stress (Esiegbe et al., 2012). Oxidative stress is a state in which the balance between the production of reactive oxygen species (ROS) and their removal by antioxidant defences before they can cause damage is upset (Collins, 2009). Although biological systems, are constantly exposed to free radicals and ROS, there exist a repertoire of anti-oxidative stress enzymes which naturally serves to minimize oxidative damage to cells (Azqueta et al., 2009).

High concentrations of toxicants or chronic exposures may overwhelm the anti-oxidative stress mechanisms resulting in oxidative stress (Reznick et al., 1998). Oxidative damage to cell membranes leads to the release of by-products such as alkanes, ketones and aldehydes including 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal and malondialdehyde (MDA) (Zielinski and Portner, 2000). The presence and activities of enzymes such as superoxide dismutase, catalase and glutathione constitutes a formidable defence system against oxidative stress (Brucka-Jastrzębska, 2010). Superoxide dismutase (SOD) catalytically breaks down super oxide radicals generated in peroxisomes and mitochondria into oxygen and hydrogen peroxides (Li et al., 1995) making them less lipid soluble and more liable to biochemical action.

The consistency in reports regarding the link between exposures to pollutants and subsequent lipid peroxidation damage (Sreejai and Jaya, 2010; Brucka-Jastrzębska, 2010; Esiegbe et al., 2012) implies that they could be suitable candidates for use as biomarkers. Given the simplistic nature of overall measured responses in making toxicological deductions, the use of biomarkers has become commonplace.

This study investigates the acute toxicity and level of

oxidative damage in tadpoles of the African common toad, *Amietophrynus regularis* (Reuss, 1833) following exposures to acute and sub-lethal concentrations of petroleum products (diesel, kerosene and petrol) and engine oils (spent and unused) to explain the possible mechanism of toxic action as a continuation of the discourse on potential biomarkers for environmental pollution monitoring. *A. regularis* is a common tadpole in the rainforests and mangrove swamps of southern Nigeria (Onadeko, and Rodel, 2009). Urbanization and forest clearance destroys their natural habitats and brings them closer to sites of human activities which further threaten their survival. Amphibians worldwide are reported to be on the decline and the drivers have been reported to include global warming (Houlahan, 2000), disease (Kiesecker, 2001) and in some cases aquatic pollution (Ezemonye and Tongo, 2010). It is commonplace to sight their tadpoles in ponds and open gutters which are receptacles and easy dumping sites for spilt petroleum products and spent engine oils. This therefore justifies a study evaluating the toxicities of these products to the tadpoles as well as the indicators of oxidative stress so as to ascertain the extent to which they pose threats to their survival. The enzyme, SOD together with levels of some metabolites, such as MDA are used as biomarkers of oxidative stress (Idowu et al., 2014). Thus, their levels in fishes exposed to petroleum products and engine oils would give an indication of the stress levels in the exposed toads.

## MATERIALS AND METHODS

### Collection and acclimatization of tadpoles

Tadpoles of African common toad, *A. regularis* (Approximate Average length = 0.80±0.15 cm), which are commonly found in ponds and gutters were collected from an undisturbed pond (N 6° 31' 1.5960", E 3° 23' 59.7840" ) at the University of Lagos campus, Lagos, Nigeria, during the breeding season (July 2013), 1 to 2 days after hatching. Hand nets were used in the collection of the tadpoles and care was taken not to agitate them during the process. The tadpoles were transferred into plastic cans containing their habitat water collected from the same pond before transporting to the Ecotoxicology laboratory about 50 m away. The natural pond water was also used during the acclimatization of tadpoles in the laboratory. They were kept in large plastic tanks (l x w x h = 60 cm x 35 cm x 30 cm), half-filled with water and aerated with a 220 v air pump so as to maintain dissolved oxygen levels in the tank. They were left to acclimatize to laboratory conditions (temperature, 26 to 28°C; humidity, 65 to 75%; Light: dark, 8:14 h) for a minimum of 72 h before using them in bioassays. Only tadpoles in tanks having mortality of less than 1% were employed for the study.

### Test compounds

Refined petroleum products (Diesel (Automotive Gas Oil- AGO), kerosene (Dual Purpose Kerosene- DPK), petrol (Premium Motor Spirit- PMS) approved for use in automobiles in Nigeria by the Department of Petroleum Resources (DPR) and engine oils (Motor



Oils- SAE 40: unused and spent) were used for this study. The petroleum products and unused engine oil were of the global grade imported for use in Nigeria. Their specific gravities were diesel: 990 mg, kerosene: 1000 mg, petrol: 990 mg, unused engine oil: 1010 mg and for spent engine oil, 1100 mg. They were purchased in plastic kegs from a filling station at Bariga, near the University of Lagos campus. The spent engine (fuel) oil was collected in 1 L plastic keg from an Auto mechanic workshop also at Bariga. The collected petroleum products and engine oils were stored in the laboratory at room temperature of 26 - 28°C prior to use.

### Acute toxicity bioassay

Preliminary tests were carried out to determine suitable range of bioassay concentrations for the study in an initial test which lasted for 96 h. The range of bioassay concentrations selected for the definitive tests were as follows: diesel: 2970, 4950, 7425, 9900 mg/L and untreated control; kerosene: 1500, 3000, 5000, 6000, 7000 mg/L and untreated control; petrol: 2970, 4950, 6930, 9900 mg/L and untreated control; unused engine oil: 7070, 7575, 8080, 10100 mg/L and untreated control; used engine oil: 1100, 3300, 5500, 7700, 8800 mg/L and untreated control. Four active tadpoles (7 to 12 days old) were randomly selected into an experimental tank (L x W x H = 13.5 x 11 x 7 cm) containing 500 ml of their natural habitat water contaminated with the respective concentrations of toxicants. Each experiment was replicated twice to make a total of 16 tadpoles per concentration. Mortality assessments was carried out every 24 h over a 96 h period and tadpoles were considered dead if there were no body movements or they become turned upside down and did not respond to repeated gentle prodding with the blunt end of forceps. Bioassay conditions were same as for acclimatization.

### Assessment of sub lethal effect

The tadpoles were further exposed to concentrations equivalent to 1/100th of the calculated LC<sub>50</sub> for 28 days. Given the limitation of size, whole tadpoles were used for the determination of the activities of SOD and levels of MDA. The whole body was homogenised (9% w/v) in 100% methanol and centrifuged at 10,000 rpm for 15 min at 4°C using the technique of Hermes-Lima et al. (1995) as described in King et al. (2012) and the supernatant was used for the assays.

### Measurement of superoxide dismutase activity

The SOD enzyme activity was measured by its ability to inhibit the antioxidation of epinephrine (that is, determining the difference in the level of superoxide anion production and decomposition) at an absorbance of 450 nm, using the method of Sun and Zigma (1978). The concentrations so determined were expressed as unit/mg protein, of which one unit is defined as the amount of enzyme needed to inhibit 50% epinephrine reduction per minute and per mg of protein at 25°C and pH 7.8.

### Lipid peroxidation assay

The thiobabaturic acid reaction (TBARS) assay was used to determine the level of lipid peroxidation in the supernatant of the tissue homogenates. Specifically, MDA, the measure of lipid peroxidation damage was determined by measuring absorbance at 535 nm in a spectrophotometer (Yagi, 1998).

### Statistical analysis

The dose-response data of quantal responses (mortality) of the tadpoles to the petroleum products were analysed by probit analysis after Finney (1971). The indices calculated from the probit analysis includes: LC<sub>5</sub>: sub-lethal concentration that causes 5% response (mortality) of exposed tadpoles at 95% confidence interval; LC<sub>50</sub>: lethal concentration that causes 50% response (mortality) of exposed tadpoles at 95% confidence interval; LC<sub>95</sub>: lethal concentration that causes 95% response (mortality) of exposed tadpoles at 95% confidence interval and toxicity factor:

$$TF = \frac{LC50 \text{ of the other Chemical}}{LC50 \text{ of the most toxic chemical}}$$

The analysis of variance (ANOVA) of the SOD and MDA values were carried out at 5% (P<0.05) level of significance using SPSS version 16.

### Toxicity ranking

The respective acute toxicity, induced SOD activities and MDA levels in the tadpoles were ranked for the five test substances on a scale of 1 to 5 (1= highest, while 5= least) in order to design a uniform semi qualitative assessment to determine the extent of consistency of the measured responses.

## RESULTS

### Relative acute toxicity of the petroleum products and engine oils to *A. regularis*

On the basis of the 48 h LC<sub>50</sub>, kerosene was found to be the most toxic (LC<sub>50</sub>= 4930 mg/L) having a toxicity factor (TF) value, 1.6 times higher than the least toxic test substance, unused engine oil. The toxicity ranking at 48 h therefore was as follows: kerosene (most toxic) followed by petrol, spent engine oil, diesel, and unused engine oil (least toxic) (Table 1). By the 96th h following exposures, spent engine oil was found to be the most toxic (LC<sub>50</sub>= 2915 mg/L), being 2.5 times more toxic than unused engine oil which remained the least toxic. The order of toxicity also included spent engine oil (most toxic), followed by kerosene, spent engine oil, petrol, diesel and unused engine oil (least toxic) (Table 2).

### Lipid peroxidation

The levels of MDA was significantly lower (P<0.05) in the control tadpoles than in those exposed to the petroleum products (Figure 1). MDA levels were highest in those exposed to unused engine oil (Table 3). The next highest MDA level was recorded in tadpoles exposed to kerosene, followed by spent engine oil, while the least level was measured in those exposed to diesel.

### Superoxide dismutase activity

The results show that the SOD activity was significantly

**Table 1.** Relative 48 h acute toxicity (mg/L) of kerosene, diesel, petrol, spent engine oil, and engine oil acting singly against tadpole (*Amietophrynus regularis*).

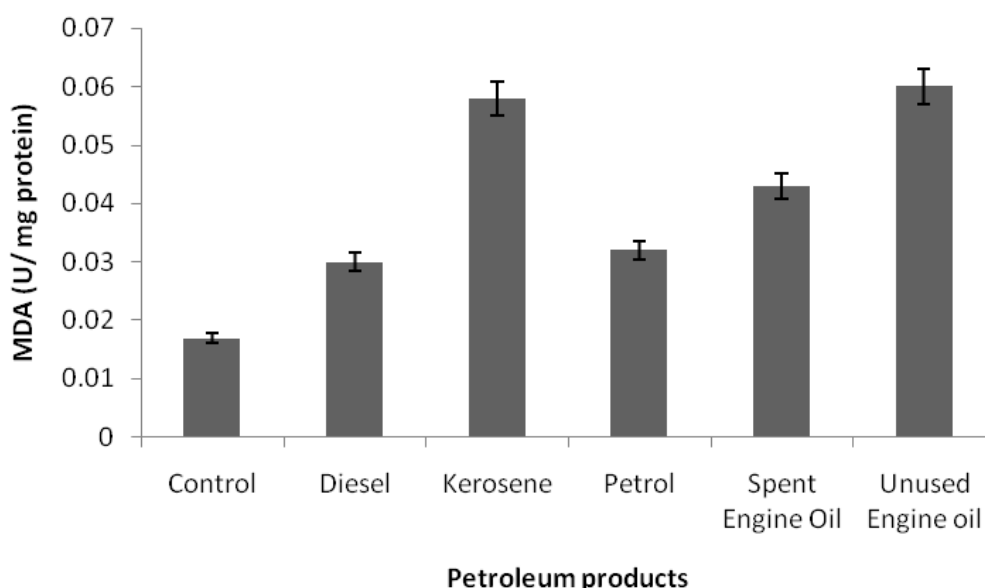
Test compounds (ml/L)	LC <sub>5</sub> (confidence interval)	LC <sub>50</sub> (confidence interval)	LC <sub>95</sub> (confidence interval)	SE	DF	Probit line equation	TF
Kerosene	2850 (1030-3720)	4930 (3830-5960)	8530 (6750-20230)	2.20	2	y=1.59+3.01x	1
Diesel	1881 (0-3445)	7286 (4455-129650)	29957.4 (13186-1156062)	1.28	2	y=1.03+2.25x	1.5
Petrol	2693 (426-3990)	6702 (4059-10949)	16682 (10474-163657)	1.46	2	y=1.17+2.95x	1.4
Unused engine oil	5555 (1071-6626)	7777 (6252-8878)	10898 (9282-46904)	4.70	2	y=4.23+2.36x	1.6
Spent engine oil	2761 (22-4301)	7524 (5478-24673)	20515 (11506-19283242)	1.65	2	y=1.28+2.46x	1.4

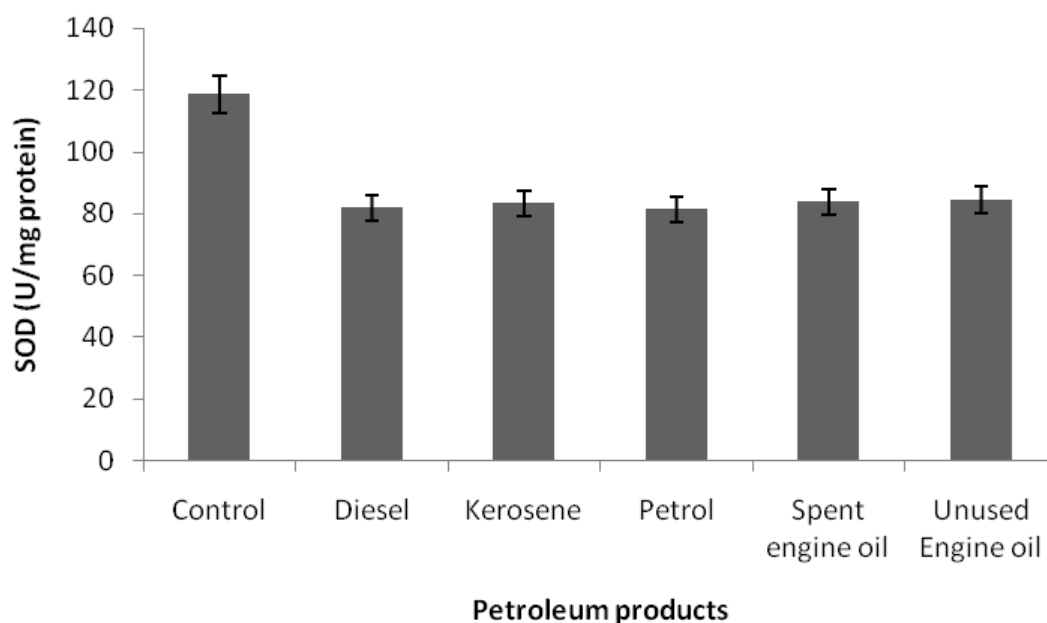
SE = Standard error; DF = degree of freedom, TF= toxicity factor.

**Table 2.** Relative 96 h acute toxicity (mg/L) of kerosene, diesel, petrol, spent engine oil, and engine oil acting singly against tadpole (*Amietophrynus regularis*).

Test compounds (mL/L)	LC <sub>5</sub> (Confidence interval)	LC <sub>50</sub> (Confidence interval)	LC <sub>95</sub> (Confidence interval)	SE	DF	Probit line equation	TF
Kerosene	1120 (70-1990)	3880 (2400-6610)	13480 (7430-258180)	1.08	2	y=0.67+2.69x	1.3
Diesel	2456 (653-3495)	5207 (3703-5801)	11207 (8088-34046)	1.51	2	y=1.17+3.07x	1.8
Petrol	1436 (39.6-2564)	3871 (1535-5267)	10454 (7019-100713)	1.45	2	y=1.05+2.16x	1.3
Unused Engine oil	6020 (111-6777)	7353 (4050-7929)	8969 (8161-171983)	9.16	2	y=8.03+2.04x	2.5
Spent engine oil	1023 (198-1727)	2915 (1727-4202)	8294 (5401-27973)	1.03	2	y=0.58+2.62x	1.0

SE = Standard error; DF = degree of freedom; TF= toxicity factor.

**Figure 1.** Comparison of the level of lipid peroxidation (MDA) in *Amietophrynus regularis* exposed to 1/100th of the respective LC<sub>50</sub> of the petroleum products and engine oils.



**Figure 2.** Comparison of the superoxide dismutase (SOD) activity in *Amietophrynus regularis* exposed to 1/100th of the respective LC<sub>50</sub> of the petroleum products and engine oils.

**Table 3.** Assessment of relationship between toxicity of the petroleum products, superoxide dismutase (SOD) activity and lipid peroxidation product (MDA- Malondialdehyde) in *Amietophrynus regularis*.

Petroleum products	96 h LC <sub>50</sub> <sup>ab</sup>	SOD activity <sup>ac</sup>	MDA <sup>bc</sup>	Remark
Diesel	4	4	5 (lowest)	Very consistent
Kerosene	2	3	2	Very consistent
Petrol	3	5 (lowest)	4	Consistent
Spent engine oil	1 (most toxic)	2	3	Consistent
Unused engine oil	5 (least toxic)	1 (highest)	1 (highest)	Inconsistent

N.B, Similar alphabets implies no significant relationship ( $P>0.05$ ).

inhibited ( $P<0.05$ ) in the tadpoles exposed to sub lethal concentrations (1/100th LC<sub>50</sub>) of the petroleum products and engine oils compared to the control after the 28 days period (Figure 2). Among those exposed to the petroleum products, petrol recorded the least activity, followed by diesel, kerosene and spent engine oil while unused engine oil had the highest activity. There was no significant difference ( $P>0.05$ ) in SOD activity among the exposed tadpoles. Overall, the assessment of the relationship between the toxicity of the petroleum products and engine oils with their respective SOD activities as well as MDA levels by way of ranking (1-5; 1= highest, 5= least/lowest) indicated that there were no significant difference ( $P>0.05$ ) between all three parameters. Summarily, the results indicated that diesel and kerosene showed very consistent relationships, petrol and kerosene showed consistent relationship while unused

engine oil which had the least toxicity but the highest levels of SOD activity and MDA was designated inconsistent (Table 3). The overall comparison of the ranks for the 96 h LC<sub>50</sub> with the respective antioxidative stress markers (that is, SOD and MDA) using independent sample t test indicated that there was no significant difference ( $P>0.05$ ) between each pair. However, there was a strong positive correlation ( $r=0.8$ ) between the SOD and MDA ranks for the test groups.

## DISCUSSION

Continued reliance of motor vehicles, generating sets, and other equipments fuelled by petroleum products make the potential for spills a continued environmental risk. There is, therefore a need for constant investigation

of their toxic effects on sensitive wildlife species such as amphibians. This study shows that petroleum products (diesel, kerosene, petrol), unused and spent engine oil are acutely toxic to the tadpoles of the common African toad (*Amietophrynus regularis*). With respect to the relative 96 h LC<sub>50</sub>, the spent engine (fuel) oil was the most toxic and this could be related to the fact that being a waste product it may contain all sorts of toxic compounds/chemicals emanating from additives and heavy metals from worn engine parts. The differential toxicity of the petroleum products and engine oils to the tadpoles can be linked to their respective physical characteristics. Refined petroleum products are more volatile than the engine oils and therefore would not be retained for long in the bioassay medium. This may account for their lower acute toxicity compared to the used and unused engine oils. However, their relative toxicity should not override the fact that they all constitute environmental hazards being rich in hydrocarbons. This raises important ecotoxicological concerns given the ubiquity of petrol filling stations and auto mechanic workshops in major cities and highways in Nigeria. These facilities often leave little consideration to waste management in their design. Surrounding drainages and ponds becomes recipients of their wastes either by deliberate introduction or when they are washed off as run off after rainfall. Dumping of spent engine oils in gutters and drains is commonplace in auto mechanic workshops in Nigeria. There are no measures put in place for collection and management of spent oils and petroleum products from these workshops which are distributed across streets corners and major roads of the country. Thus, resulting in pollution concerns to animals inhabiting urban ecosystems.

Previous investigations have evaluated the toxicity of crude oil and petroleum products on frogs (Udofia et al., 2013) and guppies (Simonato et al., 2008) linking them with acute toxicity as well as a number of sub lethal effects following long period of exposure to minute concentrations. This study confirms the toxicity of petroleum products to tadpoles, specifically of the common African toad. The toxicity of petroleum products to the tadpoles were found to increase with time of exposure, consistent with the findings of King et al. (2012) who suggested that the reason for this trend in catfishes and hermit crabs could be due to a number of factors including permeability of the skin. The LC<sub>50</sub> values obtained from this study for petrol, diesel and kerosene were lower than those reported by King et al. (2012) against early life stages of catfishes and hermit crabs. Amphibians typically have a characteristic permeable skin adapted for cutaneous respiration (Hickman et al., 2008). Lipophilic pollutants such as petroleum hydrocarbons may easily diffuse through their skin, resulting in toxic effects. This together with other physiological and morphological differences may account for the increased

toxicity to the tadpoles reported in this study. Ayoola and Akaeze (2012) however reported 96 h LC<sub>50</sub> value of 562 ml/L in catfishes exposed to spent oil, a value which is over 200 times less than that observed for tadpoles in this study. Besides differences in species susceptibility, this may be due to the wide variation in the constituents of the spent engine oils and other practices in the automobile workshops from where they were collected. Thus, the difficulty in comparing responses between species as well as used/spent engine oils is hereby noted.

The assessment of MDA, the by-product of oxidative damage to the phospholipids of cell membranes indicated significant harm to cells in tadpoles exposed to the petroleum products relative to the control individuals. Lipid peroxidation damage is one of the first indicators of damage to cells by toxicants and represents a key biomarker of oxidative stress (Cini et al., 1994). Much of the work on lipid peroxidation resulting from petroleum products and their components in Nigeria have been focused on fishes (Achuba and Osakwe, 2003; Avci et al., 2005; Doherty, 2014). Avci et al. (2005) have earlier reported lipid peroxidation in the muscles and liver of fishes obtained from a river contaminated petroleum products from a nearby refiner. This study therefore provides an opportunity to extend the knowledge of the oxidative stress impacts of petroleum products on tadpoles of the common African toad.

The results from the biochemical assays indicated that there was inhibition of SOD activities in the exposed tadpoles relative to the control. Inhibition of SOD activities have been reported in the African sharp tooth catfish (*Clarias gariepinus*) exposed to polycyclic aromatic hydrocarbons (Otitoloju and Olagoke, 2011). This gives credence to the possibility of oxidative stress resulting from the hydrocarbon fractions of the petroleum products and confirms results from the lipid peroxidation assay in this study. Specific petroleum hydrocarbons such as benzene, ethylbenzene, toluene and xylene have been also found to induce oxidative stress at sub lethal concentrations, in *Clarias gariepinus* (Doherty, 2014). SOD, though involved in the protection of biological systems from the actions of free radicals and may be overwhelmed in the event of excessive toxic onslaught, resulting in oxidative stress, a condition that may be characterized by its eventual inhibition. This therefore justifies its use as a biomarker for assessing the toxic effects and responses to toxicants in this study.

## Conclusion

The findings from this study points to a largely consistent relationship between the toxicity of petroleum products and spent engine oils and their respective SOD activity and MDA levels. This conclusion is based on the fact that

rank differences between the three parameters did not exceed 1 (one) for all toxicants except for unused engine oil. The relatively consistent relationship between SOD and MDA reported in this study was also consistent with the findings of Brucka-Jastrzębska (2010) who reported inhibition of SOD which was simultaneously associated with increase in MDA in catfishes exposed to heavy metals, lead and cadmium. The importance of antioxidative enzymes as sensitive biomarkers in monitoring environmental pollution therefore cannot be downplayed owing to the large number of investigators who have demonstrated this in a variety of animal groups as documented by Otitolaju and Olagoke (2011).

This study therefore justifies the use of MDA levels and SOD activity as suitable compliments for monitoring oxidative stress resulting from exposure to petroleum products. The consistent relationship between these biomarkers and 96 h LC<sub>50</sub> values for some of the tested products is noteworthy and presents an opportunity for more investigative studies so as to understanding the mechanisms of action and make a case for their use in routine assessments of impacts of such spills in the environment.

### Conflict of Interests

The author(s) have not declared any conflict of interest.

### ACKNOWLEDGEMENT

We acknowledge the assistance of Late Mr. E. A. Faton, who made efforts and gave advice regarding collection of tadpoles for this study.

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## Full Length Research Paper

# Statistical optimization of lactic acid production by *Lactococcus lactis* strain, using the central composite experimental design

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Received 19 January, 2014; Accepted 6 October, 2014

The individual and interactive effects of a total inoculum size (% v/v), fermentation temperature and skim milk dry matter added (% w/v) on the lactic acid production by *Lactococcus lactis* LCL strain were studied by quadratic response surface methodology. The central composite design (CCD) was employed to determine maximum lactic acid production at optimum values for process variables and a satisfactory fit model was realized. The mathematical relationship of the lactic acid production on the three significant independent variables can be approximated by a nonlinear polynomial model. Predicted values were found to be in a good agreement with experimental values ( $R^2$  of 96.7% and  $R^2(adj)$  of 92.1% for response  $Y$ ). The result of optimization predicted by the model has shown that the maximal result for lactic acid production revolved around 92°D at the optimal condition with 2% of inoculum size, temperature at 30°C and skim milk dry matter added at a central point of 2% (w/v).

**Key words:** Central composite design, *Lactococcus lactis*, lactic acid production, inoculum size, temperature, skim milk dry matter.

## INTRODUCTION

The manufacture of fermented foods has a long tradition. At first, there was a purely empirical principle without the connection between metabolic activity of microorganisms (so-called "house flora") and desired changes in the product (Geisen et al., 1992). The fermentation process was used to improve shelf-life and safety of foods enabling people in moderate and cold regions to survive

winter seasons and drought periods (Holzapfel, 1997). Spontaneous fermentation of foods is characterized by the participation of lactic acid bacteria, Gram-positive, catalase-positive cocci, yeasts and moulds (Buckenhüskes, 1993). Fermented milks are the most common products from which other products are also made (Thapa, 2000). Starter culture organisms used in

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**Abbreviations:** RSM, Response surface method; CCD, central composite design; BBD, box-behnken design; IDF, international dairy federation; DOE, design of experiment.

fermentations belongs to a family of bacteria collectively known as the lactic acid bacteria (LAB). Fermented milks are products prepared by controlled fermentation of milk to produce acidity and flavor to desired level. Modern starter cultures are selected either as single or multiple strains, specifically due to their adaptation to the substrate or raw material (Holzapfel, 2002). The inoculation of milks with a starter culture composed of selected lactic acid bacteria that improves quality, safety, properties standardization, including flavor and color, and shortening in the ripening time (Leroy et al., 2006; Rantsiou et al., 2005). On a technological standpoint, these bacteria are invited to play the technological part to which they were selected, namely; the production of lactic acid, aromatic compounds, and production of CO<sub>2</sub>, bacteriocins, resistance to phages, proteolytic activity and autolytic potential (Gibbs, 1987; Frey, 1993; Huang et al., 1994; Albenzino et al., 2001; Beresford et al., 2001; Hassaïne et al., 2007).

One of the most sought technological properties in lactic acid bacteria, is undoubtedly the production of lactic acid, because this activity is essential in the early stages of product processing and thereafter is mainly responsible for microbial stability of the final product through the pH decrease (Drosinos et al., 2007). This acid is widely employed as bacterial biopreservative in foods (Ray and Sandine, 1992) and recently, as monomer for the plastic polymer synthesis, solvents and oxygenated chemicals (Datta et al., 1982; Datta and Henry, 2006).

These last years, the lactic acid production has received increased attention sanctioned by a considerable number of publications (Yu et al., 1997; Lei et al., 2008; Plessas et al., 2008; Yu et al., 2008; Adesokan et al., 2009; Cristian et al., 2009; de Lima et al., 2009; Yao et al., 2009; Cristian et al., 2010; de Lima et al., 2010; Abdel-Rahman et al., 2011; Coelho et al., 2011; Kostov et al., 2011; Leite et al., 2012; Dwivedi et al., 2012; Tanyildizi et al., 2012; Ghaffar et al., 2014). In these studies, wide varieties of products and raw materials from the food and/or agriculture industries have been employed for microorganism growth due to their considerable availability and low cost. Examples include cheese whey, corn steep liquor, corn syrup, distillery yeast and molasses (Lei et al., 2008; Mussatto et al., 2008; Yu et al., 2008; Ben-Kun et al., 2009; Yao et al., 2009; Abdel-Rahman et al., 2011; Gowdhaman et al., 2012).

Biotechnological processes for the production of lactic acid usually include lactic acid fermentation. There have been numerous investigations on the development of biotechnological processes for lactic acid production, with the ultimate objectives to enable the process to be more efficient and economical by using strategies for optimization, based mainly on the modeling methodology (Yu et al., 2008; Cristian et al., 2009; Yao et al., 2009; de Lima et al., 2009; Cristian et al., 2010; de Lima et al., 2010; Muthuvelayudham and Viruthagiri, 2010; Coelho et al., 2011; Kostov et al., 2011, Dwivedi et al., 2012;

Gowdhaman et al., 2012; Tanyildizi et al., 2012; Saravanan et al. 2012; Leite et al., 2012). On the other hand, an indispensable tool for the optimization, control, design and analysis of the combined production of lactic acid to industrial scale derived the development of mathematical robust models, formulated with parameters of clear biological significance and statistically consistent which can be easily implemented in miscellaneous applications. Compared with conventional methods, the response surface method, commonly called a "RSM", is a time and labor saving method, which also reveals the interaction between the components of a reacted medium and seek the physical and chemical optimum levels (Ghadge and Raheman, 2006; Tang et al., 2004). RSM mainly consisted of the central composite design, the box-behnken design, the one factor design, the D-optimal design, the user-defined design, and the historical data design. The central composite design (CCD) and the box-behnken design (BBD) were the most used response surface design methods, which had 5 and 3 levels, respectively for one numeric factor. Central composite design (CCD) (Box and Wilson, 1951) is an experimental strategy for seeking the optimum conditions for a multivariable system, and it is an efficient technique for optimization.

The method was used to evaluate the coefficients in a quadratic mathematical model. The main purpose of this study was to perform the CCD in order to investigate the effect of total inoculum size (% v/v), fermentation temperature and skimmed milk dry matter added (% w/v) on the lactic acid production and for optimization of these parameters.

## MATERIALS AND METHODS

### Bacterial strain and growth conditions

*Lactococcus lactis* LCL strain, used throughout this work belonged to the collection of "Laboratoire de Biologie des Microorganismes et Biotechnologie" of Oran University (Algeria). This strain was maintained on M17 broth or 10% (w/v) skim milk and deep-frozen at -20°C. As required, this culture was thawed and reactivated by two transfers in 10% (w/v) skim milk (30°C, 24 h).

### Acidification activity

The lactic acid concentration was measured according to the International Dairy Federation (IDF, 1995). After subculturing in M17 Broth and 10% (w/v) skim milk in succession at 30°C for 24 h, the microbial culture was inoculated in reconstituted sterile non-fat dry milk 10% (w/v) at a level described in CCD tables (Tables 1 and 2). Titrable acidity was determined after 7 h of incubation; it is followed by measuring the Dornic acidity that expressed the acidity developed in the medium by transformation of lactose into lactic acid. Experiments were carried out in triplicate.

### Design of experiment (DOE)

Experiment was conducted at "Laboratoire de Biologie des



**Table 1.** Experimental factors and levels investigated on the lactic acid production.

Variable	Symbol	Range and level				
		Lowest	Low	Center	High	Highest
		$-\alpha$	-1	0	+1	$+\alpha$
Total inoculums size (% v/v)(I)	$x_1$	0.32	1	2	3	3.68
Fermentation temperature (°C) (T)	$x_2$	21.6	25	30	35	38.4
Skim milk dry matter added (% w/v)(DM)	$x_3$	0.32	1	2	3	3.68

**Table 2.** Central composite design (CCD) for optimization of three variables (each on five levels) in mathematically predicted and experimental values for the production of lactic acid by *Lactococcus lactis* LCL strain.

Test number	Coded level of variables			Actual level of variables			Lactic acid production (D°)	
	$x_1$	$x_2$	$x_3$	Inoculum size (I %)	Fermentation temperature (T°C)	Skim milk dry matter added (DM %)	Observed values	Predicted values
1	-1	-1	-1	1	25	1	20	25.22
2	+1	-1	-1	3	25	1	48	49.60
3	-1	+1	-1	1	35	1	66	62.19
4	+1	+1	-1	3	35	1	80	79.07
5	-1	-1	+1	1	25	3	35	38.71
6	+1	-1	+1	3	25	3	52	58.60
7	-1	+1	+1	1	35	3	53	54.19
8	+1	+1	+1	3	35	3	69	66.57
9	$-\alpha$	0	0	0.32	30	2	40	37.57
10	$+\alpha$	0	0	3.68	30	2	70	68.45
11	0	$-\alpha$	0	2	21.6	2	36	27.14
12	0	$+\alpha$	0	2	38.4	2	60	64.89
13	0	0	$-\alpha$	2	30	0.32	70	70.10
14	0	0	$+\alpha$	2	30	3.68	75	70.93
15	0	0	0	2	30	2	93	92.23
16	0	0	0	2	30	2	92	92.23
17	0	0	0	2	30	2	91	92.23

Microorganismes et Biotechnologie" and was designed by central composite design (CCD). It was chosen to show the statistical significance of the effects of total inoculums size (% v/v), fermentation temperature and skimmed milk dry matter added (% w/v) on the lactic acid production by *L. lactis* LCL strain. The experiments were designed by using the STATISTICA v.7.0 software package (StatSoft, USA).

CCD allows estimating the second-degree polynomial of the relationships between the factors and the dependent variable and gives information about interaction between variables (factors). The lowest and the highest levels of variables are shown in Table 1. A  $2^3$  factorial central composite design with eight star points, and three replicates at the center points leading to 17 runs were employed for the optimization of the culture conditions. The variables were coded according to the following equation (Equation 1).

$$x_i = (X_1 - X_0) / \Delta X \quad i = 1, 2, \dots, k \quad (1)$$

Where,  $x_i$  is the dimensionless value of a variable,  $X_1$  the real value of a variable,  $X_0$  the value of  $X_1$  at the center point, and  $\Delta X$  the step

change. The central composite design including the factors, their levels and the result from each test, is shown in Table 2. The second-order polynomial equation, which includes all interaction terms were used to calculate the predicted response (Equation 2).

$$Y_i = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} x_{ii}^2 + \sum_{ij=1}^k \beta_{ij} X_i X_j \quad (2)$$

Where,  $Y_i$  is the predicted response,  $x_i$  and  $x_j$  the input variables, the intercept term,  $\beta_0$  the linear effects,  $\beta_{ii}$  the squared effects and  $\beta_{ij}$  the interaction term. The design expert software has been used for regression and graphical analysis of the obtained data. The optimum levels of total inoculums size (% v/v), fermentation temperature and skimmed milk dry matter added (% w/v) were obtained by solving the regression equation and also analysis of the response surface contour plots.

#### Statistical data analysis

STATISTICA v.7.0 software package (StatSoft, USA) was used for the experimental design matrix, data analysis and quadratic model

**Table 3.** Model coefficient estimated by linear regression.

Factor	Coefficient	standard error	Computed t-value	P-value	Statistical significance of coefficient
Intercept	92.2345	3.368031	27.38528	0.00000	*
$x_1$	18.3806	3.165163	5.80717	0.000659	*
$x_1^2$	-27.7883	3.486988	-7.96913	0.000093	*
$x_2$	22.4730	3.165163	7.10012	0.000194	*
$x_2^2$	-32.7486	3.486988	-9.39165	0.000032	*
$x_3$	0.4984	3.165163	0.15745	0.879335	
$x_3^2$	-15.3875	3.486988	-4.41282	0.003108	*
$x_1 x_2$	-3.7500	4.133659	-0.90719	0.394464	
$x_1 x_3$	-2.2500	4.133659	-0.54431	0.603125	
$x_2 x_3$	-10.7500	4.133659	-2.60060	0.035394	*

$x_1$ : Inoculum size (I %);  $x_2$ : fermentation temperature (T°C);  $x_3$ : Skim milk dry matter added (DM%)

**Table 4.** Analysis of variance (ANOVA) for the second-order polynomial model.

Source	Sum of squares	Degrees of freedom	Mean of square	F-test	P-value
Model	7181.83	9	797.98	23.35	0.0005*
Residual error	239.22	7	34.17		
Total	7421.05	16			

\*Statistical significance;  $R^2=0.967$ ;  $R^2_{adj}=0.921$ ;  $R=0.983$  and  $R_{adj}=0.959$ .

building. Response surface and contour plots were generated to understand the interaction of different variables. The central composite design including the factors, their levels, and the result from each test is shown in Table 2.

## RESULTS AND DISCUSSION

The central composite design matrix of the studied variables: inoculum size ( $x_1$ ), temperature ( $x_2$ ) and skim milk dry matter added ( $x_3$ ) using the isolated *L. lactis* LCL. The highest lactic acid production achieved in the verification experiment was 93.00 °D (as seen in run 15). The application of multiple regression analysis methods yielded the following regression (Equation 3) for the experimental data demonstrated that lactic acid production was an empirical function of test variables in coded units.

$$Y = 92.2345 + 18.3806 x_1 + 22.4730 x_2 + 0.4984 x_3 - 27.7883 x_1^2 - 32.7486 x_2^2 - 15.3875 x_3^2 - 3.7500 x_1 x_2 - 2.2500 x_1 x_3 - 10.7500 x_2 x_3 \quad (3)$$

The quadratic model in Equation 3, with nine terms, contains three linear terms, three quadratic terms and three factorial interactions, in which  $Y$  is the predicted response, that is, lactic acid concentration and  $x_1$ ,  $x_2$  and  $x_3$  are the coded values of the test variables inoculum size, temperature and skim milk dry matter added, respectively.

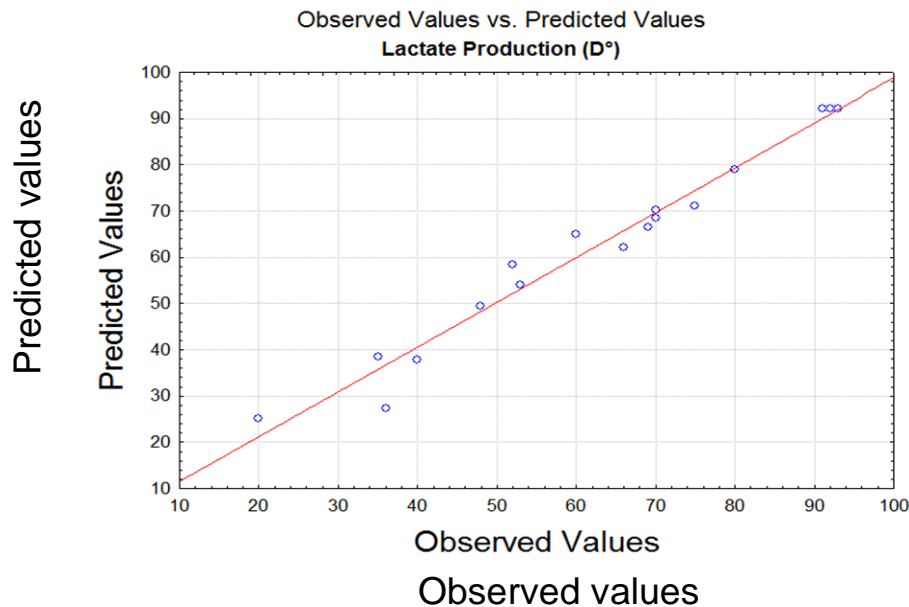
Table 3 displays the Student's  $t$ -distribution and the corresponding values, along with the estimated parameters. The probability ( $p$ ) values were used as a tool to check the significance of each coefficient. A larger magnitude of the  $t$ -test and smaller  $p$ -value denote greater significance of the corresponding coefficient (Lee and Wang, 1997; 2001; Li and Lu, 2005).

The results reveal (Table 3) that the independent variables  $x_1$  and  $x_2$  had a strong positive linear effect on the response ( $P < 0.05$ ), as an increase in its concentration led to an increased yield. The same is observed with the squared variables ( $x_1^2$ ,  $x_2^2$ ,  $x_3^2$ ) and the interaction term  $x_2 x_3$ ; the negative signs revealed a reduction in lactic acid production when its concentration was increased in the system.

Among these, insignificant terms (on the basis of  $P$ -values greater than 0.05) are neglected, that is, the case of the independent variable  $x_3$  was not significant within the range of this study. The Equation 3 model was modified to reduce the fitted model ( $Y_a$ ) (Equation 4).

$$Y_a = 92.2345 + 18.3806 x_1 + 22.4730 x_2 - 27.7883 x_1^2 - 32.7486 x_2^2 - 15.3875 x_3^2 - 10.7500 x_2 x_3 \quad (4)$$

The statistical significance of Equation 4 was checked by an  $F$ -test and the analysis of variance (ANOVA) for the quadratic response surface model is summarized in Table 4. The model  $F$ -value of 23.35 with a very low



**Figure 1.** Relation between experimental (observed) and predicted value of lactic acid production using equation 4.

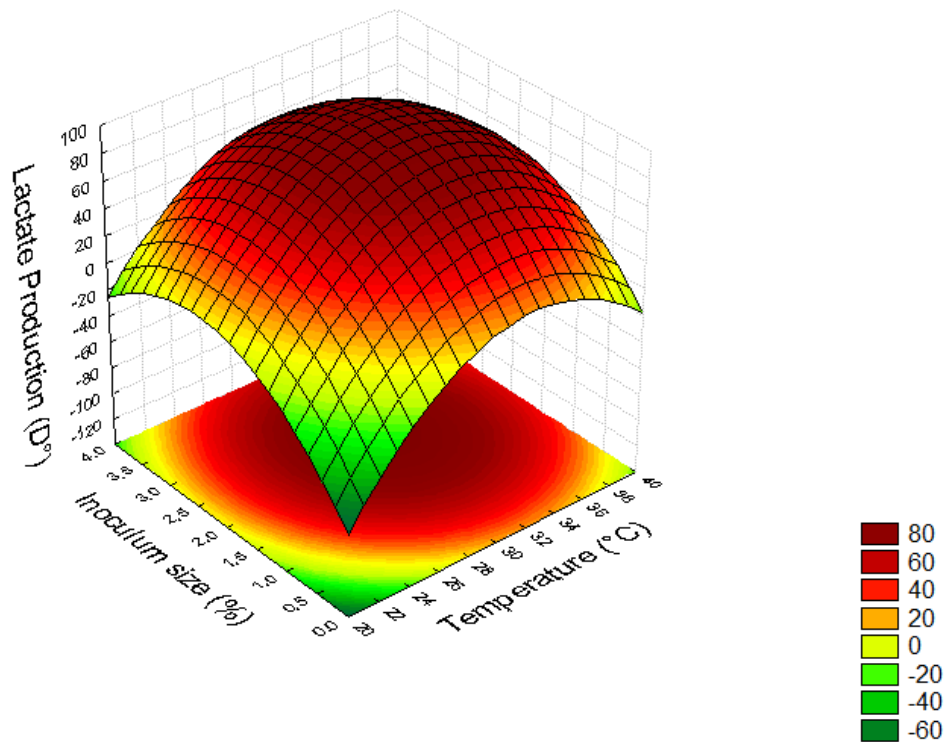
probability value ( $P$ -value = 0.0005) indicated that the model was highly significant. Experimental results and the predicted values obtained by using model (Equation 4) are shown in Figure 1. As it can be seen, the predicted values match the experimental values reasonably well with  $R^2$  of 0.957 and adjusted  $R^2$  of 0.921. The high  $R$ -value (0.983) demonstrates strong agreement between the experimental observations and predicted values. This correlation is also confirmed by the plot predicted versus experimental values of lactic acid production in Figure 1, as all points cluster around the diagonal line, demonstrating that no significant violations of the model were found. The goodness of the model was checked by the determination coefficient ( $R^2$ ). In this case, the  $R^2$ -value (0.967) for Equation 4 indicating that 96.7% of the variability in the response could be explained by the model. Normally, a regression model with an  $R^2$ -value greater than 0.9 is considered as having a very high correlation (Rao et al., 2006). The value of the adjusted determination coefficient (adjusted  $R^2 = 0.921$ ) was also satisfactory for confirming the good significance of the model. The high  $R$ -value (0.983) demonstrates a high degree of agreement between the experimental observations and predicted values.

The 3D response surface plot is a graphical representation of the regression equation. It is plotted to explain interaction of the variables and locate the optimal level of each variable for maximal response (Figures 2, 3 and 4). Each response surface plotted for lactic acid production represents the different combinations of two test

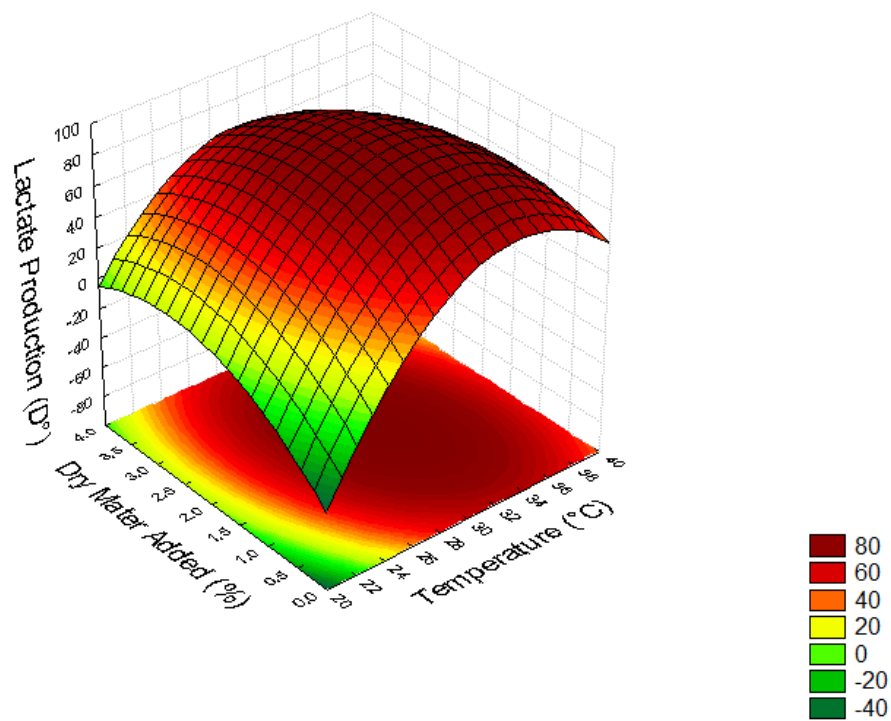
variables at one time while maintaining the other variable at the zero level. These 3D plots and its respective contour plots provide a visual interpretation of the interaction between two factors and facilitate the determination of optimum experimental conditions.

The convex response surfaces suggest that there are well-defined optimal solutions. If the surfaces are rather symmetric and flat near the optimum, the optimized values may not vary widely from single variable conditions (Rao et al., 2006). Interactions between variables can be inferred from the shapes of the contour plots. Circular contour plots indicate that interactions between variables are negligible, as shown in Figure 2. In contrast, elliptical plots indicate interactions, as it is shown in Figures 3 and 4 (Muralidhar et al., 2003). The inoculum size and the fermentation temperature seem to be dominant variables in lactic acid production model (Figures 2 and 3). Whereas, the skim milk dry matter added (on linear term) does not seem to have a notable effect on this production (Figures 3 and 4). The maximal lactic acid production occurred when inoculum size and temperature were in the neighborhood of 2% (v/v) and 30°C, respectively.

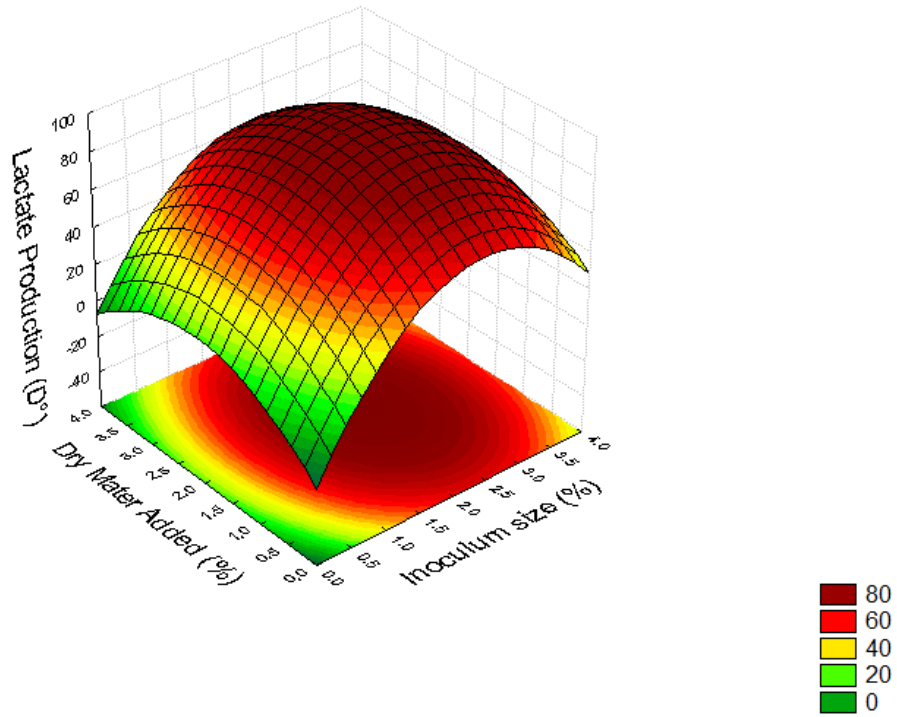
The area of optimum lactic acid production levels of the tested variables is located close to the central point, and they were represented in desirability charts (Figure 5) and isoresponse plot (Figure 6), constructed using response surface regression in STATISTICA software. The point of maximal lactic acid production was determined through canonical analysis of the adjusted



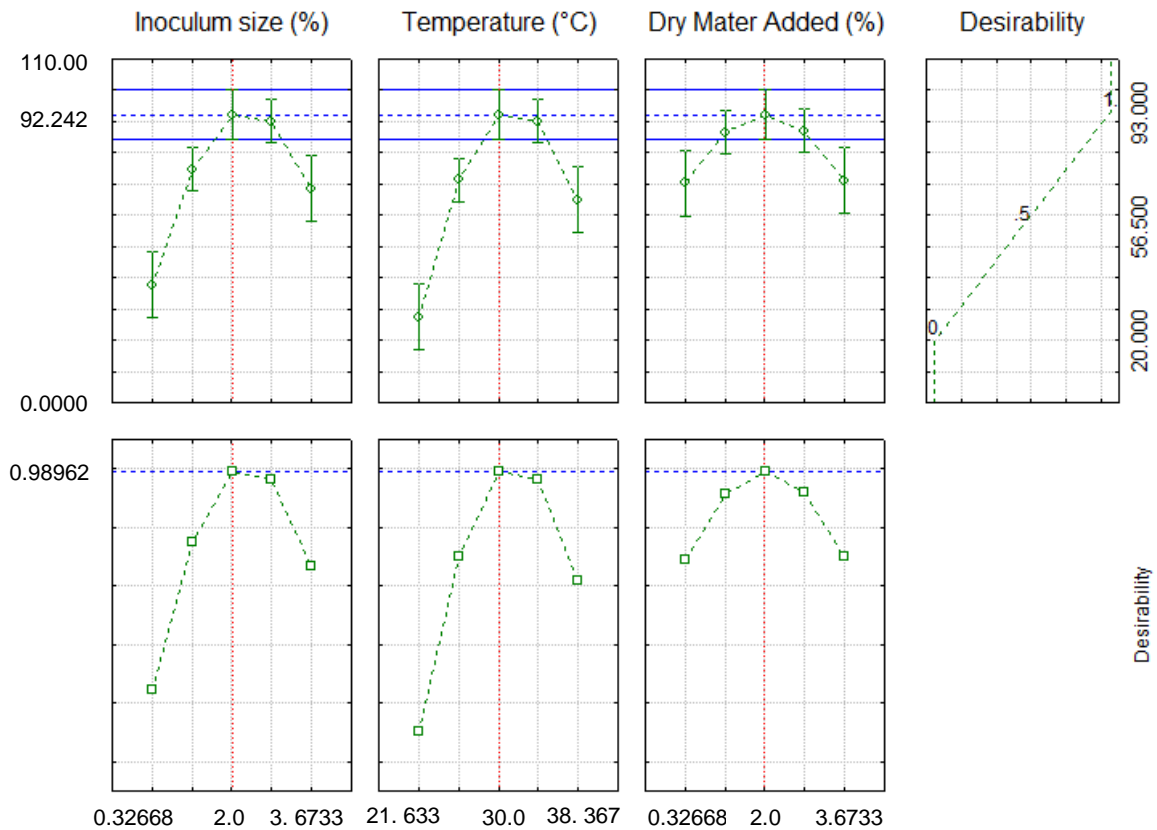
**Figure 2.** Response surface plot showing the effect of inoculums size and temperature on lactic acid production. The value of the variable skim milk dry matter added was fixed at the central point.



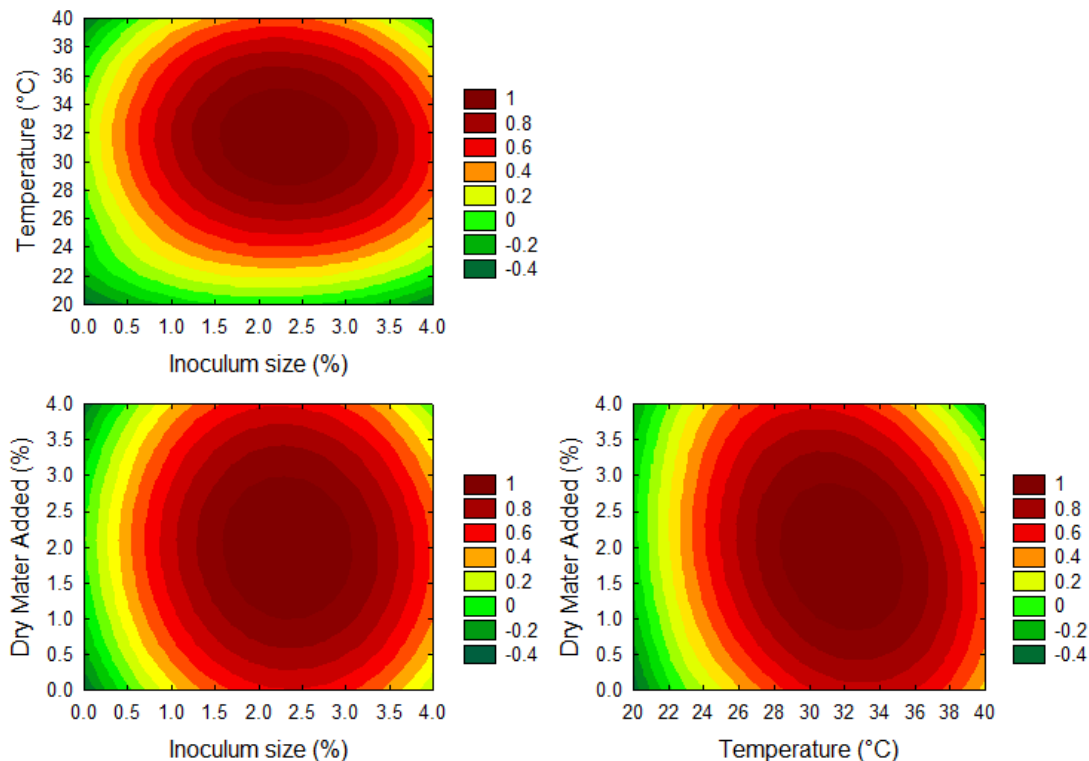
**Figure 3.** Response surface plot showing the effect of skim milk dry matter added and temperature on lactic acid production. The value of the variable inoculums size was fixed at the central point.



**Figure 4.** Response surface plot showing the effect of skim milk dry matter added and inoculums size on lactic acid production. The value of the variable temperature was fixed at the central point.



**Figure 5.** Desirability charts of variables for maximum response (lactic acid production).



**Figure 6.** Desirability isoresponse plot of variables for maximum response (lactic acid production).

model. A study was carried out to identify the nature of the stationary point (maximal point or low response or still of a saddle point). These levels were as follows: inoculum size 2% (v/v), temperature 30°C and skim milk dry matter added to 2% (w/v) for 92.24 °D predicted value of lactic acid production. To confirm the adequacy of the model for predicting maximal lactic acid production, three additional experiments were also conducted at these predicted optimum levels. The mean value of lactic acid concentration obtained is  $92 \pm 0.5$  °D, which is an excellent agreement with the predicted value.

## Conclusion

It is possible to affirm that the controlled inoculums size, the temperature of fermentation and skim milk dry matter added influenced the predictive model for maximal lactic acid production by *L. lactis* LCL strain by using the central composite design method and response surface analysis. The optimization of the analyzed responses demonstrate that the best result for lactic acid production revolves around of 92 °D was obtained with 2% (v/v) of inoculums size, temperature at 30°C and skim milk dry matter added at a central point 2% (w/v).

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

## Somatic embryogenesis and plant regeneration from leaf explants of *Rumex vesicarius* L.

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Received 19 July, 2014; Accepted 13 October, 2014

An attempt was made to study the somatic embryogenesis and plant regeneration from the *in vitro* leaf explants of *Rumex vesicarius* L. a renowned medicinal plant, which belongs to polygonaceae family. Effective *in vitro* regeneration of *R. vesicarius* was achieved via young leaf derived somatic embryo cultures. Embryogenic callus was induced from leaf explants on Schenk and Hildebrandt (SH) medium supplemented with various concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) (0.5 to 3.0 mg/l) along with Kinetin (Kn) (0.5 mg/l). High frequency of somatic embryogenesis was effective on SH medium with 2, 4-D (2.5 mg/l) + Kn (0.5 mg/l) from leaf explants. Secondary somatic embryogenesis was also observed when primary somatic embryos were subculture on the same somatic embryo induction medium. Well developed cotyledonary shaped embryos regenerate 80% of shoots on media containing 2,4-D 0.5 mg/l + 2.0 mg/l BA. The regenerated shoots transferred to rooting media containing Indole- 3-butyric acid (IBA). Efficient rooting of 90% was noted on SH media with 1.0 mg/l IBA. Finally, these *in vitro* regenerated plantlets were hardened, acclimatized and successfully transferred to the field. The post transplantation survival rate of these regenerated plants was 65 to 70%. The *in vitro* regenerated plants and flowers were similar to mother plants. This protocol will be useful for genetic transformation experiments in *R. vesicarius* L.

**Key words:** *Rumex vesicarius* L, 2,4-dichlorophenoxy acetic acid (2,4-D), kinetin (Kn), Benzyl adenine (BA), Indole- 3- butyric acid (IBA).

### INTRODUCTION

Somatic embryogenesis (SE) is the ultimate developmental pathway by which somatic cells develop into structures that resemble zygotic embryos (that is, bipolar and without vascular connection to the parental tissue) through an orderly series of characteristic embryological

stages without fusion of gametes (Jimenez, 2005). SE has been traditionally divided into two main stages, namely induction and expression. In the former, somatic cells acquire embryogenic characteristics by means of gene expression (Feher et al., 2002). The ontogeny,

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physiological, biochemical and media properties are required for somatic embryogenesis (Victor, 2001). Typical globular, heart, torpedo and cotyledonary stages of somatic embryos are different from various kinds of explants. True-to-type nature of the somatic embryo derived plantlets has been reported (Tokuhara and Mii, 2001). As a result any plant which needs to be altered by genetic engineering and transgenesis requires a pre developed protocol for successful regeneration through somatic embryogenesis (Birch, 1997; Thangavel et al., 2014). Developing a protocol for plant regeneration through somatic embryogenesis will immensely benefit the plant conservation programs too as it is a shorter and viable method for producing a large number of plantlets.

The genus *Rumex* belongs to family Polygonaceae that comprises about 150 species widely distributed around the World. The main chemical constituents of *Rumex* are anthraquinones and flavanoids (Cunningham, 1993). *Rumex vesicarius* L. (English: bladder dock, Hindi: Chooka, Sanskrit: Amlavetasa, Telugu: Chukkakura) a common green leafy vegetable is also used in herbal and ayurvedic formulations. It is a branched succulent herb and is distributed throughout India (Alam, 2012). The plant extract have been used to reduce cholesterol levels, biliary disorders (Rechinger, 1984; Mona et al., 2013) and also it showed significant effect on antioxidant (Palani and Ramakrishnan, 2011; 2012; Sakkir et al., 2012) and antimicrobial activities (Al Akeel et al., 2014; Ramesh and Asha, 2013).

*In vitro* regeneration of *R. vesicarius* L. has been achieved by researchers using explants like shoot tips, nodal explants, leaves and callus (Panduraju et al., 2009; Abo El-soud et al., 2012; Nandini et al., 2013; Lavanya et al., 2013). However, there is no report on the induction of somatic embryogenesis. This is an alternative method for plant propagation over regeneration via organogenesis. The plants regenerate *via* somatic embryogenesis is of single cell origin with true-to-type and are produced in large numbers within a short period (Ammirato et al., 1983; Lavanya et al., 2014).

Many researchers have emphasized that somatic embryogenesis is preferred method for rapid *in vitro* multiplication of plants (Moon et al., 2013), production of artificially synthetic seeds (Ravi and Anand, 2012), Agro bacterium mediated genetic transformation studies and regeneration of transgenic plants (Satyanarayana et al., 2012). In the present study we made an attempt to establish a reliable and efficient protocol for the induction of somatic embryogenesis and plant regeneration from leaf explants of *R. vesicarius* L.

## MATERIALS AND METHODS

### Culture medium and conditions

The seeds of *R. vesicarius* were collected from the plants grown in the research field of Department of Biotechnology, K L University. They were soaked in sterile distilled water for 24 h, later cleaned

with 5% tween-20 (w/v) and thoroughly washed in running tap water 3 to 4 times. Subsequently, they were surface sterilized with 0.1% w/v  $\text{HgCl}_2$  for 2 to 3 min followed by rinsing with sterile distilled water for 2 to 3 times and germinated aseptically on SH medium (Schenk and Hildebrandt, 1972). Finally, these seeds were flame sterilized with Whatman filter paper and supplemented on the surface of the nutrient culture medium SH without growth regulators. Effective plantlets developed from these seeds within one week. After two weeks, leaves were taken as explants for callus induction. We found that compared to *ex vitro*, the *in vitro* leaf explants was found to be appropriate as it was responding well under *in vitro* conditions.

### Embryo germination and plantlet formation

For germination and plantlet formation cotyledonary stage somatic embryos were transferred to SH medium supplemented with 0.5 mg/l 2,4-D + 0.5 to 3.0 mg/l BA.

### Culture conditions

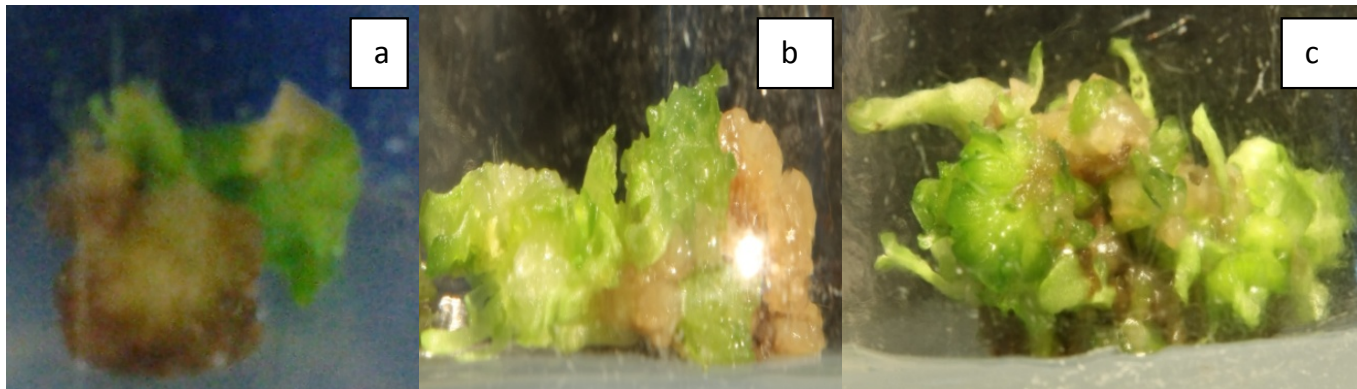
SH media were supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar (Himedia). After adding all the growth regulators, the pH of the medium was adjusted to 5.6 with 1 N NaOH or 1 N HCL and autoclaved at 121°C with 15 p.s.i pressure for 15-20 min. All the cultures were incubated at  $25 \pm 2^\circ\text{C}$  under a 16 h photoperiod. Light intensity of 40 to 50  $\mu\text{molm}^{-2}\text{s}^{-1}$  was provided by using cool white fluorescent tubes. The cultures were transferred to fresh medium after an interval of four weeks. For each hormonal treatment 20 replicates were raised and the experiments repeated at least twice. Data on somatic embryogenesis and germination were statistically analyzed using Turkey's HSD test at  $p=0.05$  with SPSS ver.13.0. The results are expressed as Mean  $\pm$  SE of two experiments.

### Acclimatization

The plants were taken out from the cultured tubes and washed with sterile distilled water under aseptic conditions to remove agar medium. They were shifted to plastic pots containing sterile vermiculate: soil (1:1), covered with polythene bags in order to maintain 80 to 85% relative humidity and kept in culture room for 3 weeks. Later, they were transferred to earthen pots containing garden soil and maintained in the research field.

## RESULTS AND DISCUSSION

The *in vitro* leaf explants were spliced at the terminal ends using scalpels and inoculated on SH medium containing different concentrations of 2,4-D (0.5 to 3.0 mg/l) in combination with Kn (0.5 mg/l). Highly differentiated, friable callus was induced from these explants in one week (Figure 1a). Within 10 to 15 days of culture inoculation greenish friable callus was observed (Figure 1b). Green nodular embryogenic callus was noticed after three weeks of culture inoculation from these explants (Figure 1c). When the explants of embryogenic callus was cut into fragments and cultured on the same induction medium for an extended period of three to four months, secondary somatic embryos with different shapes such as globular, heart, torpedo and cotyledonary



**Figure 1.** *In vitro* regeneration via Somatic embryogenesis in leaf explant cultures of *Rumex vesicarius* L. **(a).** Initiation of callus from leaf explant. **(b).** Profuse, greenish and friable callus formed from leaf explants. **(c).** A type of embryogenic callus after 3 weeks in culture, the callus was green and nodular with the presence of abundant somatic embryos.



**Figure 2.** *In vitro* regeneration via somatic embryogenesis in leaf explant cultures of *Rumex vesicarius* L. showing somatic embryogenesis in *Rumex vesicarius* L. **1.** Initiation of spherical shaped globular embryoids from leaf explants in *R. vesicarius* L. **2.** Transformation of globular embryoids into heart shaped embryo. **3.** Modification of heart shaped embryo into torpedo shaped embryonic form. **4.** Development of cotyledonary shaped embryonic buds. **5.** Nodule, shoot buds regeneration from cotyledonary buds.

embryoids were observed after four to six weeks of culture inoculation (Figure 2).

Somatic embryos proliferation occurred in two ways such as direct somatic embryos formation from explants

and indirect from repetitive organogenesis. SH medium with 0.5 mg/l 2,4-D + 0.5 mg/l Kn showed 30% of somatic embryoids induction. At 1.0 mg/l 2,4-D + 0.5 mg/l Kn 45% of embryoids were observed. 60% of somatic embryoids

**Table 1.** Effect of various concentrations of 2,4-D and 0.5 mg/l Kn on Somatic embryogenesis in leaf explants of *R. vesicarius* L.

Growth regulators mg/l 2,4-D+Kn	Percentage of response for somatic embryo formation	Average number of somatic embryos/explants (Mean $\pm$ SE)
0.5+0.5	30	8.66 <sup>A</sup> $\pm$ 0.930
1.0+0.5	45	9.44 <sup>A</sup> $\pm$ 1.125
1.5+0.5	60	16.33 <sup>B</sup> $\pm$ 1.873
2.0+0.5	75	24.20 <sup>B</sup> $\pm$ 2.464
2.5+0.5	85	36.0 <sup>C</sup> $\pm$ 3.120
3.0+0.5	65	18.46 <sup>B</sup> $\pm$ 2.036

**Table 2.** Effect of 2,4-D and BA on germination of shoots from somatic embryos in *R. vesicarius* L.

Growth regulators mg/l 2,4-D+BA	Percentage of somatic embryo germination	Average number of shoots from somatic embryos (Mean $\pm$ SE)
0.5+0.5	40	5.50 <sup>A</sup> $\pm$ 0.62
0.5+1.0	65	9.23 <sup>B</sup> $\pm$ 1.04
0.5+2.0	80	16.875 <sup>C</sup> $\pm$ 1.90
0.5+3.0	55	7.63 <sup>B</sup> $\pm$ 0.899

was observed at 1.5 mg/l 2,4-D + 0.5 mg/l Kn. Among the various concentrations of 2,4-D tested in combination with 0.5 mg/L Kn, the percentage of explants responded for somatic embryo formation was found to be higher at 2.5 mg/l 2,4-D + 0.5 mg/l Kn in leaf explants with maximum of  $36.0 \pm 3.12$  somatic embryo production. However, at the concentration of 2,4-D higher than 2.5 mg/l the percentage of somatic embryo induction was lower (Table 1). The development of somatic embryos was asynchronous. As a result, various stages of embryo development could be observed in the same cluster of embryos originally from the explants. When these embryos with different developmental stages were transferred to the same medium, further germination in them was not observed.

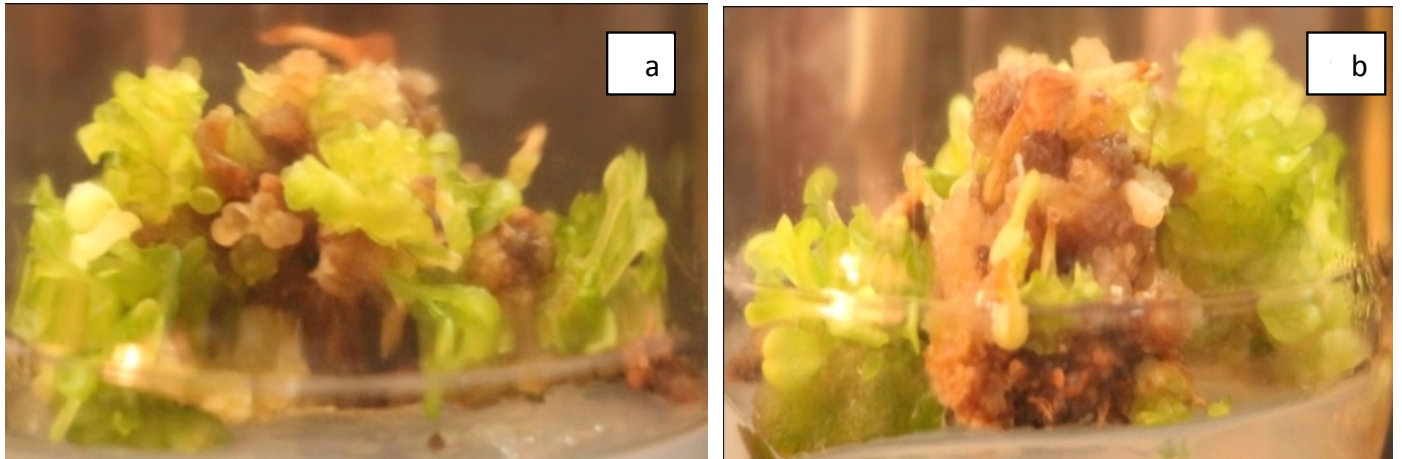
### Embryo germination and plantlet formation

The cotyledonary embryos proliferated to nodular buds with synthesis of shoot bud initiation effectively on 2,4-D 0.5 mg/l + 2.0 mg/l BA when compared to other hormonal concentrations. Highest shoot bud initiation was found to be 80% at 2,4-D 0.5 mg/l + 2.0 mg/l BA, 65% of germination was observed in 2,4-D 0.5 mg/l + 1.0 mg/l BA, 55% at 2,4-D 0.5 mg/l + 3.0 mg/l BA and 40% at 2,4-D 0.5 mg/l + 3.0 mg/l BA. Hence, it was observed that the increase in the growth hormone concentration showed gradual decrease in germination of shoots. Therefore, SH medium with 2,4-D 0.5 mg/l + 2.0 mg/l BA is proven to be effective for germination of maximum number of shoots

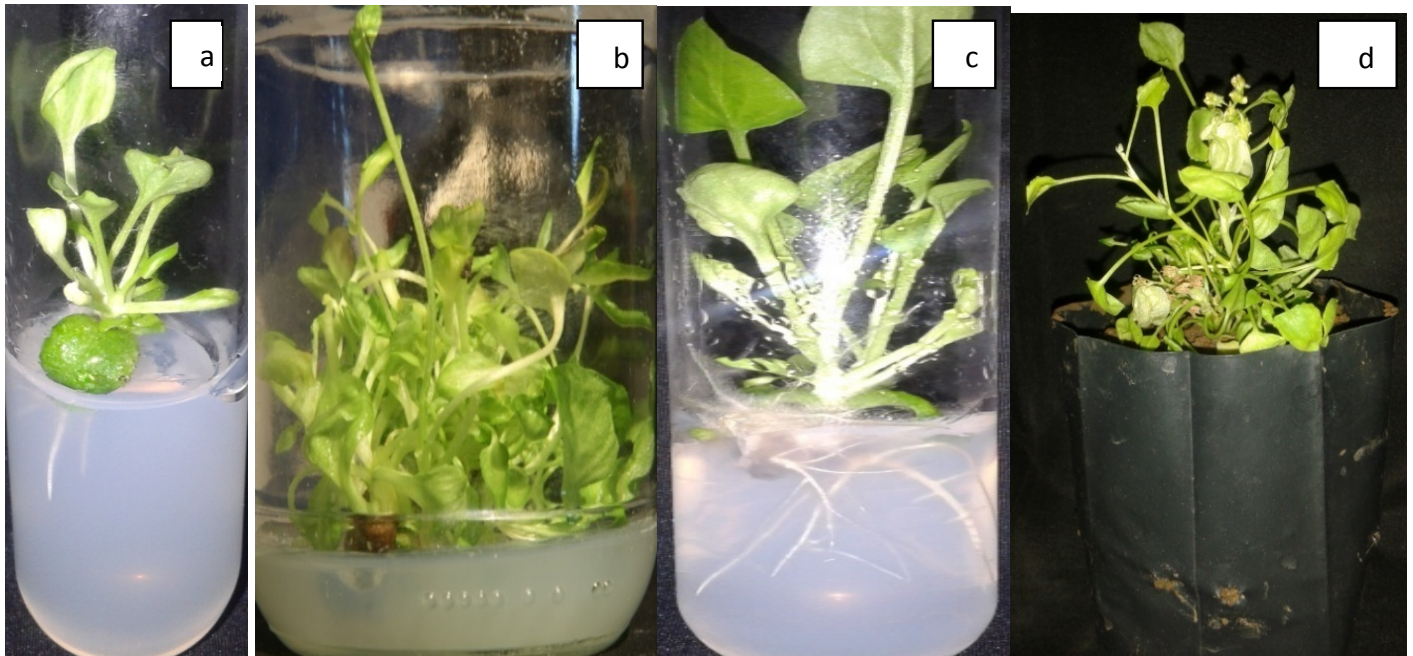
$16.875 \pm 1.90$  from cotyledonary embryoids in *R. vesicarius* (Table 2, Figure 3a and b). Later, the *in vitro* regenerated shoots were separated from the embryogenic callus and sub cultured on to fresh media containing 2.0 mg/l BA. These plantlets elongated and produced multiple shoots within two weeks (Figure 4a and b).

After elongation, the *in vitro* regenerated shoots were transferred onto rooting media containing IBA (0.5 to 2.0 mg/L). The highest rooting (90%) was noted on SH medium containing 1.0 mg/L IBA with average number of roots ( $6.38 \pm 0.687$ ) (Table 3, Figure 4c). Increasing or decreasing the concentrations of IBA resulted in lower rooting. Later, these *in vitro* regenerated plantlets were transferred to plastic pots containing sterile vermiculite: soil (1:1) mixture. Finally, they were shifted to earthen pots after hardening in the culture room and maintained in the research field under shady conditions. The survival percentage of plants was found to be 70 to 80%. The plants were normal; morphological and floral characters were found to be similar to the donor plants (Figure 4d).

In the present investigation, the results on somatic embryogenesis have shown that auxin, such as 2,4-D along with cytokinin BA are essential for inducing the somatic embryogenesis from leaf explants of *R. vesicarius*. The auxin/ auxin in combination with cytokinin used in the medium can greatly influence the frequency of induction and also on maturation of somatic embryos. The requirement of cytokinin in addition to auxin was observed in *Solanum surattense* (Rama swamy et al., 2005) whereas, somatic embryogenesis was reported on



**Figure 3.** *In vitro* regeneration via Somatic embryogenesis in leaf explant cultures of *Rumex vesicarius* L. (a and b): Regeneration of shoots from cotyledonary stage embryos in media containing 0.5 mg/l 2,4-D + 2.0 mg/l BA.



**Figure 4.** *In vitro* regeneration via Somatic embryogenesis in leaf explant cultures of *Rumex vesicarius* L. (a and b). Elongation and multiplication of shoots regenerated from somatic embryos in *Rumex vesicarius* L. (c). Rooting from *in vitro* regenerated shoots in media containing 1.0 mg/l IBA. (d). Acclimatization of plantlet.

**Table 3.** Effect of IBA on induction of roots from shoots in *R. vesicarius* L.

Growth regulators mg/l IBA	Percentage of rooting	Average number of roots from <i>in vitro</i> shoots (Mean $\pm$ SE)
0.5	60	3.33 <sup>A</sup> $\pm$ 0.423
1.0	90	6.38 <sup>C</sup> $\pm$ 0.687
2.0	75	4.8 <sup>B</sup> $\pm$ 0.554

Values are expressed as mean  $\pm$  SE (n=10 in replicate). Mean followed by same letters do not differ significantly at  $p \geq 0.05$  by Tukey's HSD test.

medium containing 2,4-D alone in *Capsicum annuum* L. (Marla et al., 1996). Direct somatic embryogenesis was also reported by adding Kn to the medium and also the number of embryos further increased by enriching the medium with 2,4-D in leaf explants of *Cicer arietinum* L. (Dinesh et al., 1994).

New gene products are needed for the progression from the globular to the heart shaped stage and these new products are synthesized only, when exogenous auxin is removed (Zimmerman 1993). But according to our observation in *R. vesicarius* for morphogenesis of somatic embryos, auxins and cytokinins combination is required. At higher concentration of auxin, probably the population of embryogenic cells drops due to their disruption and elongation and the embryogenic potential of the culture are lost (Aboshama, 2011). Maturation process is critical step in somatic embryogenesis. Similarly, somatic embryo maturation on MS medium containing the combination of 2,4-D and Kn was observed in *Brassica oleraceae* and *Oryza sativa* L (Siong et al., 2011; Verma et al., 2011).

## Conclusion

For induction of *in vitro* somatic embryogenesis, the type of primary explants, choice of genotypes and hormonal concentration plays an important role (Josephina and Van Staden, 1990). During the present investigation it was found that the high concentration of auxin in combination with less concentration of cytokinin induced the somatic embryogenesis and maturation of somatic embryos in *R. vesicarius*. However, for germination of somatic embryos, low level of auxin and high concentrations of cytokinin combination is required. Secondary embryogenesis observed in *R. vesicarius* has great potential for its mass propagation and repetitive embryogenesis can also be used for synthetic seed production and genetic transformation.

## Conflict of Interest

The author(s) have not declared any conflict of interest.

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## Full Length Research Paper

# Noble silver nanoparticles (AgNPs) synthesis and characterization of fig *Ficus carica* (fig) leaf extract and its antimicrobial effect against clinical isolates from corneal ulcer

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Received 27 August, 2014; Accepted 13 October, 2014

Nanotechnology is rapidly growing with nanoparticles produced and utilized in a wide range of pharmaceutical and commercial products throughout the world. In this study, fig (*Ficus carica*) leaf extracts were used for ecofriendly extracellular synthesis of stable silver nanoparticles (AgNPs) by treating an aqueous silver nitrate (1 mM) solution and using the plant *F. carica* leaf extracts as reducing agents. The bioreduced silver nanoparticles were characterized by ultra violet visible (UV-Vis) spectrophotometer, Fourier transform infra-red (FTIR) spectroscopy and transmission electron microscopy (TEM). The average particle size ranged from 5 to 40 nm. The particle size could be controlled by changing the reaction temperature, leaf broth concentration and AgNO<sub>3</sub> concentration. Further, these biologically synthesized nanoparticles concentration of 50 µl were found to be highly effective and exhibited maximum microbial activity with mean zone of inhibition 20.33±1.00 mm and 18.00±1.00 against *pseudomonas aeruginosa* and *Aspergillus fumigatus* isolated from human corneal ulcer patients. This environmentally friendly green synthesis is an eco-friendly approach to conventional chemical synthesis and can potentially be used in various areas such as food, cosmetics, and medical applications and hope the recent technology can provide next generation of antimicrobials.

**Key words:** *Ficus carica*, silver nanoparticles, characterization, antimicrobial activity.

## INTRODUCTION

In 21<sup>st</sup> century, the development of green processes for the synthesis of nanoparticles is evolving into an important branch of nanotechnology (Raveendran et al., 2006; Armendariz et al., 2002). Today, nanometal particles especially Silver have drawn the attention of

scientists because of their extensive application in the development of new technologies in the areas of material sciences, electronics, medicine and biolabelling as well as antimicrobials (Magudapathy et al., 2001; Panacek, et al., 2006). Silver has been used as an antimicrobial agent

for centuries, the recent resurgence in interest for this element particularly focuses on the increasing threat of antibiotic resistance, caused by the abuse of antibiotics (Panaek et al., 2006; Sambhy et al., 2006). The use of environmentally benign materials like plant leaf extract, bacteria and fungi for the synthesis of silver nanoparticles offers numerous benefits of eco-friendliness and compatibility for pharmaceutical and biomedical applications as they do not use toxic chemicals in the synthesis protocols (Upendra Kumar et al., 2009). Synthesis of nanoparticles provides advancement over chemical and physical methods as it is a cost effective and environmentally friendly and in this method there is no need to use high pressure, energy, temperature and toxic chemicals (Goodsell, 2004).

Corneal ulceration continues to be one of the most important causes of ocular morbidity and blindness worldwide, bacterial keratitis is considered a leading cause of monocular blindness in the developing world (Solomon et al., 2006). The incidence of infection by specific organisms varies by region, and practitioners should be aware about the local epidemiological patterns of corneal infection. Suppurative corneal ulcers may be caused by bacteria, fungi and protozoa. Moreover, the increasing resistance of many bacteria and the side effects to the currently used antibiotics are documented (Yang et al., 2009), although there were a significant proportion of corneal ulcers reported from Saudi Arabia (Khairallah et al., 1992).

The genus *Ficus* constitutes about 750 species found in tropical and subtropical regions (Subramanian et al., 2013). *Ficus carica*, commonly called "fig" plant, is known to harbor diverse chemical compounds with proven medicinal importance figs (*F. carica*) are cultivated in the Kingdom of Saudi Arabia and the leaf has been reported to have health benefits including anti-diabetic property of *F. carica* are traditionally used to cure throat diseases, constipation, hemorrhoid and high cholesterol. Several researchers demonstrated the medicinal importance of fig plant as an antioxidant (Gond and Khadabadi, 2008), antidiabetic (Patil et al., 2010), hepatoprotective (Jeong et al., 2009), antipyretic (Rubnov et al., 2001), and antimicrobial (Aref et al., 2011). Latex of fig suppresses cancer cell proliferation and has an antiviral potential (Shankar, 2004). Several plants have been utilized for the production of silver nanoparticles (Parashar, 2009; Tripathi et al., 2009). Much attention is now required for synthesis of nanoparticles using biological sources due to limitations associated with chemical and physical methods of nanoparticle synthesis.

In the present study, reducing silver ions present in the

aqueous solution of silver nitrate by the help of *F. carica* extract and their antibacterial assessment was performed to produce novel drugs to overcome drug resistance and adverse reaction. This research study was undertaken to determine the effect of fig leaf extract as antimicrobial against local bacterial and mycotic infectious agents in corneal ulcer, it will be helpful in planning of corneal ulcer management strategy.

## MATERIALS AND METHODS

### Collection of *F. carica* leaf

*F. carica* leaves were collected from Riyadh market, and the species was identified with the authenticated specimen from the Department of Agriculture, Qassim University, Kingdom of Saudi Arabia.

### Preparation of fig leaf extract

The silver nitrate ( $\text{AgNO}_3$ ) was purchased from Sigma-Aldrich chemicals and the fresh leaf extract used for the reduction of  $\text{Ag}^+$  ions to  $\text{Ag}^0$  was prepared by taking 20 g of thoroughly washed finely cut leaves in 500 ml Erlenmeyer flask along with 100 ml of distilled water and then boiling the mixture for 5 min before decanting it. Further, the extract was filtered with Whatman No. 1 filter paper and stored at 4°C and used for further experiments.

### Synthesis of silver nanoparticles

In a typical experiment, *F. carica* leaf extract (0.5 ml) was added to 10 ml of 1 mM  $\text{AgNO}_3$  aqueous solution. The bioreduced aqueous component (0.5 ml) was used to measuring UV-Vis spectra of the solution. The particle suspension was diluted 10 times with distilled water to avoid the errors due to high optical density of the solution.

### UV-Vis spectral analysis

Synthesized silver nanoparticles was confirmed by sampling the aqueous component of different time intervals and the absorption maxima was scanned by UV-Vis spectrophotometer at the wavelength of 300 to 800 nm on Perkin-Elmer Lambda 25 spectrophotometer.

### FTIR spectral analysis

The bioreduced silver nitrate solution was centrifuged at 10,000 rpm for 15 min and the dried samples were grinded with KBr pellets used for FTIR measurements. The spectrum was recorded in the range of 400 to 4000  $\text{cm}^{-1}$  using Thermo Nicolet Nexus 670 spectrometer in the diffuse reflectance mode operating at resolution of 4  $\text{cm}^{-1}$ .

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**Abbreviations:** UV-Vis, Ultra-violet visible; FTIR, Fourier transform infra-red; TEM, transmission electron microscopy.



### TEM analysis of silver nanoparticles

Morphology and size of the silver nanoparticles were investigated by TEM images using Phillips, TECHNAI FE 12 instrument. Thin film of the sample was prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid and drying under lamp.

### In-vitro antibacterial and fungal activity of silver nanoparticles

#### Test microorganisms

Microbial cultures of six different strains from bacterial and fungal isolates were used for determination of antimicrobial activity. Gram-positive: *Staphylococcus aureus*, *Streptococcus pneumoniae*, Gram-negative: *Pseudomonas aeruginosa*, *Proteus vulgaris* as well as *Aspergillus fumigates* and *Fusarium* spp. clinical isolates were used. All the strains were sub-cultured at 37°C on Mueller-Hinton agar and potato dextrose agar (Oxoid, Hampshire, UK) every 15 days and stored at 4°C. The isolates were obtained during parallel studies from clinical cases suffered corneal infections and have been subjected to several hospitals at Qassim region during 2012. Sampling, culturing, isolation and identification were done in the Department Medical Laboratories, Qassim University, Kingdom of Saudi Arabia.

#### Antibiotic susceptibility testing

The test microorganisms were also tested for their sensitivity against the bacterial and fungal drugs Ciprofloxacin (5 µg) and Ketoconazole (30 µg). The cultures were enriched in sterile Mueller-hinton broth for 6 to 8 h at 37°C. Using sterile cotton swabs, the cultures were aseptically swabbed on the surface of sterile Mueller-hinton agar plates and potato dextrose agar (Guzmán et al., 2008). Using an ethanol dipped and flamed forceps, the antibiotic discs were aseptically placed over the seeded agar plates sufficiently separated from each other to avoid overlapping of inhibition zones. The plates were incubated at 37°C for 24 h for bacteria and seven days for fungi the diameter of the inhibition zones was measured in mm. All media used in the present investigation were obtained from Oxoid, Hampshire, UK.

#### Antibacterial activity by well diffusion method

Antibacterial activity of AgNPs was carried out by agar well diffusion method. Each microorganisms were grown overnight at 37°C in Mueller-Hinton Broth. 100 µl of standardized inoculum (0.5 MacFarland) of each test bacterium were inoculated on molten Mueller-Hinton agar, homogenized and poured into sterile Petri dishes. The Petri dishes were allowed to solidify inside the laminar hood. Standard cork borer of 16 mm in diameter were used to make uniform wells into which was added (50 µl) synthesized silver nanoparticles. Zones of inhibition for control, AgNPs and silver nitrate were measured. The experiments were repeated thrice and mean values of zone diameter were presented.

#### Antifungal activity by well diffusion method

Potato dextrose agar plates were prepared, sterilized and solidified. After solidification, fungal cultures were swabbed on these plates. Three cavities were made using a cork borer (10 mm diameter) at an equal distance and were filled with the Silver nanoparticle solution (50 µl), then were incubated at 37°C After seven days zone of inhibition was measured, the formation of a clear zone (restricted growth) around the cavity is an indication of antifungal activity.

## RESULTS AND DISCUSSION

### Synthesis of silver nanoparticles (AgNP's)

After the addition of the extract to the silver nitrate solution, the solution changed from colourless to pale yellow within 2 min, the final colour deepening to brown within 30 min. Figure 1 shows the *F. carica* leaf extract with silver nitrate at initial point of time and after 30 min reaction end point, similar results were reported (Balaji et al., 2008). The brown colour indicated surface plasmon vibrations, typical of silver nanoparticles (Saxena, et al., 2010).

### Characterization of silver nanoparticles (AgNP's)

UV-vis is the most widely used technique for the structural characterization of nanoparticles, so the sizes of the synthesized nanoparticles were provisionally predicted on the basis of UV-vis spectrum in the range of 200 to 800 nm. A distinct peak with smooth shoulder was observed at 432 nm (Figure 2). Thus, the UV-vis absorption spectrum reveals the formation of nanoparticles by showing surface Plasmon absorption maxima at 432 nm. Plasmon resonance in nanoparticles is strongly depends on the shape, size and dielectric constant. Noble silver nanoparticles exhibit a strong absorption band in the visible region and giving specific color to the solution (Khandelwal et al., 2010).

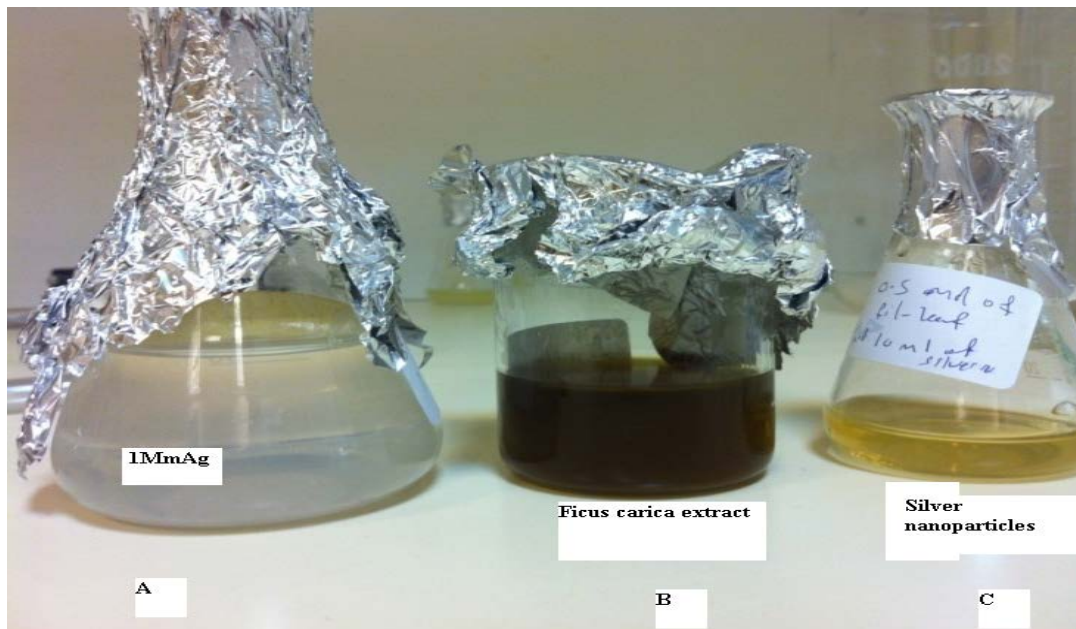
Fourier transform infrared spectroscopy (FTIR) measurements are carried out to identify the possible biomolecules responsible for the reduction of the Ag<sup>+</sup> ions and capping of the bio-reduced AgNP's synthesized by *F. carica* leaf extract. The FTIR spectra of *F. carica* leaf extract and biosynthesized nanosilver are depicted in Figure 3. The appearance of peaks in the amide I and amide II regions is the characteristic of proteins/enzymes that have been found to be responsible for the reduction of metal ions. FTIR analyses confirm that the larger size of the nanoparticles might be due to the capping of nanoparticles by proteins (Warisnoicharoen et al., 2011).

### Antibacterial and antifungal analysis

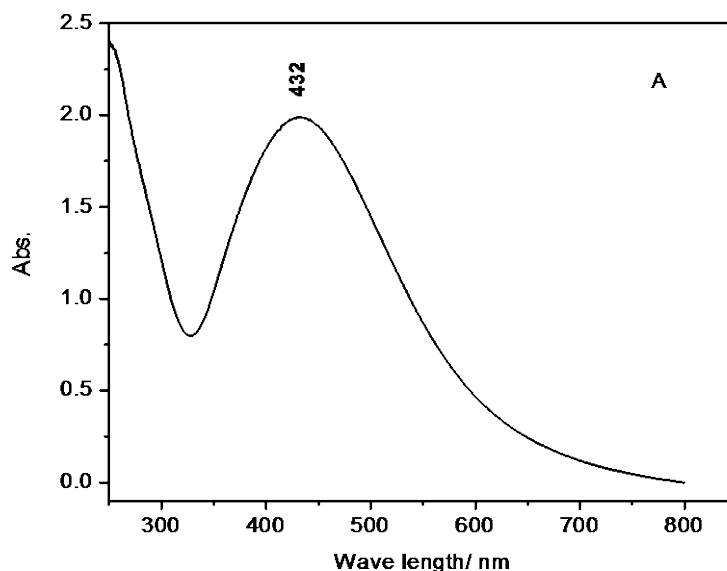
The antimicrobial activity of synthesized silver nanoparticles was investigated using the well diffusion method against different bacterial and fungal such as *S. aureus*, *S. pneumoniae*, *P. aeruginosa*, *P. vulgaris*, *A. fumigates* and *Fusarium* spp these pathogens are treated with 50 µl of AgNP.

### Determination of mean zone of inhibition

The mean zone of antibacterial activity of AgNP is



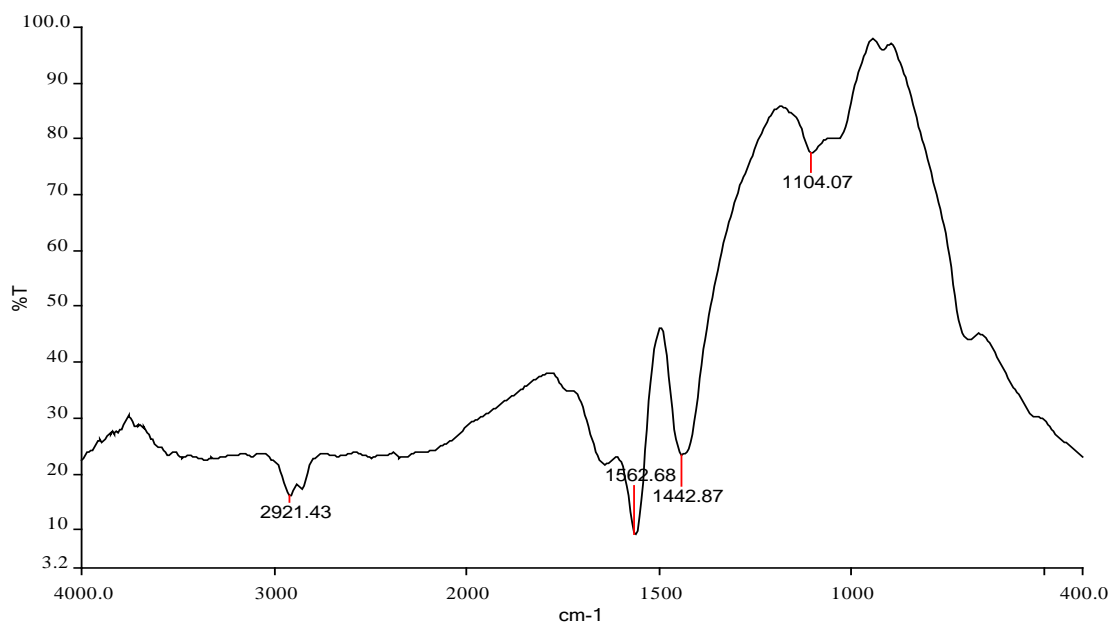
**Figure 1.** The colour change of plant extracts after addition of silver nitrate (a) 1 mM silver nitrate (b) plant extract (c) silver nanoparticles.



**Figure 2.** UV absorption spectra of silver nanoparticles. A peak was observed at 419 nm.

presented in Table 1. *P. aeruginosa*, *S. aureus* and *Aspergillus* spp. exhibited highest rate of sensitivity to aqueous extract with mean zone of inhibition of  $20.33 \pm 1.00$ ,  $19.00 \pm 1.00$  and  $15.33 \pm 0.57$  mm, respectively, at the test concentration of 50  $\mu$ l, which was comparable to standard antibiotic (Ciprofloxacin 5  $\mu$ g /disc). The AgNP exhibited lowest activity against *P. vulgaris* and *Fusarium* spp. with mean inhibition zone of,  $15.33 \pm 0.57$  and 14.66

$\pm 0.57$  mm, respectively. The biologically synthesized silver nanoparticles were found to be highly effective against different bacteria and fungi of selected species. It shows that, they have great potential in biomedical applications. Similar observation was found in *Allium cepa* (Shahverdi, et al., 2007), indication that the silver nanoparticles have an ability to interfere with metabolic pathways. The result shows the potential biocidal effect



**Figure 3.** FTIR Spectra of nanoparticles synthesized from *Ficus carica* leaf extract.

**Table 1.** Mean zone of inhibition (mm) of silver nanoparticles against bacterial and fungal isolates in comparison with standard antibiotic.

Fungus	Mean zone of inhibition (mm) (mean $\pm$ SD)	
	Silver nanoparticles (50 $\mu$ l)	Standard antibiotic (ciprofloxacin 5 $\mu$ g)
<i>Staphylococcus aureus</i>	19.00 $\pm$ 1.00	27.33 $\pm$ 0.57
<i>Streptococcus pneumoniae</i>	15.33 $\pm$ 0.57	28.66 $\pm$ 1.52
<i>Pseudomonas aeruginosa</i>	17.00 $\pm$ 1.00	30.33 $\pm$ 1.52
<i>Proteus vulgaris</i>	15.33 $\pm$ 0.57.	26.00 $\pm$ 1.52
<i>Aspergillus fumigates</i>	18.00 $\pm$ 1.00	27.66 $\pm$ 0.57
<i>Fusarium spp.</i>	14.66 $\pm$ 0.57	28.33 $\pm$ 1.52

against clinical bacterial and fungal isolates.

### Transmission electron microscope (TEM) analysis

TEM image of silver nanoparticles derived from *F. carica* leaf extract is shown in Figure 4. The morphology of the nanoparticles was spherical in nature. Under careful observation, it is evident that the silver nanoparticles are surrounded by a faint thin layer of other materials, which we suppose are capping organic material from *F. carica* leaf broth. The obtained nanoparticles are in the range of sizes approximately 5 to 40 nm and few particles are agglomerated. Figure 5 shows the histogram of silver nanoparticles, it is evident that there is variation in particle sizes and the average size estimated 9.5 nm. It may be noted that, the size of the silver nanoparticle obtained from TEM is similar with the size obtained from the FTIR determination. Same phenomenon was reported for the silver nanoparticles synthesized using *P.*

*graveolens* leaf broth.

### Conclusion

Simple, efficient and stable silver nanoparticles were synthesized by using *F. carica* leaf extract. These particles are of uniform size and shape has the potential to kill a broad range of bacteria and fungi. The bioreduced silver nanoparticles were characterized using UV-Vis, FTIR, and TEM techniques and estimated approximately as 5 to 40 nm. These particles may be useful in pharmaceutical area with potential of future development in nano preparations.

### Conflict of Interests

The author(s) have not declared any conflict of interest.

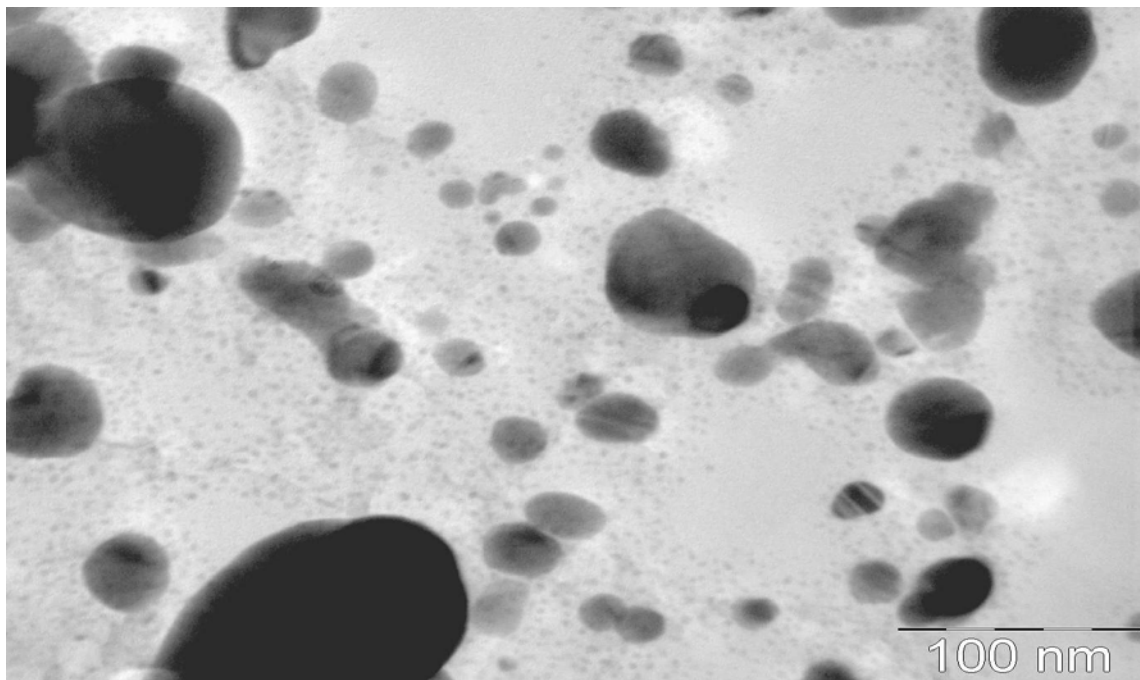


Figure 4. TEM Images of silver nano particles.

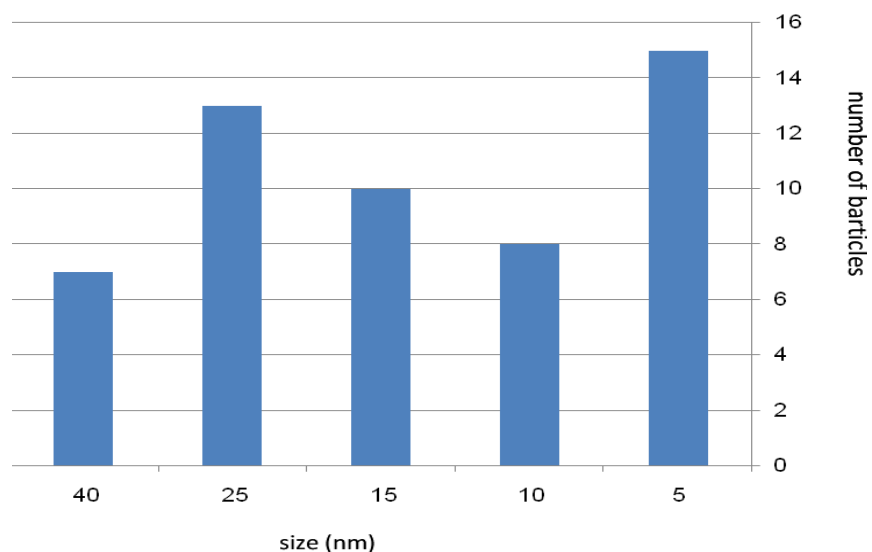


Figure 5. Histogram of synthesized silver nanoparticles.

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## Full Length Research Paper

# A novel polymerase chain reaction (PCR) for rapid isolation of a new *rbcS* gene from *Lemna minor*

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Received 14 December, 2012; Accepted 22 September, 2014

This study developed a novel polymerase chain reaction (PCR) method, ligation-mediated self-formed panhandle PCR, for gene or chromosome walking. It combined the advantages of ligation-mediated PCR in its specificity and of panhandle PCR in its efficiency. Self-formed panhandle PCR was used for a new *rbcS* gene walking to isolate 3' downstream and 5' upstream sequence; 1292 bp DNA *rbcS* gene was obtained via 3' walking of *Lemna minor* genomic DNA and 5' upstream sequence of the new *rbcS* gene with a length of 1543 bp was isolated from *L. minor* via self-formed panhandle PCR. A novel *rbcS* gene with the size of 2835 bp, which was confirmed by nested-PCR, was obtained by ligation-mediated self-formed panhandle PCR. Ligation-mediated self-formed panhandle PCR was simple and efficient and should have broad applications in the isolation of unknown sequences in genomes.

**Key words:** Chromosome walking, *Lemna minor*, polymerase chain reaction (PCR), *rbcS* gene, self-formed panhandle.

## INTRODUCTION

Over the past years, several strategies have been developed that aimed at identifying genomic fragments adjacent to known DNA sequences, without going through the process of screening genomic libraries (Wang and Guo, 2010). Polymerase chain reaction (PCR)-based methods have increasingly been applied for gene on chromosome walking. Several PCR methods were available for this purpose: i. Inverse PCR (Uchiyama and Watanabe, 2006; Huang and Chen, 2006; Liu et al., 2004; Keim et al., 2004); ii. Ligation-mediated PCR (LM-PCR) (Tonooka et al., 2008; Villalobos et al., 2006; Ren et al., 2005; Yuanxin et al., 2003; Dai et al., 2000); randomly primed PCR (RP-PCR) (Tanabe et al.,

2003).

We report here a simplified and effective PCR method, ligation-mediated self-formed panhandle PCR (SEFP-PCR). SEFP-PCR strategy is based on these principles: i. Restriction sites disperse throughout the genomes of double strand DNA in organisms are natural candidacy for panhandle adaptor pairing; ii. A panhandle adaptor can be a combination of a 3' end pairing with the bases of selected restriction sites in genomic DNA to self-form panhandle, and to limit non-specific amplifications, two-round PCR amplifications were employed in the SEFP-PCR protocol: amplify the target template by using a specific primer and panhandle primer to accumulate the

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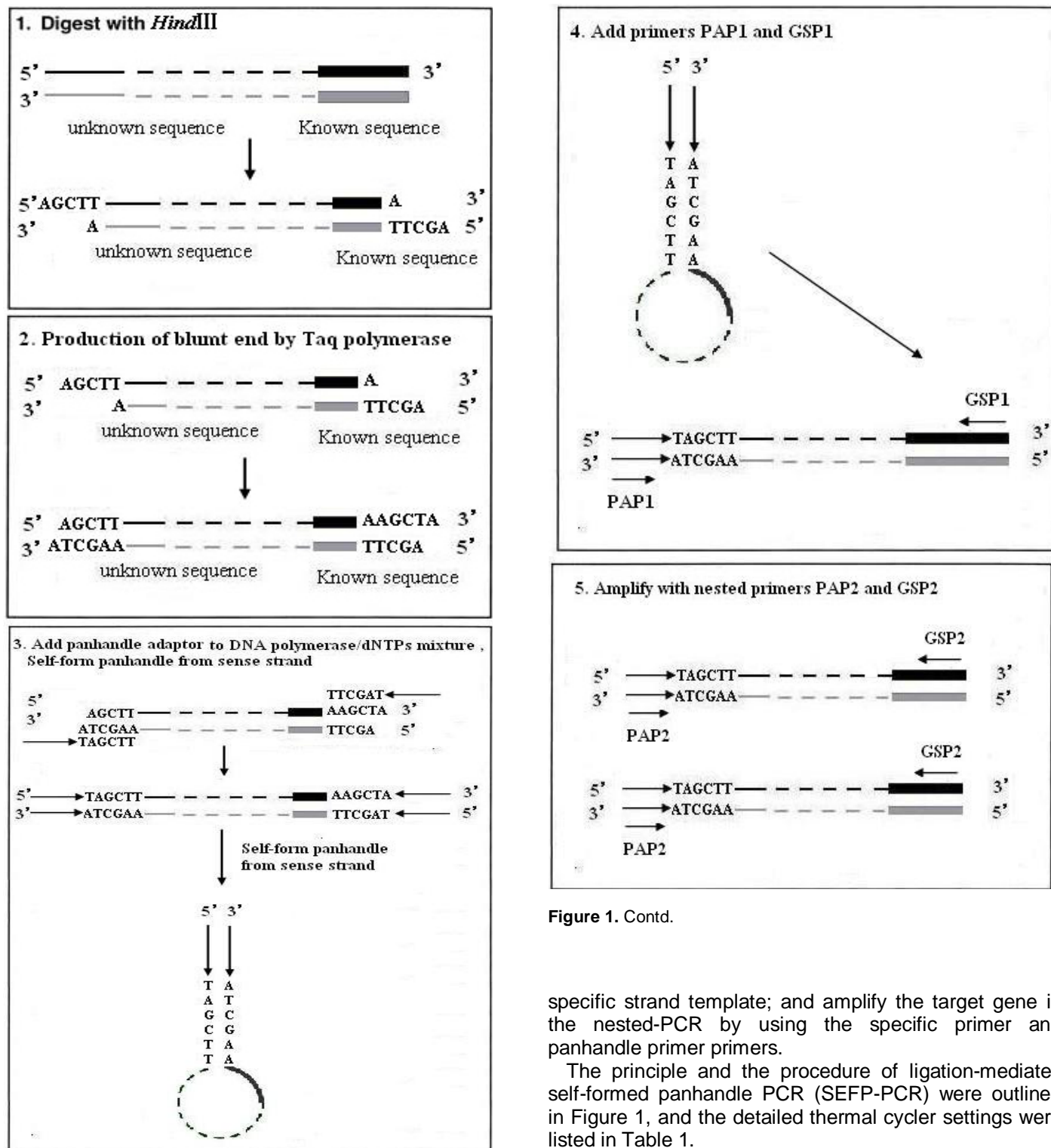


Figure 1. Contd.

**Figure 1.** Self-formed panhandle PCR procedures for 5' walking. **Step 1.** *LmrbcS* gene contained *Hind*III site, and *L.minor* genomic DNA was digested with *Hind*III. **Step 2.** production of the sticky blunt end of *Hind*III by PCR; **Step 3.** Add panhandle adaptor to DNA polymerase/dNTPs mixture and form panhandle template. **Step 4.** primary PCR using primers PAP1 and GSP1; **step 5.** isolation of 5'flanking region of *LmrbcS* gene by nested PCR using primers PAP2 and GSP2.

specific strand template; and amplify the target gene in the nested-PCR by using the specific primer and panhandle primer primers.

The principle and the procedure of ligation-mediated self-formed panhandle PCR (SEFP-PCR) were outlined in Figure 1, and the detailed thermal cyclers settings were listed in Table 1.

## MATERIALS AND METHODS

### Plant materials and genomic DNA

*L. minor* was cultured aseptically in SH liquid medium supplemented with 1% sucrose and maintained in a growth chamber at 25°C under cool white fluorescent lighting (90-100 μmol

**Table 1.** Cycling conditions used for self-formed panhandle PCR

Reaction	Step	Primer	Cycles	Cycling conditions
Self-formed panhandle	2		1	(a) 70°C ( 3 min);
			1	(a) 94°C (3min); (b) 25°C (3 min), then ramping to 70°C at 0.2°C per second; (c) 72°C ( 4 min);
	3	1 µl of 5 µM panhandle	1	(a) 94°C (3min), then cool down to 4°C at 0.2°C per second; (b) 4°C ( 5 min),
Primary	4	3 µl of 5 µM PAP1 and GSP1	20	(a) 94°C (2min); 94°C (1 min) (b) 60-50°C ( 1 min), touch down (c) 72°C ( 3 min);
			15	(a) 94°C (2min); 94°C (1 min) (b) 50°C ( 1 min), (c) 72°C ( 3 min);
Secondary	5	3 µl of 5 µM PAP2 and GSP2	20	(a) 94°C (2min); 94°C (1 min) (b) 60-50°C ( 1 min), touch down (c) 72°C ( 3 min);
			15	(a) 94°C (2min); 94°C (1min) (b) 50°C (1 min), (c) 72°C ( 3 min);

**Table 2.** Primers used for cloning of *rbcS* gene from *L. minor*.

Primer	Primers sequence ( 5' to 3')	Purpose
PF1	MGATAAGRTGTAATCCW	cloning of <i>rbcS</i> gene
PR1	TGGAAGCCATCATCGACGAAGCCAT	from <i>L. minor</i>
PAP 1	CACGACACGCTACTCAACAC	Adaptor primer
PAP 2	CTCAACACACCACCTCGC	Adaptor primer
3GSP1	GCTTCGTCGATGATGGCTTCCACCG	3'-walking
3 GSP 2	CGGCGACGAGGAAGGCTAACGATCTGT	3'-walking
5 GSP 1	CGGACCTCAGCCCGTTGAAGGGTGC	5'-walking
5 GSP 2	ACGCTGGCCACGGCGGGTGGAGC	5'-walking

photons m<sup>-2</sup> s<sup>-1</sup>) in a 18/6 h (light/dark) photoperiod. Genomic DNA was isolated from fronds of *L. minor* using the method described by Youru and Sandui (2011).

#### Isolation of the partial *rbcS* gene from *L. minor*

Based on the known *L. minor rbcS* gene sequences, degeneracy primer pairs (5'- MGATAAGRTGTAATCCW-3') and (5'-TGGAAGCCATCATCGACGAAGCCAT-3') were designed to amplify a new partial *rbcS* gene by PCR. About 400 bp DNA fragment was obtained by PCR.

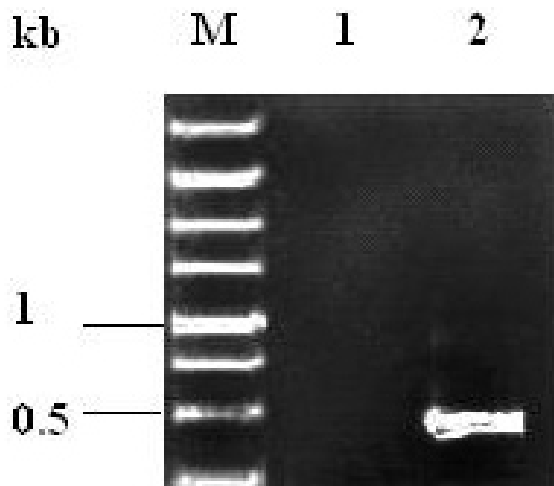
This DNA fragment was cloned into the pMD18-T vector (TakaRa) for sequencing. Sequence analysis of this DNA fragment

revealed that it shared 82-85% identity with the known *L. minor rbcS* gene, which indicated that the 400 bp DNA fragment was partial *rbcS* gene.

#### Self-formed panhandle PCR

According to the sequence of new *rbcS* gene, primers were designed for self-formed panhandle PCR (Table 2). The steps of self-formed panhandle PCR (SEFP-PCR) for 5'walking are summarized in Figure 1, and the steps of SEFP-PCR for 3'walking were the same as those for 5'walking except that genomic DNA was digested with *Bam*HI; panhandle adaptor sequences and primer sequences are shown in Table 2.





**Figure 2.** PCR amplification of partial *rbcS* gene from *L. minor*. M, Marker ; 1, negative control; 2, cloning of *LmrbcS* gene from *L. minor*.

#### Step 1: Digest genomic DNA with *Hind*III

5  $\mu$ g genomic DNA was digested with 20 units of *Hind*III (Takara) at 37°C for 2 h. The digested DNA between 2 and 5 kb was purified using DNA extraction kit (*sigma*) and resuspended in ddH<sub>2</sub>O for DNA template.

#### Step 2: Make the sticky end of *Hind*III blunt by PCR

The PCR mixture included 4  $\mu$ L of 10°C long Taq DNA polymerase buffer, 2  $\mu$ L of mixed dNTP solution (2.5 mM each of dATP, dTTP, dCTP and dGTP), 1.5 U of long Taq DNA polymerase (*Takara*), 20  $\mu$ L (10-200 ng) template DNA. PCR cycle was run, the detailed thermal cycling conditions for PCR was listed (Table 1).

#### Step 3: Add panhandle adaptor to DNA polymerase/dNTPs mixture and form panhandle template

Then 1  $\mu$ L of 5  $\mu$ M handle adaptor (CACGACACGCTACTCAACACACACCTCGCACA GCAGTCCNNNNNGGATCC) was added to the PCR mixture, and then PCR cycles were run. The detailed thermal cycling conditions for PCR is listed (Table 1).

#### Step 4: Add primers PAP1 and GSP1

After heating the reaction mixture at 80°C for 5 min, primers PAP1 and GSP1 were added. Each final 30  $\mu$ L PCR reaction mixture (30  $\mu$ L final volume) was the same as that for step 2 except for the template and primers: 0.1  $\mu$ L of the above PCR product (or diluted 10 times) and 3  $\mu$ L of 5  $\mu$ M PAP1 and GSP1 were added to the reaction mixture, then the PCR was run for 35 cycles (Table 1).

#### Step 5: Add primers PAP2 and GSP2 for nested PCR

Nested PCR was performed after preheating a 29  $\mu$ L PCR mixture containing all of the reagents except the DNA to 80°C for 5 min to prevent nonspecific priming, and then 1  $\mu$ L of the diluted PCR pro-

ducts (1000  $\times$ ) was added from Step 4 as template. PCR mixture contained 25  $\mu$ M dNTPs, 5 U of long Taq polymerase, 3  $\mu$ L of 5  $\mu$ M of gene specific primer (GSP2) and panhandle adaptor primer (PAP2) nested PCR conditions were listed (Table 1).

#### Cloning and sequencing of PCR products

The nested PCR products were separated on 1.2% agarose gels, then the specific product was purified by PCR purification kit (*Sigma*) and cloned to T-vector (*Takara*), and the positive clones were selected for sequencing (*Shanghai Baosheng Biotechnology Co. Ltd, China*).

#### Sequence analysis of the 5' flanking region of *LmrbcS* gene

DNA sequence analyses were carried out using the BLAST program (<http://ncbi.nlm.nih.gov>). The location and distribution of cis-regulatory sequence elements in the *LmrbcS* promoter were analyzed by a signal scan search in the PLACE database (<http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html>). The closest homologues to the *RBCS* promoter were identified by a homology-based search in the PLACE database. The identified *RBCS* homologous fragments were aligned to the *L. minor RBCS* gene promoter using the software program MegAlign and subsequently manually improved.

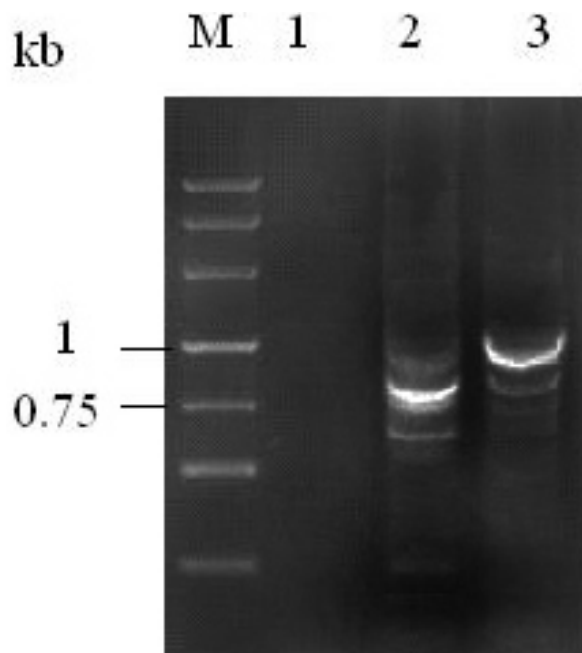
## RESULTS

#### Cloning of 3' unknown region of *LmrbcS* gene from duckweed by SEFP-PCR

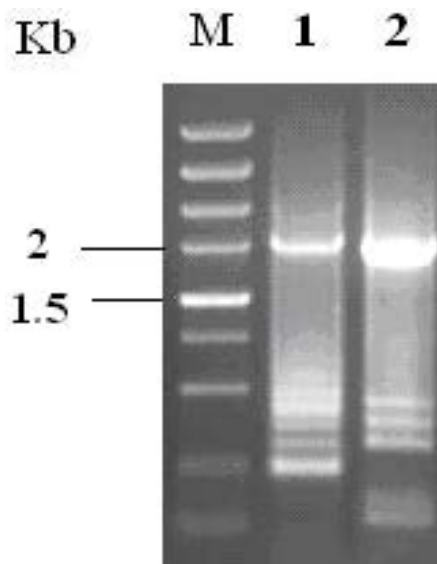
Based on the known *rbcS* gene sequence (GenBank accession No. X17231.1, X17230.1, X17232.1, X17235.1, X17234.1 X17233.1, and X00137.1) from *L. minor*, degeneracy primers were designed to obtain a partial *rbcS* gene with a length of 400 bp (Figure 2). Blast analysis showed that this *rbcS* gene (termed *LmrbcS*) shared 80% identity with the known *rbcS* genes from *L. minor*. Another 922 bp DNA fragment was obtained from 3' flanking region of *LmrbcS* via SEFP-PCR (Figure 3). Blast analysis *LmrbcS* gene with the length of 1292 bp was a new *L. minor rbcS* gene since it shared 95% identity with the known *rbcS* genes from *L. minor*.

#### Identification of 5' flanking region of *LmrbcS* from *L. minor* by SEFP-PCR

In order to allow chromosome walking into the unknown 5' flanking region of *LmrbcS* sequences, according to the DNA sequence of *L. minor LmrbcS* gene, gene-specific primers in nested positions close to the 5' end of the coding regions were designed and synthesized. After two rounds of SEFP-PCR, about 2000 bp fragment was cloned from the 5' upstream region of *LmrbcS* gene by SEFP-PCR (Figure 2). The sequencing results (supplementary material) showed that the cloned product was 1870 bp in length (Figure 4) and DNA sequence analysis (Figure 5) indicated that 1543 bp, which was the



**Figure 3.** Cloning of 3'flanking region of *LmrbcS* gene from *L. minor* via SEFP-PCR. M, DNA maker; 1, negative control; 2, 2nd round SEFP-PCR; 3, 1st round SEFP-PCR.



**Figure 4.** Isolation of 5'flanking region of *LmrbcS* gene from *L. minor* by SEFP-PCR. M, Marker; 1, 1st round I-PCR; 2, nested PCR.

5' flanking region of *LmrbcS*, contained several putative cis-elements, such as sugar responsive elements as well as circadian-box; all present in this *LmrbcS* promoter. The integrity of the genomic DNA (2835 bp in length) was

confirmed by nest PCR. Isolated 5' flanking regions were fused to *gus* gene, and tested for expression in tobacco; the isolated 5' flanking regions were shown to drive reporter gene expression in green tissues (data not shown).

TTCAATATCGTAAGCCAGTAATTTTTGACGAATCCCTCTCTCTATATTTGTAGGAAACCCTATTTTTGTCAAATCCCTCACATTTTCA  
 AATTTGGTATTGATTATTGAATTGGGTACTTCGAATATGTAATTGAGTCAAAATGTCCATCCACGTCCCTTAGGAAGTCAATGCATGGT  
 ACAACTATTGGGCACCAATTCATTAACAATGCTCAATAGAAAATTCATGCCTAATATTTAACTAGATATCACTTTCTCCCTTCATGGGGT  
 ATCAGTGTGCGTGTACCAGGATCAGGGCAAGGATCGAACGACGAACACTAGTTGGAATTTATATAGAGCCTTTGTTAATGAGGC  
 ATTCATGTCTTCTTTGGAACACAACTAGTTGTCACCTATCTCATATCTTCGTTGAAAACAAACCAATATCTCACTAATCACGCCGC  
 TATTGCTTGTGAAACCAACTAATGACCACTCCTTAATCCTTAGGCCAAATCAACATTGCCTCCTGGAAGAAGTACCAAGCCTCT  
 CCCACTATCAACATTACCCTGTATGCCATCAAAATTCACAGCAAATTCACCTTCTCCCACCATGTGTTCTTAGCTACATGAACCTT  
 AAGTGTCAATTATACTATGCCCCCTAACAAAACACACCCAAGACAATAATTGGATTGAAATAGGTGAGTTAATTTATTTTGAATATT  
 TAAATCCACCTGACTCCAATAATATCTCACTCTATCACAACAAAGACAAAATCTTCTTATTTTTTAAGTGTATAATGAAAGTGAAGCAA  
 GCAAATAAACTTATCCCTAGATAGTAATTCATACCCTTATCCCATCCCACCTAACACAAGTATCCCCTACTCACTCTCCCCTCC  
 AATAAAACCACTCAAATCCATCAAAACCATACATCTCAAGATAAAGTAATGCAACCCCTAAACCACATATCCCTAGGCTTTGTCTTTTC  
 CCACAGATAGCCTCTCCATAAAAGTGTAGCCCTTGAGATAAGACAGTTGTGGAGGAGCGGGTGGATTGTCCCCCTTGCAGAAG  
 ATCGGACCATGGCAGCCATAGAATACCCAAACCCTAAGACAGTACTGTCCCTTATTTAATCAGACCAAGAGCTCTCTCAGAGACC  
 CCAGAGCTTCCGAGAAGAAGAGGAAGAGAGAGAAGGAGAGTGACCATGCTGCCTCCATGATGAGCTCCACCGCCGCCGTG  
 GCCAGCGTTGCCAAGACCAGCATGGTGCACCCCTCAACGGGCTGAGGTCCGCCGTGCCTTCCCGCGACCAGGAAGGCCAA  
 CGATCTGTGACTCTGCCAGCAATGGCGGGAGGGTGTAGCTGCATGCAGGTGTGGCCGCCGAGGGGGTGAAGAAGTTCGAGA  
 CCTCTCCTACCTCCCTCCCTCTCCGTGAGGCTCTCTCAAGGAGGTGACTACCTCCTCCGCAACGGCTGGATTCCCTGCG  
 TTGAGTTCTCAAGGTATAACAAAACCACTTCTATCGGTTATCGGATCCTTATCGAATATCATACCCTTTTTGGAATTCTCCAAGGCG  
 AAAATCTTCCGATTTTGGTCATCCTTATTGGTACCATTTTCGATAACAATTGGGCCCGTTTCAGTTAATGGGTCCCATTTTGATACC  
 GAATCCGTATTGATATCTCCTCAGTACCATTGTGTTGCGAAACGAGTGTCTATACTTGATTGATCAGACCCATGTTTTGGTTG  
 CTAATAATTGATTGGTATCGGATGCCGAAACGAAACTTTTGATTTGCGGTTCTTATCGGTACCATTTTCGACCAAAAGTTTCCGTTA  
 ACGGGTCCCGCTTTGATACCACTATCCGTTATCGGTTACACGATCCAATTCTGATATTGGACCTGTACCAATATCTTCTAGTAACG  
 ATGGCGATAGATTGCTATCCAATTGCGATATCCATATCAAAATGACTAGTTCGATAGTCGATATCCAGACCCATGTTTGGTATCCAA  
 TATTGATTGATATTGAATACAAAAGAGAAAGTATCAGTATTCAATCTTATCGGTATTGCTACTAGATGCGATGTCGGTACCTGGATG  
 GGCGAACCCGCAAAGGATTATGAAGGAAGTGGTGCCGTATCCTTATCGATACCGGTTCAATACCGGTATCCAATACCGATAACAG  
 CTCCACTATCCAAAGTCAATCGAAGCCGCAAAACCAAGTGTGATTAGTATCTAAATCCGATATTCGTTAACAAAAGGGTTCGTGT  
 ACCGCCAATACCACGCTCCCCGGGTACTACGATGGGCGTACTGGACCATGTGGAAGCTGCCATGTTCCGGTGCACCGACG  
 CCAGCCAGGTGATCGCCGAGGTGGAGGAGGCCAAGAAGGCCTACCCCGAGTATTTGTCAGAAATCATCGGCTTCGACAACAAGC  
 GCCAAGTCCAGTGCATCAGCTTCATCGCCTACAAGCCCACCTAA

**Figure 5.** The isolated *rbcs* gene from *Lemna minor* by SEFP PCR.

## DISCUSSION

We have shown that the self-formed panhandle PCR (SEFP-PCR) is an effective method for DNA walking to an unknown genomic region from a known sequence. SEFP-PCR was successful to isolate 1292 bp *rbcs* gene and its 5'upstream sequence of 1543 bp from *L. minor*.

SEFP-PCR combined the advantage of ligation-mediated PCR in its specificity and the advantage of panhandle PCR in its efficiency. Compared with the other existing PCR methods for walking, Self-formed panhandle PCR has many advantages: (i) High specificity; (ii) a high positive rate of a specific band; (iii) Long length of a walking step, theoretically,  $4^6$  bp (4096 bp) fragment can be obtained by SEFP PCR; and (iv) a high success rate to walk down in one direction.

The advantages of this method are the easy implementation of the procedure, the use of common

materials, the relatively few steps needed to amplify the target region, and the high level of specificity achieved in target sequence amplification.

The following aspects of Self-formed panhandle PCR that are different from normal panhandle PCR should be noted: (i) The DNA template concentration should be high to facilitate the creation of the panhandle adaptors; and (ii) Panhandle adaptor should be added at annealing temperature to improve its specificity.

For the general application of this method, it should be noted that the length and efficiency of walking to an unknown region depend on the restriction enzyme used and the frequency of restriction sites in the genomic DNA of the target organism.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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