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Full Length Research Paper

Effect of different concentrations of bush passion fruit pulp and temperature in the production of beer

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Among many species of passion fruit, the bush passion fruit (*Passiflora cincinnata Mast.)* is a wild, edible fruit, of exotic flavor and has good acceptability for consumption. This study aimed to determine the physico-chemical characteristics of the bush passion fruit pulp, and to produce and characterize physico- chemically the ale beer made with bush passion fruit as a malt adjunct, using different percentages (10, 29, 39 and 49%) of this adjunct and temperatures (15 and 22°C) to evaluate its fermentative potential for production of beer and to verify the influence of the soluble solids content and the specific gravity decrease during the fermentation. The results indicated that the bush passion fruit is suitable to carry out the fermentative process and the bench test media containing 29% of malt adjunct obtained best results in fermentation at both 15 and 22°C, with alcohol yields of 7.61 and 8.29%(v/v), respectively.

Key words: Beer, adjunct, bush passion fruit, fermentative potential.

INTRODUCTION

Biochemically, the fermentation is an energy production process from organic compounds like carbohydrates by microorganisms towards alcohols, organic acids and related catabolites. The process of fermentation in the food production generates various benefits, such as food conservation and security by production of compounds that inhibit the proliferation of other microorganisms including pathogens; increase of the nutritional value in addition to the improvement of the sensory quality of the product thanks to flavoring substances (Bourdichon et al., 2012).

Nowadays, biotechnology includes a wide range of different processes, which can be applied in nutrition and agriculture sectors (Carvalho et al., 2009). There is need of new processes and development allowing the use of fruits which are characteristic for particular geographic places of the world. Passion fruit is a delicacy from tropical regions due this tasteful and sugary mucilage around the seeds although the major part corresponds to the soft underskin pulp.

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Fermented fruits are promising products due to the tendency of high consumer acceptance. Traditionally, grapes and apples are used for fermented beverages. Many countries, mainly European, can produce fruit wines by manufacturing similar to processes made with the grape. Most of them use apple, pear, blackcurrant, raspberry or cherry. In tropical countries, there is a wide variety of fruits, such as orange, guava, pineapple, passion fruit and mango, which are in economic terms, poorly exploited for the production of beverages from fruit. Brazil has a wide variety of fruits with great potential to be exploited economically for the production of beverages.

The adjuncts for brewing can be defined as any different carbohydrate source from the barley malt which contributes fermentable sugars to the wort (Stewart, 2000).

Adjuncts used in beer production allow the reduction of costs of raw materials, since the barley produced in Brazil does not meet the demand. Therefore, the use of domestic raw materials reduces costs in the production of beer extract, however, this practice should not interfere with the beer quality, giving inadequate characteristics to the product or not providing conditions for the development of the features that are expected.

It is possible to find in Brazil many types of fruit for most of the year due to a great extent and the varied climate that allows the cultivation of tropical fruits as much fruits of temperate or cold climate.

The passion fruit is classified as a tropical fruit whose aroma and taste are highly appreciated by the Brazilian consumer. Brazil occupies the first position as a producer and consumer of passion fruit (Monteiro et al., 2005). There are about 400 tropical and subtropical species of passion fruit (*Passiflora*), more than 150 are native to Brazil, of which about 60 produce fruit that can be eaten raw or in the form of juices, soft drinks, sweets and liqueurs. The juice of the passion fruit is a source of ascorbic acid, characteristic this combined with the aroma and distinctive taste, allows many placement opportunities in the international market (Sato et al., 1992).

In the wide biodiversity of passion fruit, the bush passion fruit (*Passiflora cincinnata* Mast.) from Passifloraceae family stands out due to its edible and wild fruit, of exotic flavor and good acceptability for consumption. The fruit *in natura* is very appreciated by populations from northern Brazil. This fruit has potential functional components, such as fibers, vitamins, carotenoids and inorganic compounds (calcium, iron, phosphorus) flavonoids, steroids and fatty acids.

In this present study, the bush passion fruit pulp was characterized physico-chemically and it was evaluated for the production of improved beers with bush passion fruit, as malt adjunct, using different percentages of this adjunct and fermentation temperatures to verify the influence of soluble solids and specific gravity decrease during fermentation.

Figure 1. Bush passion fruit.

MATERIALS AND METHODS

Physico-chemical characterization of bush passion fruit pulp

The bush passion fruit (Figure 1) purchased at local fairs in the city of Feira de Santana, Bahia, Brazil, were selected, cleaned and processed to obtain the pulp by albedo, skin and seeds removing device (Macanuda brand, DBM-73 NR12 model). It is important to report that the pulp was made only from the mucilage. The pH was measured in digital apparatus, and soluble solids in a digital refractometer (Reichert brand, AR200 model).

Preparation of wort

The beer wort with original extract 12^{o}P (approximately 12^{o}Brix) and pH=5.0 was produced to obtain a clear *pilsen* beer in the pilot plant facilities, located at the Department of Technology of the Laboratory Fermentation of the State University of Feira de Santana, BA, Brazil. The bush passion fruit pulp with amount of soluble solids corrected was used as adjunct.

Sampling of the pulps

Pulp samples, previously filtered, were prepared to be fermented with yeast *Saccharomyces cerevisiae* Safale S-04, type *Ale* in different pulp percentages (10, 29, 39 and 49%), and at different temperatures (15 and 22°C) that were based on literature, in order to evaluate what percentage would produce higher amounts of ethanol in the same fermentation conditions and what would be the ideal temperature for fermentation. The samples were prepared with filtered fruit pulp. The amount of soluble solids content of the samples was corrected by sugaring process with commercial sucrose to the value of 12°Brix, value traditionally used in the industry in the production of conventional beer wort resulting in beer between 4 and 5% v ethanol/v. Following the same aim, the pH of the sample was adjusted to 5.0 using deacidification technique with $CaCO_3$ at a concentration in 100 mg/L. SO_2 was added in the form

of potassium metabisulphite ($K_2S_2O_5$), as antimicrobial, since the pulp was not pasteurized. The techniques of sugaring, deacidification and microbial decontamination were performed according to Carvalho et al. (2016).

Propagation of yeast

Before starting the fermentation, the yeasts were hydrated and propagated in wort according to the specifications of the manufacturer Fermentis, Division of S. I. Lesaffre. After the hydration, the propagation of yeast was performed by adding hydrated yeast in an Erlenmeyer flask containing wort and it was incubated at 30°C for 24 h in a rotary shaker at 150 rpm, in Shaker ACB Labor.

Wort fermentation

In order to begin a fermentative process, a volume of 10% of the total volume to be fermented was removed from the propagation and added in the mixture of wort and adjunct previously prepared to ferment and with content of soluble solids and pH adjusted.

The fermentations with different percentages of adjunct (10, 29, 39 and 49%) added to the wort were conducted in Erlenmeyer flasks of 500 mL containing 250 mL of the mixture of wort, adjunct and propagation, placed in biochemical oxygen demand (BOD) incubator, Quimis Greenhouse Incubator brand, Q315M25 model, where the temperature was stabilized at 15 and 22°C. Every 12 h, samples were collected to carry out the analytical monitoring of the fermentation. The total time of fermentation was 120 h.

After the fermentation, the samples were kept for maturation, performed in a BOD incubator, stabilizing the temperature in 10° below the fermentation temperature (12°C for fermentation at 22 and 5°C for fermentation at 15°C), in order to clarify the beer. The total time of maturation was 360 h.

Determination of apparent extract, ethanol concentration and specific gravity

The values of specific gravity (g/mL), which correspond to relative density; apparent extract (°Plato), which is the extract measured during the fermentative process, and alcohol (% v/v) were determined through DDM 2911 bench densitometer from Rudolph Analytical Research. For transformation of the ethanol concentration of % v/v to g/L the following equation was used (EBC, 2005):

$$Ethanol\,(g/L) = Ethanol\,(\%\,v/v)\;x\;0{,}789\;x\;\rho$$

For transformation of apparent extract content of °Plato to g / L, the following equation was used:

$$App.Extr.\,(g/L) = app.extr.\,(°P)\;x\;\rho\;x\;10$$

Where: 0.789 g/cm^3 is the specific gravity of ethanol at 20°C and ρ is the wort specific gravity (g/mL).

Statistical analysis

The results obtained in the determinations in triplicate of soluble solid content, specific gravity, ethanol content and apparent extract of the last point of fermentation, were submitted to statistical analysis, being performed the analysis of variance by F test and comparisons between the means by the Tukey test at 5% level of probability.

Table 1. Physico-chemical analysis of the bush passion fruit pulp.

Physico-chemical analysis	Bush passion fruit
Soluble solids (°Brix), at 20ºC (TSS)	10.06 ± 0.15
pH	2.85 ± 0.11

TSS, Total soluble solids.

RESULTS AND DISCUSSION

Physico-chemical characterization of bush passion fruit pulp

The physico-chemical characteristics of bush passion fruit pulp were evaluated in order to analyze whether it is appropriate to start the procedures for the preparation of a beer using it as adjunct. The results of the physico-chemical analysis pulp are shown in Table 1.

The results obtained for physico-chemical characterization of bush passion fruit pulp revealed the potential of this fruit for the brewing industry as a malt adjunct.

In relation to the chemical characteristics of the bush passion fruit, it was observed that the average value of pH was 2.85. The determination of the pH is very important because it is necessary to verify whether the microorganism used in fermentation resists the fed substrate pH.

The total soluble solids content presented an average of 10.06 °Brix in *P. cincinnata* Mast., fruit. The amount of soluble solids is used to indicate the degree of ripeness of the fruit, the lower this degree, the pulp or juice will be more acid, and less sweet, that is, the higher the degree, the higher amount of sugars in the fruit, what in this case is a positive factor for fermentation.

The found physico-chemical characteristics demonstrated passion fruit pulp potential for industrial use as a barley malt adjunct and for *in natura* consumption; it is an already explored alternative to the juice industry due to the exotic flavor and sour sweetness.

Analytical monitoring of the fermentation process

During fermentation, samples were taken periodically every 12 h for monitoring the fermentation process. The final values of the variables are shown in Tables 2 and 3. The results obtained in the determinations in triplicate of soluble solid content, specific gravity, ethanol content and apparent extract of the last point of fermentation, were submitted to statistical analysis, being performed the analysis of variance by F test and comparisons between the means by the Tukey test at 5% level of probability. It can be seen that the samples did not show statistically significant differences in values for the mean concentration of soluble solids, specific gravity, ethanol

Table 2. Values of soluble solids, specific gravity, alcohol content and apparent extract for fermentations conducted at 15ºC.

Adjunct (%)	TSS (°Brix)	Specific gravity (g/mL)	Álcohol (% v/v)	Álcohol (g/L)	Apparent extract (g/L)
10	2.6[a]±0.01	1.0101[a]±0.02	7.47	58.94[a]±0.01	26.26[a]±0.02
29	2.3[a]±0.02	1.0090[b]±0.01	7.61	60.04[b]±0.02	24.72[b]±0.02
39	2.4[a]±0.00	1.0094[b]±0.01	7.56	59.65[a]±0.01	25.23[b]±0.03
49	2.7[a]±0.01	1.0105[a]±0.00	7.52	59.33[a]±0.03	27.28[a]±0.01

*****Media followed by same letter in a column did not differ statistically among themselves by the Tukey test at 5% probability. TSS, Total soluble solids.

Table 3. Values of soluble solids, specific gravity, alcohol content and apparent extract for fermentations conducted at 22°C.

Adjunct (%)	TSS (°Brix)	Specific gravity (g/mL)	Álcohol (% v/v)	Álcohol (g/L)	Apparent extract (g/L)
10	2.0[a]±0.02	1.0078c±0.00	7.79	61.46[b]±0.00	21.87[c]±0.01
29	1.2[b]±0.03	1.0047[d]±0.01	8.29	65.41[c]±0.02	14.07[d]±0.00
39	1.3[b]±0.01	1.0050[d]±0.02	8.25	65.09[c]±0.02	14.47[d]±0.02
49	1.3[b]±0.01	1.0050[d]±0.01	8.22	64.85[c]±0.02	15.08[d]±0.03

*****Media followed by same letter in a column did not differ statistically among themselves by the Tukey Test at 5% probability.

content and apparent extract, after fermentation and maturation.

Tables 2 and 3 showed the concentration of soluble solids, specific gravity, alcohol content and apparent extract to different percentages of malt adjunct and temperatures of 15 and 22°C .It can be seen that there was higher consumption of the fermentation substrate when it was used 29% of bush passion fruit pulp under the same conditions of fermentations for others adjuncts percentages. The substrate consumption can be observed through the values of content of soluble solids, which initiated the fermentation in about 12°Brix and in the end of fermentation, it was 2.3°Brix in experiments at 15°C and 1.2°Brix in the experiments at 22°C. From these values it emphasizes the value of the alcohol content, 7.61 and 8.29% (v/v) for tests at 15 and 22°C, respectively.

In 120 h of fermentation, the yeasts produced 60.04 g/L of ethanol (7.61% v/v) in the test using 29% of adjunct to 22°C, consuming 14.07 g /L (92.7%) of the initial apparent extract while that the yeasts in the test using 49% adjunct at 15°C produced 59.33 g/L (7.52% v/v) alcohol, with a consumption of 27.28 g/L (85.8%) of initial apparent extract. The results showed that the increase of amount of adjunct does not favor the increase of ethanol production during fermentation. The initial sugar concentration of worts in relation to study in question may have contributed to the high production of ethanol, since it contained an original extract of 16.5°P (12 °Brix). The increase in ethanol production with a high initial substrate concentration in the beer wort was verified by Dragone et al. (2003). Most beers produced worldwide has an alcohol content of around 3 to 6% (v/v), while a "weaker" beer contains about 2 to 3% alcohol, an "average" beer is about 5% and a "strong" beer is between 6 to 12% v/v alcohol (Sohrabvandi et al., 2011). Thus, the bush passion fruit is able to produce beers with high alcohol content.

The incomplete fermentation observed in the tests may result from lack of nutrients in the medium provided by the high concentration of adjunct. The majority of apparent initial extract may have been used by yeast to generate secondary fermentation products such as glycerol, organic acids, acetaldehyde, acetoin, butylene and others.

Reduction of specific gravity and cell growth

In the fermentations of worts with bush passion fruit pulp they were also studied the substrates reduction by decrease of the wort specific gravity and the cell concentration during fermentation. The specific gravity of the wort decreased with the production of ethanol, which has specific gravity of 0.789 g/L, by consumption of sugars such as glucose (specific gravity 1.54 g/L), fructose (specific gravity 1.69 g/L) maltose (specific gravity 1.54 g/L) and sucrose (specific gravity 1.59 g/L) for yeast and also by formation of volatile CO_2.

The graphical representations in Figures 2 to 5 showed that from the same specific gravity values, 1.0484 g/mL for all samples in the fermentations conducted at 15°C, at the end of fermentation (120 hours), there was a percent reduction of the specific gravity of test 2 (1.0090 g/mL), which was lower than of the other tests according to Figure 3 showing the in same conditions the yeast *Saccharomyces cerevisiae* Safale S-04 type *Ale* produced more ethanol with 29% of adjunct.

They can be observed the graphic representations of

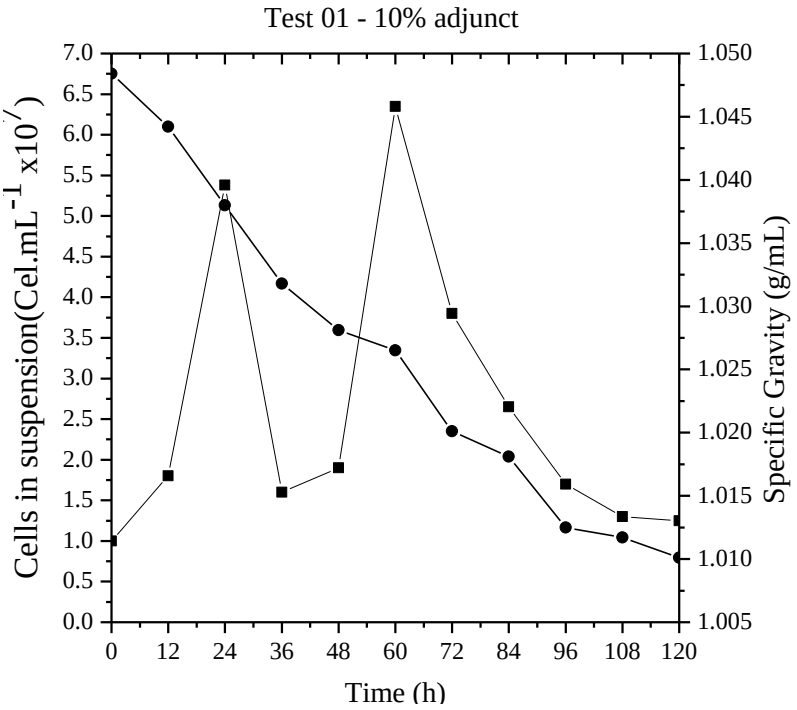

Figure 2. Graphical representation of the cell concentration in suspension (cel/mL) (▪) and specific gravity decrease (g/mL) (•) during the fermentation of the test with 10% of adjunct at 15°C.

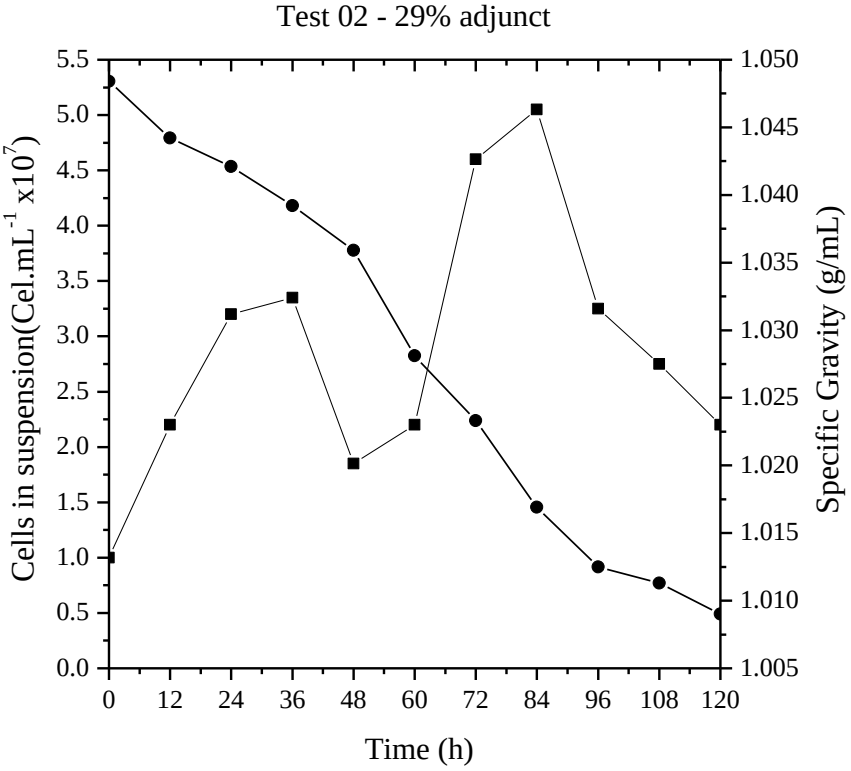

Figure 3. Graphical representation of the cell concentration in suspension (cel/mL) (▪) and specific gravity decrease (g/mL) (•) during the fermentation of the test with 29% of adjunct at 15°C.

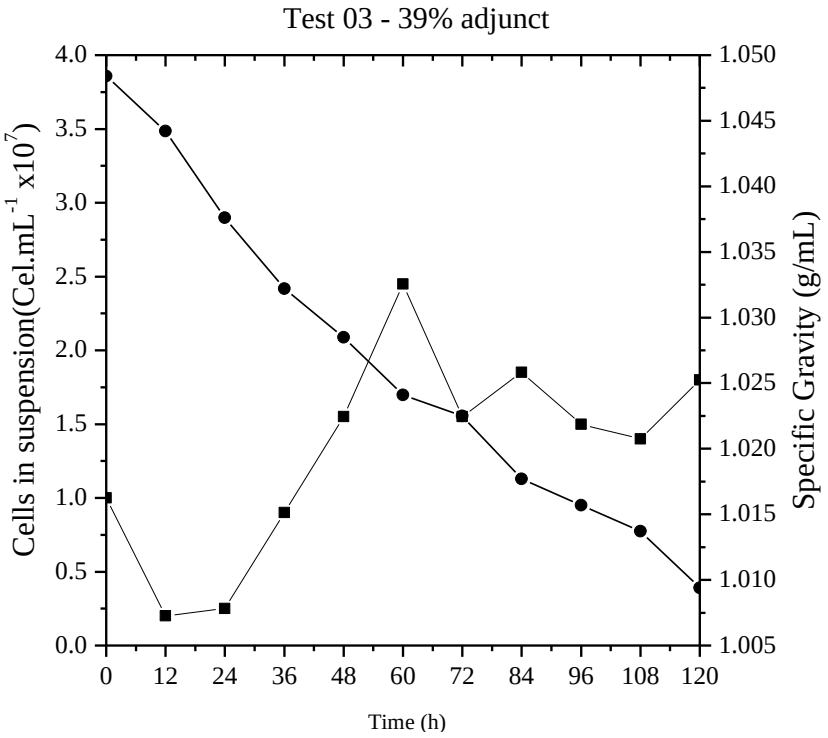

Figure 4. Graphical representation of the cell concentration in suspension (cel/mL) (▪) and specific gravity decrease (g/mL) (•) during the fermentation of the test with 39% of adjunct at 15°C.

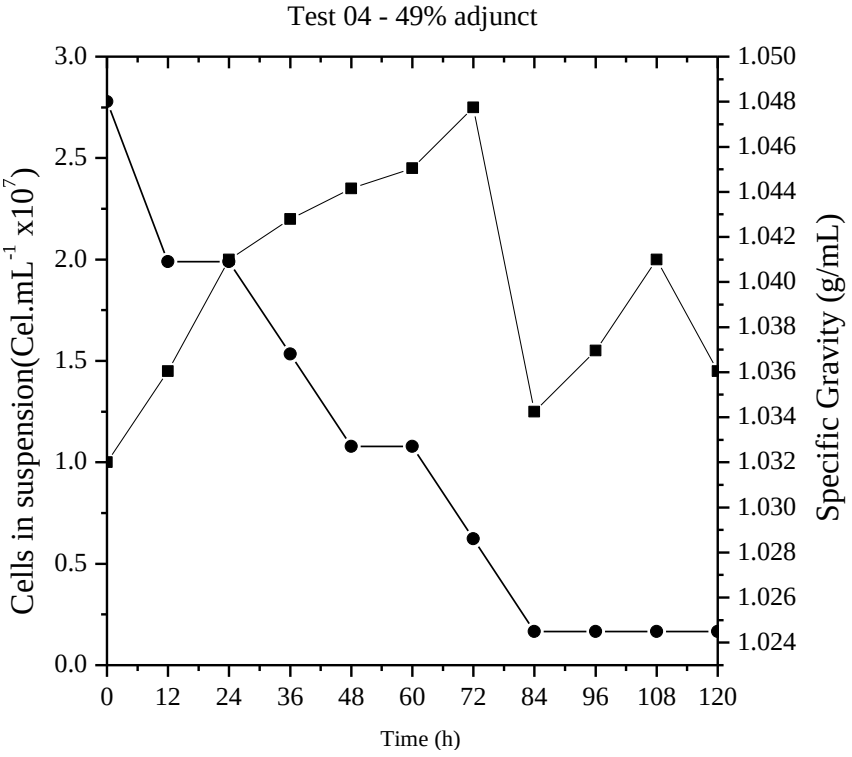

Figure 5. Representation of the cell concentration in suspension (cel/mL) (▪) and specific gravity decrease (g/mL) (•) during the fermentation of the test with 49% of adjunct at 15°C.

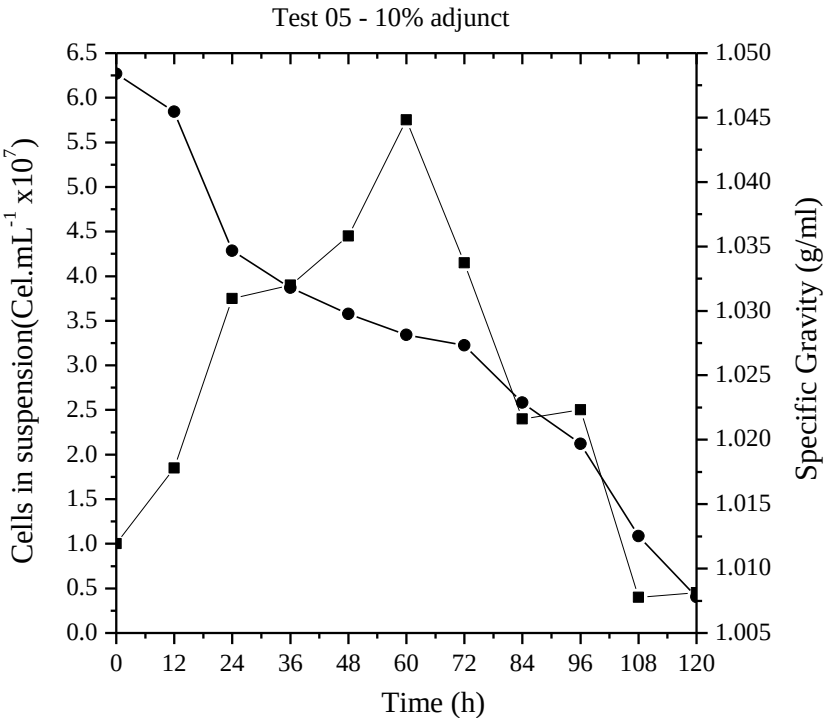

Figure 6. Graphical representation of the cell concentration in suspension (cel/mL) (▪) e specific gravity decrease (g/mL) (•) during the fermentation of the test with 10% of adjunct at 22°C.

the cell concentration obtained by cell count in Neubauer chamber for different adjuncts percentages (10, 29, 39 and 49%) and at temperatures of 15 and 22°C. In the experiments conducted at 15°C it is observed that the yeasts reached maximum cell concentration after 60 h of fermentation, obtaining 6.35×10^7 (cells/mL) by method of cell viability in the test using 10% of bush passion fruit pulp (Figure 2). In the test using 29% pulp, it is observed that yeasts reached a maximum cell concentration after 84 h of fermentation, obtaining 5.05×10^7 (cells/mL), as shown in Figure 3. In the test using 39% of pulp, the yeasts reached a maximum cell concentration after 60 h of fermentation, obtaining 2.45×10^7 (cell /mL), according to Figure 4. And in the test using 49% of pulp, the yeasts reached a maximum cell concentration after 72 h fermentation, obtaining 2.75×10^7(cells/mL) (Figure 5). It is also possible to notice that from these times the yeasts began to enter in the impracticability phase (or the death phase).

The efficiency of fermentation and the profile of the end product quality are closely linked to the amount and health of the used yeast (Briggs et al., 2004). So, evaluating and predicting the viability of the cell, and the yeast performance during fermentation phase is an important condition (Carvalho et al., 2009).

It can be observed that during the first hours of fermentation in all tests, the number of viable cells increases and then decreases quickly. This behavior can be explained by the flocculation, a reversible process, it makes some yeast cells adhere to each other forming aggregates (Guido, 2003). According Dequim (2001), flocculation, in beer production, is important to obtain a clear product and good aroma.

In the same way of the experiments performed at 15°C, from the experiments conducted to 22°C it was observed that for fermentation with 10% of adjunct, the yeasts reached a maximum cell concentration after 60 h, obtaining 5.75×10^7 (cel/mL) (Figure 6). In the fermentation using 29% adjunct, after 72 h, the yeasts reached a maximum cell concentration corresponded to 13.5×10^7 (cells/mL), according to Figure 7. In the test using 39% adjunct, the yeasts reached a maximum cell concentration after 84 h, obtaining 20.0×10^7 (cells/mL), as shown in Figure 8. In the test using 49% of adjunct, the yeasts reached a maximum concentration after 72 h of fermentation, obtaining 16.5×10^7 (cells/mL) (Figure 9). It can also be noticed in the graphs that from these times, the yeasts begin to enter in the impracticability phase.

As well as the tests performed at15°C, the graphs in Figures 5 to 8 showed that, starting from specific gravity values equal to 1.0484 g/mL in fermentations conducted at 22°C, at the end of fermentation (120 h) there was a percentage reduction in the specific gravity of the test 6 (1.0047 g/mL) (Figure 6), which was lower than that of test 5 (1.0078 g/mL) (Figure 5), and of tests 7 and 8 (1.0050 g/mL) (Figure 7 and 8, respectively), showing

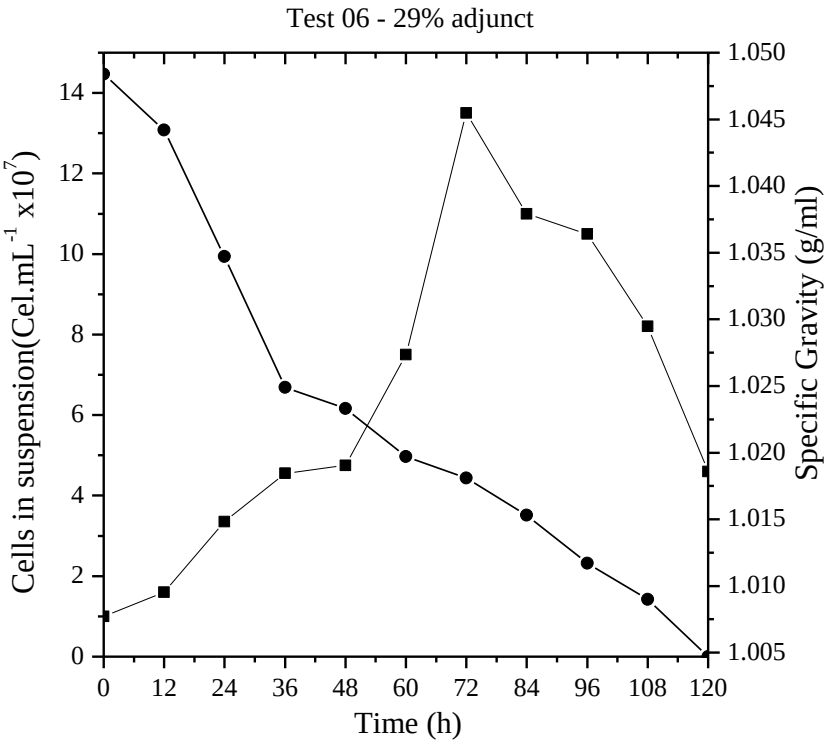

Figure 7. Graphical representation of the cell concentration in suspension (cel/mL) (▪) and specific gravity decrease (g/mL) (•) during the fermentation of the test with 29% of adjunct at 22°C.

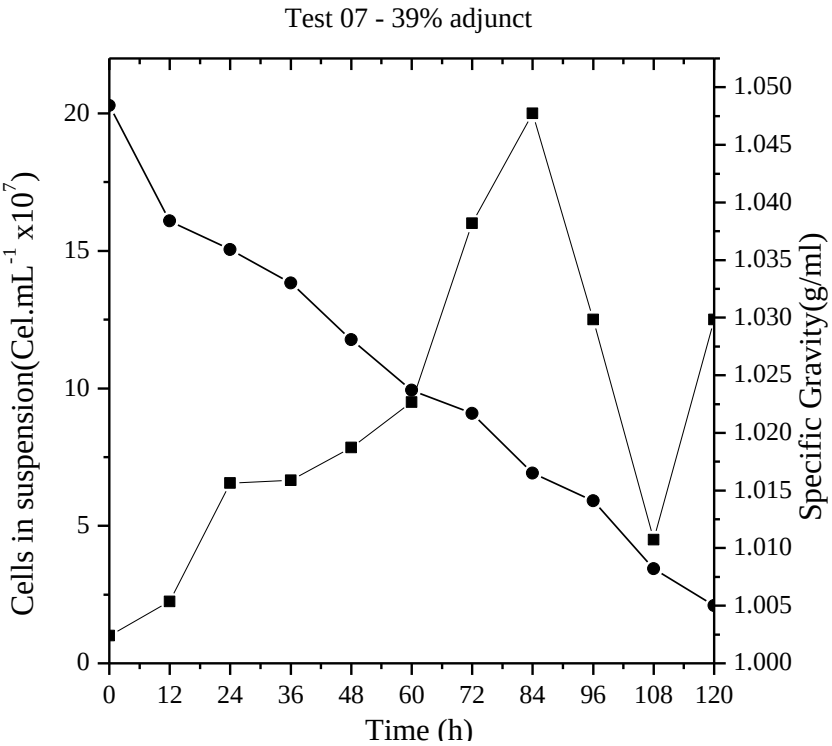

Figure 8. Graphical representation of the cell concentration in suspension (cel/mL) (▪) and specific gravity decrease (g/mL) (•) during the fermentation of the test with 39% of adjunct at 22°C.

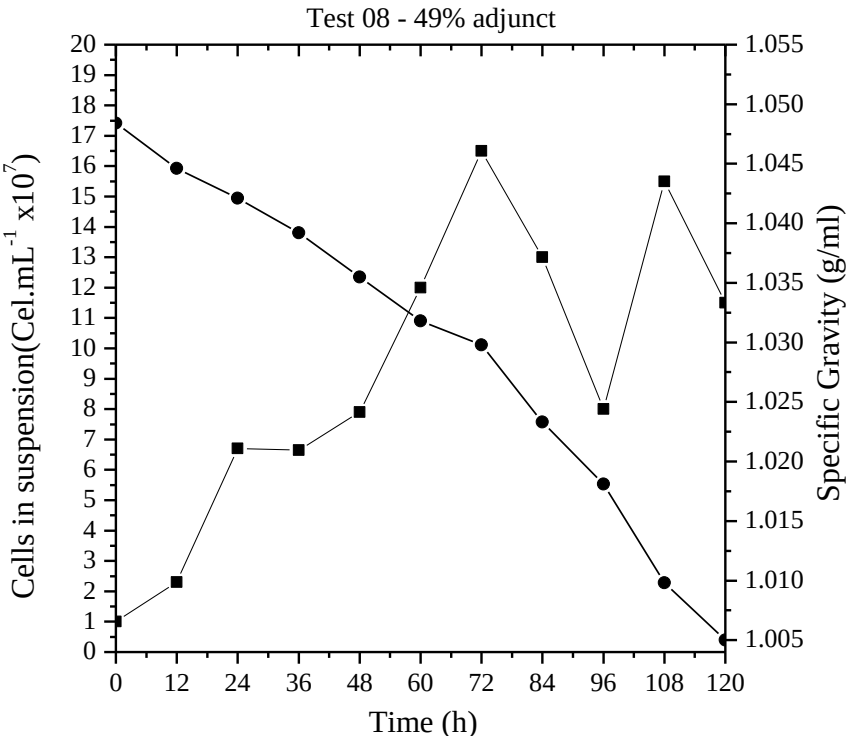

Figure 9. Graphical representation of the cell concentration in suspension (cel/mL) (▪) and specific gravity decrease (g/mL) (•) during the fermentation of the test with 49% of adjunct at 22°C.

once again that in the same conditions, the yeast *S. cerevisiae* Safale S-04 type *Ale* produced more ethanol with 29% adjunct.

This work demonstrates that the bush passion fruit has favorable characteristics that can be used in the fermentation of the beer. The increase of adjunct amount (39 and 49%) did not favor the fermentation of the wort, between different temperatures and adjunct percentages used, the wort containing 29% of bush passion fruit pulp conducted at 22°C showed better values of ethanol production (8.29% (v/v)), consuming 14.07 g/L (92.7%) of the apparent initial extract, and to have better cell growth and reduced specific gravity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Association of growth hormone gene polymorphism with quantitative characteristics of thin-tailed sheep using PCR-RFLP in Jambi province

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The objective of this research was to obtain quantitative growth hormone gene polymorphism association between growth hormone gene genotype with quantitative characteristics of the thin-tail sheep in the highlands and lowlands of Jambi Province. Two phases of research were done on the field and in the laboratory. Field research which was conducted to obtain the quantitative characteristics data includes; withers height (WH), body length (BL), chest grid (ChG), chest depth (ChD), chest width (ChW), body weight (BW) and body weight gain (BWG). Laboratory research included: DNA isolation, amplification and gel purification, characterization and identification using PCR-RFLP with the MspI, and AluI. Quantitative characteristics data was collected from 240 heads of thin-tailed sheep. Blood samples were collected from 160 of them and all were between the ages of 1 and 2 years (I1 = pair of permanent teeth). Field research was conducted in the Kerinci District and Sungai Penuh City (Highlands) and Muara Jambi Districts and Batanghari Districts (lowlands). The purposive sampling technique used in this research revealed that: 1) quantitative characteristics (BW, BWG, BL, ChG, ChD, and ChW) of thin-tailed sheep both male and female in highland were better than in the lowlands; 2) studied locus was polymorphic on the highlands and lowlands; 3) the highest of genotype and allele frequencies of both highlands and lowlands were related to the genotype (+/+) and allele (+), respectively; 4) the highest quantitative characteristic (BW BW, BWG, BL, ChG, ChD, and ChW) of thin-tailed sheep was genotype (+/+); and 5) the diversity of GH genes MspI and AluI were associated with BW, BWG, BL, ChG, ChD, and ChW of thin-tailed sheep both on the highlands and lowlands of Jambi Province.

Key words: Characterization, local sheep, thin-tailed sheep, diversity, growth hormone (GH) gene, lowlands, highlands, PCR-RFLP.

INTRODUCTION

Small ruminants, especially native breed types, play an important role in the livelihoods of a considerable part of human population in the tropics from socio-economic aspects. Thus, integrated attempt in terms of management and genetic improvement to enhance production is of crucial importance (Mohammadabadi and Sattayimokhtari, 2013). Economical and biological efficiency of sheep production enterprises generally

improves by increasing productivity and reproductive performance of ewes. There is a fairly even spread of thin-tailed sheep in the Jambi Province ranging from lowlands to highlands. They have development potential because they adapt to various environmental conditions. High demand for meat is not followed by an increase in population, this thus leads to a gap between production and demand for meat between 2010 to 2014. There was population increase of only 3.84% per year, while demand (slaughter) increased by an average of 6.93% per year (Department of Animal Husbandry and Animal Health, Jambi Province, 2014). These conditions led to decrease in population of TTS in Jambi Province and consequently extinction. Approximately 30% of the original animal world is categorized as tending to extinction.

One effort to preserve TTS is to find the database through quantitative characterization of the characters that have economic value. However, quantitative characterization of the characters are generally less effective because it requires a large number of animals, take a long time and recording must be complete. Advances in science and technology fields of molecular genetics and molecular biology with genome sheep from time to time (Crawford et al., 1995) are expected to contribute significantly to the progress and development of animal husbandry in particular breeding program.

Applications of molecular genetics have many important advantages. One such significant advantage is the genotyping of individuals for specific genetic loci (Javanmard et al., 2008; Mousavizadeh et al., 2009). The genes that affect a polygenic trait are not exactly known, however a number of candidate genes with major effects have been recognized. In candidate gene approach to identify genes responsible for variation in a polygenic trait, the process is selection of candidate genes based on the relationship between physiological or biochemical processes involved in the expression of the phenotype then testing the selected genes as putative quantitative trait loci (QTL) (Mousavizadeh et al., 2009). Furthermore, the study of native breeds is necessary for conservation of genetic resource in livestock (Mohammadi et al., 2009) and a species without enough genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites. In addition, the ability of a population to respond adaptively to environmental changes depends on its level of genetic variability or diversity (Khodabakhshzadeh et al., 2016a, b).

Characterization of genetic diversity associated that the production traits that is related to economic traits such as growth can be done through in-depth analysis of the structural genes or other parts which are crucial for the growth of livestock such as growth hormone (GH) gene (Di Stasio et al., 2005; Kumari et al., 2014). GH gene has been used as a candidate gene in finding a link between genotype with phenotype in several species, including Bos taurus and Bos indicus (Ge et al., 2003; Beauchemin et al., 2006; Mohammadabadi et al., 2010), goat (Mousavizadeh et al., 2009; Mohammadabadi, 2012) and sheep (Honarvar et al., 2012; Ghazi et al., 2014). The presence of polymorphisms in the GH gene is also associated with production properties (Pereira et al., 2005), and carcass weight (Beauchemin et al., 2006).

GH gene is the controller of the existence and growth properties polymorphism important in supporting selection of the nature of the growth. It is interesting to study the existence and diversity of GH gene associated with quantitative characteristics of the thin-tailed sheep in the province of Jambi. Is diversity a difference that is due to differences in GH gene owned or variety of environmental variation? The answer to this question is necessary in order to study molecular genetics at present and in the future.

Evaluation of GH gene polymorphism need to be done to encourage selection of the growth traits, especially associated with the quantitative characteristics of the thin tail sheep (TTS) in Jambi Province, whether the difference is due to differences in diversity of GH gene or of environmental variation.

The objectives of this research were to investigate the quantitative characteristics of local TTS, TTS growth hormone gene polymorphism and linkage relationship between polymorphism of growth hormone and quantitative characteristics of TTS at highlands and lowlands in Jambi Province.

MATERIALS AND METHODS

The research material was local thin tail sheep (TTS) in the highlands and lowlands in Jambi Province. Quantitative characteristics data were collected from 240 heads of TTS. Blood samples were collected from 160 sheep between age 1 and 2 years old (I1 = a pair of permanent teeth). Research was done in the Kerinci District, Sungai Penuh City (Highlands), Muara Jambi Districts and Batanghari Districs (lowland).

Data were collected quantitatively using the following characteristics : withers height (WH), the body length (BL), chest grid (ChG), the chest depth (ChD), chest width (ChW), body weight (BW), body weight gain (BWG) and blood sample were collected in all location. Blood sampling of TTS was taken through the jugular vein with no heparin venoject vacuum tubes. The Blood samples were then preserved with absolute ethanol. Thereafter, absolute ethanol in the ratio 1:1 was added to the blood samples and stored at room temperature. Thereafter, blood samples were added to absolute ethanol in the ratio 1: 1 and stored at room temperature. The observed variables associated with DNA analysis of GH gene fragment of MspI and AluI included: (1) the frequency of the gene, (2) GH's gene allele obtained from the analysis of PCR-RFLP MspI and AluI, (3) the balance of genes in the population, (4) heterozygosity, (4) the value of Polymorphic Informative Content (PIC), (5) the presence or absence of mutations, (6) homology of GH's gene

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Table 1. The length and location of *the GH* gene and primers used for PCR analysis.

Position of the segment	Long (Bp)	Primers	Sequence (5 '3')	Annealing temperature (°C)
175-774	579	GHY1-Fwd.	5 'CAT TTG AAA ATA TGT GAG CAC ACA G 3'	60.3
		GHY1-Rev.	5 'CCC CAC CTC TAG GAC ACA TC 3'	
671-1361	690	GHY2-Fwd.	5 'CTG TTT GCT GTG GCC AAC 3'	60.3
		GHY2-Rev.	5 'CCA AAG AAG CGA CTG GAT GA 3'	
1248-1927	679	GHY3-Fwd.	5 'CCG AGG CAG CAG ACA TTG 3'	62.5
		GHY3-Rev.	5 'GAA CAT GCG GCG CTT ACA	

sequence, and (7) linkage of GH's gene genotype and quantitative trait (8) the relationship between GH's gene and quantitative characteristics.

Purposive sampling in survey method was applied on field study; in the laboratory,the polymorphism of GH gene was detected with PCR-RFLP technique.GH gene amplification, which has further isolated DNA was amplified using 3 pairs of primer: the length of each primer was 579 , 690 and 679 bp. They were located in E1-E5 and I1-I5. More details of the primers used are presented in Table 1.

Amplification begins with denaturation at 94°C for 2 min, followed by 40 amplification cycles; each cycle was programmed for 30 min at 94°C denaturation, annealing of 62°C for 80 s, and extension of 72°C for 90 s. The amplification process ends with a final extension at 72°C temperature for 5 min. Amplification results can be viewed by performing electrophoresis with agarose of 2%, which is colored with ethidium bromide. Furthermore, the bands are visible on gel formed in each groove of wells containing DNA samples of PCR products. Determination of the size of each fragment of GH was formed on agarose gel by comparing the position of band formed by positioning the ladder of DNA bands. DNA is visualized, documented by Gel Documentation system (Biometra-German) and then the picture was taken and stored on Compact Disc.

For the polymorphism detected by PCR-RFLP, the PCR amplification products obtained were then digested with AluI restriction enzyme cutting sites AG*CT and MspI cutting site C*CGG (Promega). Total volume for the digestion consists of 50 ml of nuclease free water (ddH_2O) (17.5 ml), 25 ml PCR product, 5 ml enzyme buffer, enzyme MspI or AluI (2.5 ml). This mixture was then incubated for approximately 12 h, and then migrated to agarose gel (2%) by ethidium bromide. Furthermore, it went through electrophoresis, which was done with Thermo Scientific models A5, power supply of EV 231 Consort, USA, of 100 V, 74 mA for 2 h. It was also further examined by gel documentation system (Biometra-German), photographed and stored on Compact Disc.

In the results of the sequence of GH's gene fragment of thin-tailed sheep, its similarity (homology) was analyzed with the sequence contained in GenBank using software (software) computer programs Basic Local Alignment Search Tool (BLAST) (https: //blast.ncbi.nlm.nih. Gov / BLAST. cgi, 2015),which ensures that GH's gene fragment sequence is analyzed. BLAST results had the highest similarity of at least 97%. Furthermore, alignment is done to look for mutations, deletions and insertions in order to obtain similarity (homology) by using software (software) ClustalW computer (http://www.ebi.ac.uk/Tools/ MSA / clustalw2, 2015). Genotype frequencies from PCR-RFLP assumed from the combination of different alleles produced based on the presence or absence of one or more sites if the ribbon is cut, marked (-/-), if cut off all (+ /+) and if the number of truncated and not cut (+/-) (Kumari et al., 2014) (https://www.ncbi.nlm.nih.gov/probe/docs/, 2015).

Genotype frequency was calculated based on the number of alleles of a genotype divided by the number of samples. Allele frequency is calculated by summing all alleles divided by 2N. GH gene allele frequencies derived from the analysis of PCR-RFLP MspI were calculated using the formula (Nei, 1987). The genetic diversity (genetic variability) is done through observation of the estimated frequency of heterozygosity (Ho), heterozygosity expectations (Hi) and standard error heterozigous expectations (Weir, 1996; Nei, 1987). An allele informative level is calculated using a value approach informative polymorphic content (PIC) (Botstein et al., 1980). Hardy-Weinberg Equilibrium was tested with *Chi-square* ($X^{2)}$ (Hartl and Clark, 1997). Analysis of the influence of the GH gene genotype weight gain, body weight and quantitative characteristics was performed by t-test (Gasper, 2006). PCR products were used for sequencing amplification product nukleotid, using the method of Sanger et al. (1977), with the recommended procedure of Sambrook and Russel (2001). Results sequences were obtained and the variability between the highlands and lowlands was tested using DNA Star program.

RESULTS

Phenotype characteristics

Average quantitative characteristics of DET (both males and females) in the highlands and lowlands of Jambi Province are presented in Table 2. In Table 2, the average of all the quantitative traits of both males and females in the highlands (Kerinci distric and Sungai Penuh city) was better than that of the lowlands (district Muaro Jambi and Batanghari). T-test showed that quantitative characteristics of thin-tailed sheep in the highlands were significantly different ($P < 0.05$) from those of the lowlands in Jambi Province.

DNA isolation and amplification

The concentration of DNA isolation makes highlands and lowlands vary from 100 to 400 ng/ml. High and low concentrations of DNA produced are highly dependent on the ability of the cell nucleus to lyse. Yurnalis et al. (2013) state that if the nucleus of the cell lysis process can run well, then the resulting DNA concentration is high enough and the quality of the DNA will be good or otherwise. PCR results performed to amplify DNA in the highlands and lowlands showed that the length of the fragments was similar in each primary GHY1, GHY2 and GHY3 that are 599, 690 and 679 bp, respectively. More detail is shown in Figure 1.

Table 2. Mean quantitative characteristics of the thin tail Local sheep males and females at highlands and lowlands in Province of Jambi.

Site	Karakteristik Kuantitatif						
	BW	BWG	BL	WH	ChG	ChD	ChW
Highland							
Male	20.24±2.44	75.67±13.12	56.80±2.56	53.41±2.40	62.57±2.17	24.45±2.54	14.90±2.44
Female	18.08±2.65	51.28±16.26	54.50±2.69	52.57±2.76	61.35±2.56	21.89±2.38	14.65±2.44
Lowland							
Male	18.69±3.06	60.89±16.96	53.25±3.00	50.25±2.82	59.30±1.92	20.56±2.34	12.11±2.36
Female	16.01±3.67	36.50±15.69	51.00±2.71	49.68±2.48	58.46±1.82	19.10±2.12	11.33±2.04

BWG = body weight gain; BW = weight; BL = body length; WH = wither high; ChG = chest circumference; ChD = chestdeep and the ChW = chest width.

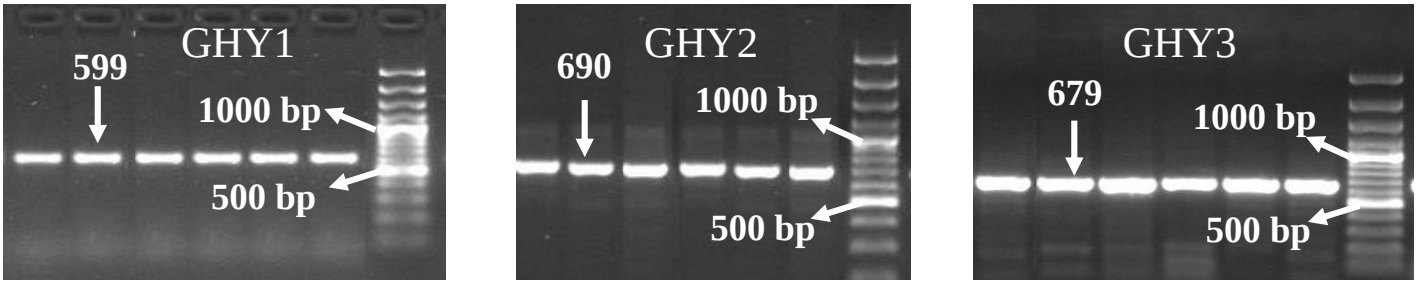

Figure 1. Result of PCR using primer GHY1, GHY2 and GHY3.

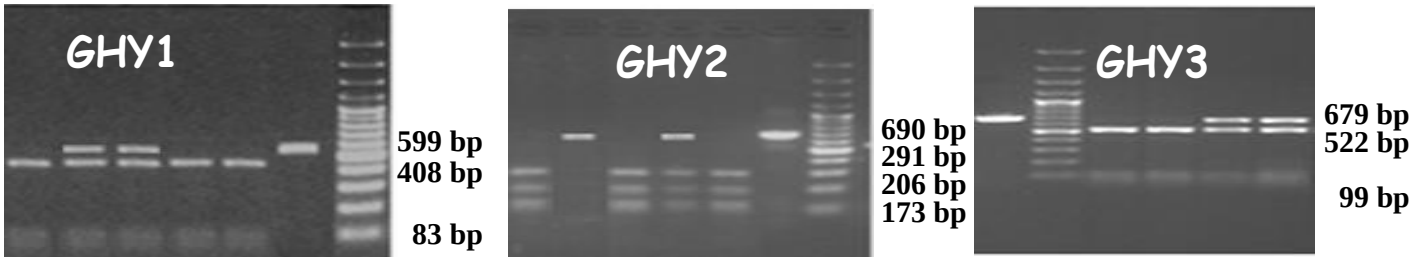

Figure 2. Results of electrophoresis of PCR-RFLP on growth hormone gene of thin-tailed sheep using enzyme MspI at Primary GHY1, GHY2 and GHY3 in the highlands and lowlands.

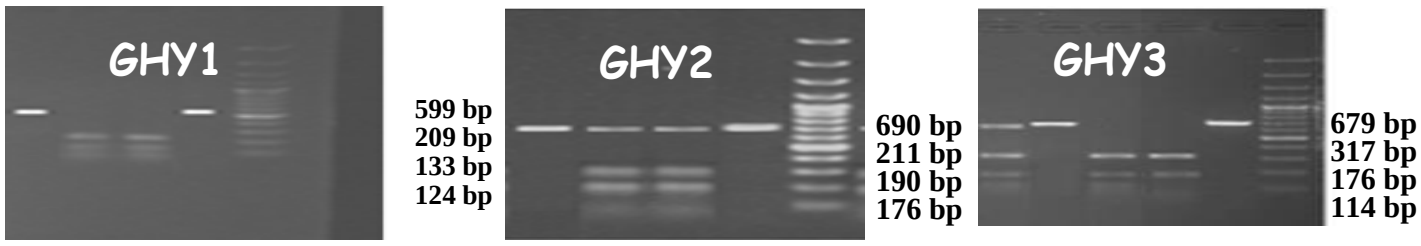

Figure 3. Results of electrophoresis of PCR-RFLP on growth hormone gene of thin-tailed sheep using enzyme *Alu*I at Primary GHY1, GHY2 and GHY3 in the highlands and lowlands.

Results of electrophoresis of PCR-RFLP on growth hormone gene of thin-tailed sheep in the highlands and the lowlands using MspI, and AluI enzymes showed the same results in each primary GHY1, GHY2 and GHY3. More details are presented in Figures 2 and 3.This result showed that there was no difference in band number of GH

Table 3. GH gene MspI genotype frequencies and AluI TTS at a lowland and highland in Province of Jambi.

Loction/Enzyme	No. of sample	Genotype	Genotype frequency	Alelle frequency
Highlands		**+/+**	132 (0.5500)	**+**= 0.6938
*Msp*I	240	**+/-**	69 (0.2875)	**-**= 0.3063
		-/-	39 (0.1625)	
Lowlands		**+/+**	126 (0.5250)	**+**= 0.6625
*Msp*I	240	**+/-**	66 (0.2750)	**-**= 0.3375
		-/-	48 (0.2000)	
Highlands		**+/+**	168 (0.7000)	**+** = 0.8125
*Alu*I	240	**+/-**	54 (0.2250)	**-** = 0.1875
		-/-	18 (0.0750)	
Lowlands		**+/+**	147 (0.6125)	**+** = 0.7125
*Alu*I	240	**+/-**	48 (0.2000)	**-** = 0.2875
		-/-	45 (0.1875)	

Table 4. Values of observed heterozygosity (Ho) and expectation heterozygosity (He) of GH gene of PCR-RFLP *Msp*I dan *Alu*I.

Site	n	PCR-RFLP marker	Heterozygosity	
			Ho	He
Highlands	240	*MspI*	0.2875	0.4257
Lowlands	240	*MspI*	0.2750	0.4481
Highlands	240	*AluI*	0.2250	0.3053
Lowlands	240	*AluI*	0.3250	0.4015

gene on restriction point CC*GG and AG*CT in both locations.

Genotype frequencies, allele, Hard-Weinberg equilibrium, estimation of heterozygosity and PIC

Polymorphism or genetic diversity can be determined by analysis of the frequency of genotype and allele frequencies. Results of the cutting with the enzyme MspI at GH gene fragment in both the highlands and lowlands of genotype frequency (+/+) were higher than genotype frequency (+/-) and (-/-). In the highlands the obtained genotype frequencies were (+/+) 0.5500, (+/-) 0.2875 and (-/-) 0.1625 to the frequency of allele (+) 69.38 and (-) 30.63%. In the lowlands, obtained genotype frequencies were (+/+) 0.5250, (+/-) 0.2750 and (-/-) 0.2000 to the frequency of allele (+) 66.25 and (-) 33.75%.

Results cutting with the enzyme AluI for the GH gene fragment in both the highlands and the lowlands genotype frequency (+/+) were higher than the genotype frequency (+/-) and (-/-). In the highlands, obtained genotype frequencies were (+/+) 0.7000, (+/-) 0.2250 and (-/-) 0.0750 to the frequency of allele (+) 81.25% and (-) 18.75%. In the lowlands, the obtained genotype frequencies were (+/+) 0.6125, (+/-) 0.2000 and (-/-) 0.1875 to the frequency of allele (+) 71.25% and (-) 28.75%. More details can be seen in Table 3.

Test of the Hardy-Weinberg equilibrium law on thin-tailed sheep population in the highlands and lowlands on the GH gene PCR-RFLP MspI and AluI was performed using chi-square test. GH gene MspI allele frequency and AluI were tested on thin-tailed sheep in the highlands and lowlands in Hardy-Weinberg imbalance ($P < 0.01$).

Result of heterozygosity estimation with PCR-RFLP marker of MspI and AluI at all study sites showed that the value of expected heterozygosity (He) was higher than observed values (Ho). More details can be seen in Table 4.

Local sheep PIC values thin tail to fragment GH gene MspI and AluI in the highlands and lowlands are presented in Table 5.

Based on the PIC value, it can be stated that marker of PCR-RLFP of gene fragment GH AluI is not different with PCR-RFLP of gene fragment GH MspI in the lowlands and highlands. PIC values obtained are categorised in the medium (moderate), so that these values can be expressed quite informatively as a GH gene fragment marker on MspI and AluI.

Relationship between polymorphisms of GH genes with quantitative characteristics

The average quantitative characteristics of DET in the

Table 5. Estimation of the value of polymorphic information content (PIC) DET gene fragment of GH MspI and AluI in lowland and highland province of Jambi.

Site	n	*Polymorphic Informative Content* (PIC)	
		GH Gene Fragment MspI	GH Gene Fragment AluI
Highlands	240	0.3797	0.2815
Lowlands	240	0.3975	0.3677

Table 6. Means of characteristic GH gene fragment of thin-tailed sheep using *Msp*I and *Alu*I in highlands and lowlands.

Site/characteristics	Genotype		
	+/+	+/-	-/-
Highlands MspI			
BWG	0.0899±0.0114[a]	0.0759±0.0064[b]	0.0645±0.0075[c]
BW	22.2519±2.5286[a]	21.1458±1.7935[b]	20.5311±2.6702[c]
LB	58.8688±2.2773[a]	58.1198±2.0864[b]	56.8361±3.0573[c]
WH	55.4084±2.6777[a]	54.9001±1.9705[b]	54.4013±2.9319[c]
ChG	64.4261±2.4150[a]	63.9291±1.8306[b]	63.0101±2.5642[c]
ChD	25.5693±1.6429[a]	25.0791±1.1344[b]	24.7515±1.8240[c]
ChW	15.4803±1.1689[a]	15.1133±0.8910[b]	14.9986±1.0956[c]
Lowlands MspI			
BWG	0.0764±0.0158 [a]	0.0591±0.0047 [b]	0.0481±0.0058 [c]
BW	21.1274±2.2441 [a]	18.5504±1.6586 [b]	16.6256±1.1799 [c]
LB	56.5173±2.3176 [a]	54.0838±1.6564 [b]	53.1163±1.3145 [c]
WH	52.3217±2.2593 [a]	50.1349±1.5269 [b]	49.1647±0.7901 [c]
ChG	60.9372±1.9024 [a]	59.4086±1.0630 [b]	58.7667±0.4279 [c]
ChD	22.9243±1.3990 [a]	21.0286±1.5550 [b]	21.1440±0.8657 [c]
ChW	13.8673±1.6257 [a]	12.1718±1.4473 [b]	11.7451±0.8958 [c]
Highlands MspI			
BWG	0.0861±0.0131 [a]	0.0741±0.0069 [b]	0.0625±0.0095 [c]
BW	21.9588±2.4528 [a]	21.0263±2.2706 [b]	19.1183±0.6954 [c]
LB	58.6303±2.3587 [a]	57.9232±2.1549 [b]	54.0423±1.9300 [c]
WH	55.3233±2.5692 [a]	55.1358±1.9046 [b]	52.3763±1.8106 [c]
ChG	64.1657±2.3955 [a]	63.9673±2.0305 [b]	61.1494±1.9163 [c]
ChD	25.3835±1.6619 [a]	24.9335±1.6760 [b]	23.2856±2.1599 [c]
ChW	15.4032±1.1677 [a]	15.0934±0.7866 [b]	14.4004±0.6543 [c]
Lowlands AluI			
BWG	0.0717±0.0160 [a]	0.0583±0.0092 [b]	0.0478±0.0101 [c]
BW	20.5331±2.3714 [a]	17.7270±1.6610 [b]	17.0133±1.9803 [c]
LB	55.9954±2.3897 [a]	53.8307±1.4541 [b]	52.7504±1.3055 [c]
WH	51.8231±2.3417 [a]	49.7834±1.1692 [b]	49.2165±0.7234 [c]
ChG	60.6445±1.7416 [a]	58.9798±1.3423 [b]	58.7706±0.6189 [c]
ChD	22.4275±1.6705 [a]	21.3825±1.1932 [b]	21.0119±1.4642 [c]
ChW	13.4447±1.8072 [a]	12.1186±1.1632 [b]	11.8340±0.6611 [c]

Different letter in same row showed significant difference, db = n -2 t_{tabel} α 5% = 1.645.

highlands and lowlands of GH gene fragments using PCR-RFLP MspI, and AluI, is presented in Table 6.

TTS average quantitative characteristics, in the highlands and lowlands of GH genes using PCR - RFLP MspI identifier and AluI genotype were +/+ > +/- > - /-. Results of t test analysis between genotype (+/+ , +/- and -/-) on the quantitative characteristics of DET in the highlands and lowlands have the same pattern that is

significantly different (P < 0.05). The results obtained indicate that there is a relationship between genotype GH genes with quantitative characteristics as analyzed by TTS.

Growth hormone gene sequences

Results of alignment analysis of GH gene fragment primer GHY1, GHY2, GHY3 and its combined with BLAST good primer on the highland and lowland showed high similarity (homology) with the gene sequences of ovis aries growth hormone. Homology analysis of primer GHY1, GHY2 and GHY3, and combined with the same accession number are EF077162.1-1973bp, DQ461677, 1-2987bp, DQ461669.1-bp, DQ461667.1-4804 bp, and X12546.1- 2162 bp obtained at 99, 99, 98 and 98%, respectively. These results indicate that the GH gene amplified fragment was GH gene fragment of sheep (Ovis aries).

Multiple alignment is used to predict the structure and function of proteins. One method used is ClustalW. Primary clustalW results for GHY1, GHY2, GHY3 and combined sequentially obtained similarities were 98.57, 98.19, 98.30 and 98.86%.

Based on analysis of the sequences obtained that purportedly mutations occur at both sites is a mutation between the bases cytosine (C) to thymine (T) and the base cytosine (C) to guanine (G). Based on the results analysis, mutation sequences GH gene PCR - RFLP MspI (C*CGG) is C➡T at position 319 bp in exon 1, while the GH gene PCR- RFLP AluI (AG*CT) is C➡G at a position 983 bp in exon 3.

DISCUSSION

Effect of altitude

Kerinci district and Sungai Penuh City are located in the highlands with an altitude of l ≥ 1500 m above sea level and affected by environmental factor, while Muaro and Batanghari district are located in lowlands with an altitude of 100-500 m above sea level. The difference of altitude will certainly lead to differences in temperature, humidity, rainfall, wind speed and irradiation time. According to Calderon et al. (2005), there is a significant difference between the performance of livestock production in the lowlands (hot area) and upland (cold area). Here in after, referred environmental factors will affect the productivity of sheep (Popoola et al., 2014; Idris et al., 2014; NseAbasi et al., 2014).

Isolation and amplification

Isolated DNA of thin-tailed sheep in highland and lowlands in jambi province range from 100 to 400. Differences in concentrations of DNA produced is highly dependent on the ability of the cell nucleus lysis. If the cell nucleus could be well lysed, then the resulting DNA concentration will be high enough in both quality and quantity. However, if the cell nucleus is not lysed perfectly, DNA concentration will be low and sometimes contaminated with other materials. This indicates that the use of genomic DNA Purification Kit (Promega - USA) is a powerful enough to isolate the genomic DNA DET.

The results of DNA amplification using primers GHY1 DET, GHY2 and GHY3 sequentially generate specific fragments because electrophoresis results produced only one band with a length in accordance with the expected sequence which were 599, 690 and 679 bp. According to Rahayu et al. (2006), primer is an essential part of the primer PCR as an initiator to the target DNA synthesis; in addition to the PCR, results were favorably affected by several factors such as purity DNA extraction result, the accuracy of the primaries used, as well as the accuracy of PCR conditions. This condition indicates that the PCR reaction conditions and primers used through design with primer3 program are quite good because it gives a very specific PCR product as expected.

Allele frequencies, heterozygosity and PIC *(Polymorphic Content Informative)*

A locus would be polymorphic when the frequency of the largest allele is equal to or less than 0.95. Conversely, a locus would be monomorphic when the frequency of the greatest allele exceeds 0.95. Proportion of polymorphic loci in a population is often used as one of the indices of genetic diversity. Other grades which are also often used as an index of genetic diversity in a population are heterozygosity average or the frequency of heterozygotes (H) on average.

PIC can be used as a basis in determining an identifier information, and determining whether there is a polymorphic allele in addition based on the value of heterozygosity. Based on this measure of a good third allele frequency, heterozygosity and PIC values, it can be stated that the population DET in lowland and high in Jambi province have genetic diversity or are polymorphic. This result does not vary much with the results of Kumari et al. (2014) against 9 nations of sheep in India who obtain the A allele frequency (+) higher than the frequency of allele (-). According to Machado et al. (2003), if the Ho value is lower than the He value, it indicates a degree of endogamy (mating within the group). Furthermore, Javanmard et al. (2005) found that heterozygosity with a value below 50% indicates the low variation of a gene in the population. Heterozigosyty value is one of parameter used to determine population polymorphism (Ahmaed et al., 2014). Botstein et al. (1980) stated that the PIC can be used as a basis in determining

whether an identifier information, and determine whether there is a polymorphic allele in addition based on the value of heterozygosity, then Puja et al. (2013) stated that the PIC is high enough to give an indication that the sample population is very heterogeneous and indicated little going selection for certain characteristics while PIC small value indicates that the sample population is very homogeneous and indicated their selection for certain characteristics. Diversity or variation in a species would be very useful in the field of genetics or for the benefit of selection. These variations can be used to identify and locate the origin of a particular type of animal, knowing the kinship between species to the preparation of the gene map. Genetic variation information can be used as the basis for selection to improve livestock production and conservation goals.

Relationship between polymorphisms of GH genes with quantitative characteristics

The result shows that there is a relationship between genotype of GH gene with quantitative characteristics of thin-tailed sheep. This is consistent with the statement of Hajihosseinlo et al. (2013) that stated that genotype frequencies have a relationship with the characteristics of the sheep makooe. Research of Hua et al. (2009) explained that the haplotip diversity of GH's gene of HaeIII on Boer goats had effect on birth weight, weaning weight, body weight gain per day before weaning and body weight till 11 months. Furthermore, according to Alakilli et al. (2012), genotype frequencies can be used as molecular marker genes growth properties on goats. Marker of PCR-RFLP could be used as a selection tool. Polymorphisms of genotype frequency could be used as a basis of selection and breeding programs that very are useful in order to increase the population of thin-tailed sheep in Jambi Province.

Growth hormone gene sequences

There was a change in the base using the restriction enzyme MspI or AluI, although there is a chance of change (mutation) with other bases. This is due to the limited number of samples in sequence analysis. However, these results are in accordance with those reported by Malewa et al. (2014). Mahrous et al. (2014) reported no change between the cytosine (C) to thymine (T) and the cytosine (C) to guanine (G) in the Ovis aries, fat-tailed sheep, Egypt sheep and Saudi Arabia sheep. Furthermore, according to Jakaria (2008), the base nucleotide changes that occur are in the site MspI (CT) and AluI (CG). Ge et al. (2003) suggest changes in the AluI site which can alter the amino acid leucine (L) (CTG) to valine (V) (GTG) on growth hormone.

Mutations obtained in this study were categorized as substitution mutation, where mutation transition type for restriction enzymes MspI and transversion mutation types for restriction enzyme AluI. This is consistent with the statement of Li and Gaur (1991) that the mutations occur as a result of the substitution of adenine to guanine bases (purines) or between cytosine and thymine (pyrimidine). Transversion mutation occured due to the exchange between purine bases (A, G) with a pyrimidine (C, T). It is important to study the mutation as causes of genetic diversity, which can be used to determine the genetic distance (Mahrous et al., 2014), the characteristic of breed (Elkorshy et al., 2013), the relationship with growth traits (Moradian et al., 2013; Jia et al., 2014) and the ability of reproduction (Moradband et al., 2011).

Conclusion

Based on the research, it can be concluded that quantitative characteristics (BW, BWG, BL, ChG, ChD, and ChW) of thin-tailed sheep in both male and female in highland was better than in the lowlands. The marker of PCR-RFLP MspI and AluI GH gene were polymorphic on the highlands and lowlands. The highest genotype frequency and frequencies of allele in both the highlands and the lowlands were the genotype frequency (+/+) and the frequency of allele (+). The highest quantitative characteristic were BW BW, BWG, BL, ChG, ChD, and ChW of thin-tailed sheep was genotype (+/+). The diversity of GH genes MspI and AluI are associated with BW, BWG, BL, ChG, ChD, and ChW of thin-tailed sheep both on the highlands and the lowlands of Jambi Province.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Exploration of Sri Lankan soil fungi for biocontrol properties

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Sri Lankan soil is a rich source of fungi with new strains that are not well explored to date. In the present study, a total of 83 soil fungi were isolated from different locations of Sri Lanka using chitin and β-1,3-glucan selective media. Of the 83 isolates, 22 isolates showed high biocontrol activities against seven selected plant pathogenic fungi in dual culture plate assay. These 22 isolates were further analyzed for chitinase, glucanase production and for antifungal activities. *Trichoderma* sp. 1 showed significantly high mean chitinase (0.60±0.012 nkat/ml) and glucanase (0.42±0.031 nkat/ml) activities after 24 h and 96 h of incubation respectively compared to other isolates. Furthermore, it revealed the highest mean percentage (76.66±7.6) of inhibition against *Phytophthora meadii* in the well diffusion method using chitinase filtrates. *Penicillium* sp. 1 showed the highest mean percentage (64.75±1.6) of inhibition against *Rigidoporus microporus* in well diffusion method with glucanase filtrates. Three *Trichoderma* isolates that unveiled high enzyme activities as well as high antifungal activities were identified as *Trichoderma erinaceum* (*Trichoderma* sp. 1), *Trichoderma virens* (*Trichoderma* sp. 5) and *Trichoderma asperellum* (*Trichoderma* sp. 8) using molecular characterization.

Key words: Chitin, glucan, antifungal, *Trichoderma*, *Phytophthora, Rigidoporus.*

INTRODUCTION

Soil fungi such as *Trichoderma, Rhizopus, Aspergillus* and *Penicillium*, that have strong biocontrol activity which produced chitinases and glucanases which have been studied extensively. In Sri Lanka, current estimates suggest that there could be as many as 25,000 of fungi species, of which only a little more than 2,000 are presently known (Karunarathna et al., 2012). Having special environmental characteristics and being rich in soil fungi populations, the mycology of Sri Lankan soil has not been well explored, constituting an excellent source for the search of new strains. The soil samples used in this study were collected from a range of locations in Sri Lanka (9 districts) including natural forests, a compost heap, chitin bated soil, market garden and from rubber plantations.

Chitin and β-1,3-glucan selective media were used to

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isolate soil fungi, that produced chitinases and glucanases. The importance of β-1,3-glucanase (EC 3.2.1.39) and chitinase (EC 3.2.1.14) as key enzymes responsible for pathogenic fungal cell and sclerotial wall lysis and degradation has been reported (EL-Katatny et al., 2000). Therefore, use of chitinase and glucanase producing micro organisms as biological control agents against many fungal pathogens has been reported.

Most of the previous investigations indicated that, *Trichoderma* species are the best biocontrol agents among other soil fungi (Bell et al., 1982; Elad and Kapat, 1999; Ramezani, 2009). However there is still considerable interest in searching for new biocontrol fungi isolates especially in Sri Lankan soil which has not been well explored although it is known to be rich in biodiversity. By considering all these facts, the present study was undertaken to explore Sri Lankan soil, to isolate fungi that have strong biocontrol activities with high chitinase and β-1,3-glucanase production.

MATERIALS AND METHODS

Collection sites

For isolation of glucanolytic and chitinolytic fungi, soil samples were obtained from five different types of locations in Sri Lanka covering 9 districts including rubber plantations (Mathugama, Kuruwita and Kegalle), natural forests (Dambulla, Sigiriya, Polonnaruwa and Mathara), compost heap, market garden (Kandy) and from a baited method.

Isolation of chitinolytic and glucanolytic fungi

The soil samples were collected into sterile polypropylene bags, brought to laboratory within 12 h and used to isolate the fungi using the soil dilution plate method, with either chitin or glucan selective media. Chitin selective medium contained; yeast extract (5 g/L), $(NH_4)_2SO_4$ (1 g/L), $MgSO_4.7H_2O$ 0.3 (g/L), KH_2PO_4 (1.36 g/L) and Agar (20 g/L) with 1.5% colloidal chitin as the main carbon source (Severgnini, 2006).

Colloidal chitin was prepared as described by Ahmad et al. (2010). The glucan selective medium contained; $(NH_4)_2SO_4$ (1 g/L), $MgSO_4.7H_2O$ (0.3 g/L), KH_2PO_4 (0.8 g/L), KNO_3 (0.2 g/L) and agar (20 g/L) with 0.5% β-glucan prepared using dry yeast cells according to the method described by Zechner-Krpan et al. (2010) which is used as the main carbon source.

The pH of both media was adjusted to 5.5. To prevent bacterial growth culture media, 50 mg/L were amended with ampicillin. The pure cultures of fungi isolates obtained were maintained on potato dextrose agar during the period of study.

Screening of soil fungi isolates for biocontrol activities against pathogenic fungi using dual culture plate assay

Soil isolates (83) were tested on seven selected plant pathogenic fungi, using the dual culture method (Matroudi et al., 2009; John et al., 2010). Pure cultures of plant pathogenic fungi *Fusarium oxysporum, Rhizoctonia solani, Colletotrichum gloeosporioides, Curvularia lunata, Corynespora cassiicola*, *Rigidoporus microporus* and *Phytophthora meadii* were obtained from the Department of Plant Sciences, University of Colombo.

Potato Dextrose Agar (PDA) plates were inoculated, by placing a 9 mm diameter mycelial disc of the pathogenic fungus on one side, obtained from a 4 days old culture on PDA. A similar disc of soil isolate, obtained from the growing edge of 4 days old culture on PDA was placed on the opposite side of the pathogenic fungus.

The plates were incubated for 3 days at room temperature (28±2°C). The contact zones of the two colonies were observed under the light microscope for any interactions between the two fungi. For controls, only the pathogenic fungi were inoculated separately on a one side of the Petri plates, containing PDA media. In this dual culture plate assay, soil isolates were identified as effective biocontrol agents based on their ability to overgrow, form an inhibition zone and then growth inhibition of pathogen.

Morphological identification of soil isolates

Fungal mycelia and their reproduction structures were examined microscopically and identified upto genus level, using the mycological key (Coomaraswamy and Fonseka, 1981). Spore size, shape and nature of conidiophores were considered to identify fungi species.

Measurement of enzyme activities

The chitin and glucan liquid media were prepared as previously described without adding agar to solid media was added to 100 ml conical flasks. The liquid media in flasks were inoculated with a 9 mm diameter mycelial disc, obtained from the growing edge of a 4 day old culture of isolates.

The inoculated flasks were incubated on a rotary shaker of 120 rpm at room temperature. The cultures were harvested at 24, 42, 72 and 96 h intervals, by filtration through whatman no 1 filter paper. Resulting filtrates were stored at 4°C and was used to determine enzyme activity.

The activities of enzymes were determined by dinitrosalicylic acid (DNS) assay (EL-Katatny et al., 2000). The chitinase assay was conducted using 500 µl of 1% (w/v) colloidal chitin in sodium acetate buffer (pH 5.5) and 1 ml of culture filtrate. The mixture was incubated at 45°C for 30 min followed by incubation at 90°C for 10 min. Then, the mixture was centrifuged at 13,000 rpm for 10 min in a water bath.

To the supernatant, 1 ml of DNS reagent was added followed by 300 µl of potassium sodium tartrate. Thereafter, the mixture was heated in a boiling water bath for 5 min and after, cooled to room temperature. The absorbance was recorded using a spectrophotometer at 540 nm. The enzyme blank was distilled water and the control was uninoculated liquid medium.

The glucanase activity was also assayed similarly by incubating 1 ml of 2.5% β-glucan in sodium acetate buffer (pH 5.5), with 200 µl of enzyme solution. All other steps followed were exactly as for chitinase assay. The amount of reducing sugars released was calculated from standard curves for glucose and the activities of chtinase and glucanase were expressed in pkat (pmol/s). All experiments were carried out in triplicate. The data obtained were statistically analyzed using MINITAB 14. One way ANOVA and Tukey test were performed using 95% simultaneous confidence intervals.

Well diffusion method to detect antifungal activity of isolates against pathogenic fungi

Petri plates containing 25 ml of PDA were prepared and 4 wells were made with an equal distant to each other and 2 cm from the center. Then an 8 mm diameter agar plug obtained from the edge of 4 days old culture of the pathogenic fungus on PDA was placed

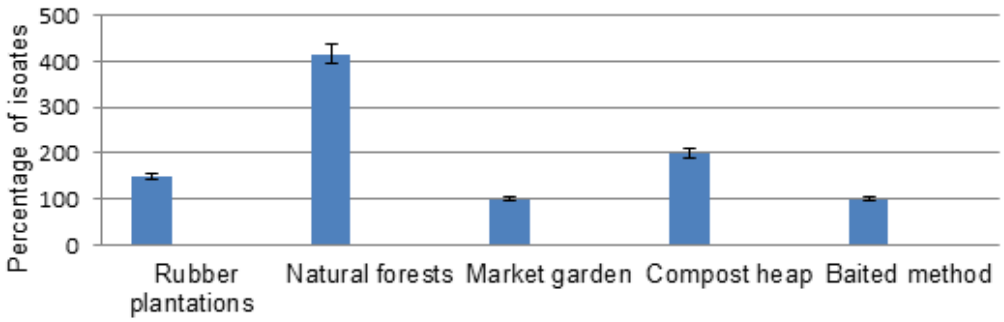

Figure 1. Percentage values of soil isolates of different sites.

at the center of the plate.

Thereafter, 3 of the 4 wells on each plate were filled with 50 µl of fungal filtrates and the 4th well was filled with 50 µl of the boiled fungal filtrate, which was the control. The plates were inoculated at room temperature for four days and radial mycelia growth of the test pathogen was determined by measuring the radius. Percent inhibition (PI) was calculated as; PI (%) = (γ°-γ/ γ°) x 100 where, γ° is the radius of test pathogen in the control (liquid medium) and γ is the radius of test pathogen in the enzyme solution.

All tests were carried out with three replications, for both chitinase and glucanase filtrates of all 22 isolates against 7 pathogenic fungi. The data obtained were statistically analyzed using MINITAB 14.

Molecular identification of *Trichoderma* isolates

Genotypic identification was carried out by PCR amplification and sequencing of ITS region. The rDNA sequence of ITS region using universal primers; ITS 1(5''- TCC GTA GGT GAA CCT GCG G- 3'') and ITS 4 (5''- TCC TCC GCT TAT TGA TAT GC- 3'') (Chakraborty et al., 2010), were used to amplify a ~ 600 bp fragment of the ribosomal DNA (rDNA), including the 5.8S gene and the flanking intergenic transcribed spacers ITS1 and ITS2.

The amplified fragments (~600 bp) were separated by agarose gel (1%) electrophoresis and the excised fragments were purified using PCR Clean-Up System-Wizard®SV Gel (Promega), according to the manufacturer's instructions. The purified amplicons were bidirectionally sequenced using ITS1 and ITS4 primers. The resultant sequences were edited using Bio Edit version 7.2.0 and were subjected to BLAST search analysis at NCBI. DNA sequences were submitted to GenBank under accession numbers; KJ381061, KP641614 and KY270875.

RESULTS

In this study, 83 chitinolytic and glucanolytic fungi were isolated from soil samples, collected from different locations in Sri Lanka, using chitin and glucan based selective media. Out of 83, 48 were isolated from glucan selective medium and 35 were isolated from chitin selective medium.

According to the results, the highest percentage of isolates was obtained from the natural forests (Figure 1). Out of 83 isolates, 22 showed biocontrol activity against seven pathogenic fungi in dual culture plate assay. They produced inhibition zones with pathogenic fungi colony, mycelia degradation and in some cases coiling structures were observed when examined under the microscope (Figure 2).

Enzyme assay

Chitinase activity of the isolates ranged from (0.60 - 0.00) and (0.54 - 0.00) nkat/ml, respectively (Table 1). In most of the species the optimum chitinase activity after 24 h of growth and optimum glucanase activity after 96 h of growth. *Trichoderma* sp. 1 and *Penicillium* sp. 1 (0.60±0.012 nkat/ml and 0.55±0.042 nkat/ml), showed significantly high mean chitinase activities after 24 h of incubation. *Trichoderma* sp. 1 and *Trichoderma* sp. 8 (0.42±0.031 nkat/ml and 0.54±0.009 nkat/ml) showed significantly high mean glucanase activities after 96 h of incubation.

Well diffusion method to detect antifungal activity of isolates against pathogenic fungi

In chitinase filtrate assay, the highest mean percentage was observed in *Trichoderma* sp. 1(76.66 ± 7.6) against *P. meadii*. *Trichoderma* sp. 1 and *Penicillium* sp 4 showed significantly high mean percent inhibition values than others isolates, against the seven tested pathogenic fungi ($P<0.05$) (Table 2). In glucanase filtrate assay, the highest mean percentage was observed in *Penicillium* sp 1(64.75±1.6) against *R. microporus*, *Trichoderma* sp 1 and *Penicillium* sp 1. *Aspergillus* sp 1 and *Trichoderma* sp 8 showed significantly high mean percent inhibition values than the rest of the isolates against seven tested pathogenic fungi ($P<0.05$) (Table 3) (Table 4).

Amplification of ITS region of *Trichoderma* isolates

The ITS regions of three *Trichoderma* isolates (Table 4) were successfully amplified and sequenced. A comparative analysis of rDNA sequences of the three Trichoderma isolates against rDNA sequences of

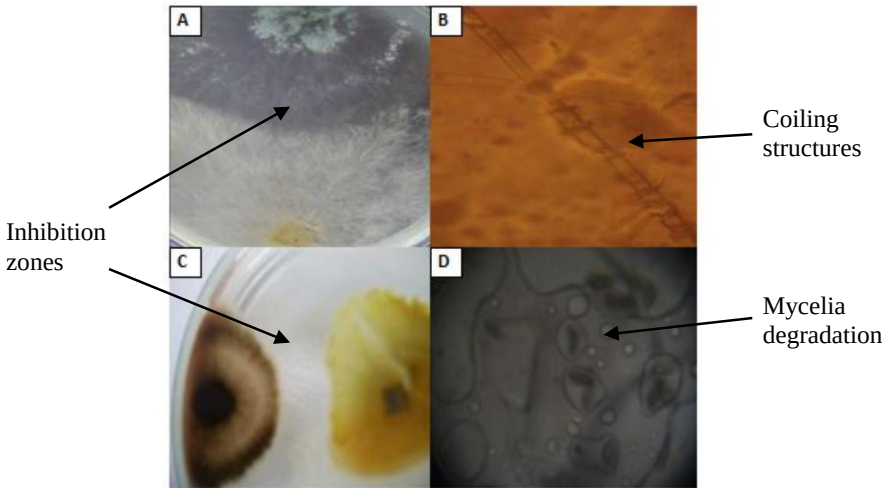

Figure 2. A. *Trichoderma* sp (1) against *Rhizoctonia solani* showing inhibition zone. **B.** Microscopic view of coiling structures between *Trichoderma* sp. (1) against *Rhizoctonia solani*. **C.** *Penicillium* sp. (8) against *Curvularia* sp showing inhibition zone. **D.** Microscopic view of mycelia degradation of *Curvularia* sp.

Table 1. Chitinase and glucanase activities of soil fungal isolates.

Soil isolate	Chitinase activity (nkat/ml)				Glucanase activity (nkat/ml)			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Aspergillus sp. 1	0.25±0.01	0.06±0.01	0.06±0.00	0.03±0.00	0.07±0.00	0.12±0.01	0.21±0.01	0.15±0.01
Penicillium sp. 1	0.55±0.04	0.24±0.00	0.12±0.03	0.11±0.00	0.11±0.01	0.14±0.00	0.16±0.01	0.15±0.00
Aspergillus sp. 2	0.48±0.01	0.09±0.02	0.03±0.01	0.01±0.05	0.04±0.03	0.02±0.01	0.03±0.03	0.12±0.00
Trichoderma sp. 1	0.60±0.01	0.23±0.00	0.15±0.02	0.06±0.05	0.01±0.00	0.08±0.10	0.24±0.01	0.42±0.03
Trichoderma sp. 2	0.19±0.04	0.06±0.00	0.03±0.00	0.03±0.00	0.01±0.00	0.17±0.05	0.14±0.00	0.24±0.00
Trichoderma sp. 3	0.04±0.01	0.02±0.00	0.02±0.04	0.02±0.00	0.00±0.00	0.01±0.00	0.00±0.00	0.03±0.01
Trichoderma sp. 4	0.29±0.09	0.14±0.01	0.13±0.01	0.12±0.00	0.00±0.00	0.00±0.00	0.06±0.05	0.19±0.00
Trichoderma sp. 5	0.19±0.00	0.11±0.01	0.09±0.00	0.07±0.00	0.04±0.03	0.19±0.01	0.23±0.01	0.24±0.00
Penicillium sp. 2	0.33±0.00	0.17±0.01	0.13±0.00	0.10±0.00	0.08±0.00	0.06±0.00	0.06±0.00	0.08±0.00
Penicillium sp 3	0.25±0.01	0.17±0.01	0.09±0.01	0.07±0.01	0.04±0.01	0.04±0.00	0.05±0.01	0.10±0.00
Trichoderma sp. 6	0.11±0.00	0.18±0.02	0.172±0.0	0.14±0.00	0.04±0.00	0.08±0.00	0.08±0.00	0.04±0.00
Penicillium sp. 4	0.30±0.01	0.16±0.04	0.12±0.01	0.10±0.00	0.03±0.01	0.00±0.00	0.03±0.00	0.01±0.00
Penicillium sp. 5	0.28±0.00	0.09±0.01	0.02±0.01	0.02±0.00	0.01±0.00	0.04±0.01	0.07±0.00	0.09±0.01
Aspergillus sp. 3	0.11±0.00	0.08±0.00	0.02±0.00	0.01±0.00	0.01±0.00	0.00±0.00	0.03±0.01	0.01±0.00
Penicillium sp. 6	0.13±0.00	0.09±0.00	0.04±0.00	0.05±0.00	0.05±0.00	0.06±0.00	0.04±0.00	0.05±0.00
Penicillium sp. 7	0.09±0.00	0.05±0.00	0.01±0.00	0.07±0.00	0.03±0.00	0.01±0.00	0.01±0.00	0.10±0.00
Penicillium sp. 8	0.07±0.03	0.04±0.01	0.04±0.00	0.00±0.00	0.03±0.01	0.02±0.00	0.00±0.00	0.04±0.00
Aspergillus sp. 4	0.05±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.00
Trichoderma sp. 7	0.19±0.07	0.20±0.00	0.11±0.02	0.09±0.04	0.12±0.00	0.11±0.01	0.17±0.00	0.24±0.01
Trichoderma sp. 8	0.04±0.00	0.06±0.02	0.24±0.00	0.35±0.00	0.53±0.00	0.52±0.02	0.47±0.00	0.54±0.00
Fusarium sp. 1	0.09±0.03	0.02±0.00	0.01±0.00	0.02±0.00	0.07±0.01	0.05±0.00	0.02±0.00	0.04±0.00
Penicillium sp. 9	0.20±0.04	0.04±0.00	0.04±0.01	0.03±0.00	0.12±0.00	0.10±0.00	0.05±0.00	0.09±0.00

Trichoderma sp. available in GenBank data base werecarried out separately for each using BLAST algorithum at the website http://www.ncbi.nlm.nih.gov. The homology search against the GenBank data base revealed a 100% similarity to the ITS region of *T. erinaceum* (*Trichoderma* sp 1):KJ38061, *T. virens* (*Trichoderma* sp 5):KP641614 and *T. asperellum* (*Trichoderma* sp 8): KY270875.

Table 2. Mean percent inhibition of chitinase filtrates of isolates on seven pathogenic fungi.

S/N	Isolates	Pathogenic fungi						
		Colletotrichum	*Corynespora*	*Curvularia*	*Fusarium*	*Phytophthora*	*Rigidoporus*	*Rhizoctonia*
1	*Aspergillus sp.1*	40.7±8.50	1.23±2.14	42.66±4.6	33.33±7.6	64.75±1.6	2.97±2.94	0.00±0.00
2	*Penicillium sp .1*	06.1±5.30	27.7±25.4	8.33±14.4	8.33±14.4	0.00±0.00	0.87±1.51	0.00±0.00
3	*Aspergillus sp. 2*	10.0±17.3	3.70±3.20	11.6±10.4	27.26±7.8	2.22±3.85	1.96±3.39	0.00±0.00
4	*Trichoderma sp.1*	16.0±6.90	41.6±7.21	48.33±2.8	31.6±16.1	76.66±7.6	38.66±2.3	3.70±6.41
5	*Trichoderma sp.2*	47.1±5.88	18.7±16.5	40.73±6.4	36.66±5.7	14.3±24.7	6.16±2.13	0.00±0.00
6	*Trichoderma sp.3*	16.6±14.4	0.00±0.00	0.00±0.00	0.00±0.00	9.00±0.00	0.00±0.00	0.00±0.00
7	*Trichoderma sp. 4*	2.22±3.85	0.00±0.00	0.00±0.00	0.00±0.00	38.33±7.6	0.00±0.00	0.00±0.00
8	*Trichoderma sp. 5*	3.70±6.41	47.9±7.21	9.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	4.00±4.00
9	*Penicillium sp. 2*	50.0±05.0	11.7±2.95	0.00±0.00	3.17±2.74	38.33±7.6	20.0±4.00	9.06±9.09
10	*Penicillium sp. 3*	3.50±3.04	0.00±0.00	6.66±8.82	5.55±4.80	0.00±0.00	16.0±4.00	46.66±2.9
11	*Trichoerma sp. 6*	04.0±04.0	21.1±7.69	28.2±22.8	18.17±4.5	5.92±4.62	0.00±0.00	26.6±15.3
12	*Penicillium sp. 4*	45.0±8.66	49.3±2.30	10.0±10.0	0.00±0.00	66.66±0.0	58.33±7.2	41.33±2.3
13	*Penicillium sp. 5*	0.00±0.00	0.00±0.00	20.0±10.0	0.00±0.00	2.74±2.71	0.00±0.00	0.00±0.00
14	*Aspergillus sp .3*	1.66±2.89	1.62±1.40	38.4±3.84	3.17±2.74	0.72±1.25	11.11±9.6	22.8±39.6
15	*Penicillium sp. 6*	13.33±7.6	45.0±6.61	32.2±1.92	20.0±8.66	7.84±4.49	21.73±2.2	0.95±1.65
16	*Penicillium sp. 7*	14.3±12.5	4.44±5.09	37.01±3.7	0.00±0.00	3.00±3.00	1.96±1.69	0.00±0.00
17	*Penicillium sp .8*	1.85±3.20	12.0±08.0	43.75±3.1	7.51±2.57	24.44±8.4	31.11±1.9	0.00±0.00
18	*Aspergillus sp. 4*	16.0±04.0	12.8±2.56	18.16±4.5	9.72±8.67	0.98±1.69	4.76±1.64	0.00±0.00
19	*Trichoderma sp. 7*	2.38±4.12	51.6±17.5	41.1±12.6	1.90±1.65	28.33±5.8	2.22±3.85	0.00±0.00
20	*Penicillium sp. 9*	2.56±4.44	13.33±1.4	3.88±4.19	7.62±5.95	0.74±1.28	6.66±6.66	0.00±0.00
21	*Trichoderma sp. 8*	56.0±04.0	11.33±2.3	18.66±2.3	67.94±2.2	2.38±2.38	22.22±5.1	0.00±0.00
22	*Fusarium sp. 1*	0.00±0.00	0.00±0.00	8.89±3.84	9.05±4.54	2.43±2.44	36.66±3.3	0.00±0.00

*Values are mean of three replicates ± SD. (P<0.05).

Table 3. Mean percent inhibition of glucanase filtrates of isolates on seven pathogenic fungi.

S/N	Isolates	*Colletotrichum*	*Corynespora*	*Curvularia*	*Fusarium*	*Phytophthora*	*Rigidoporus*	*Rhizoctonia*
1	*Aspergillus sp.1*	46.7±2.88	49.33±2.3	50.0±0.00	36.5±7.27	63.33±1.4	60.0±3.33	0.00±0.00
2	*Penicillium sp.1*	47.4±5.26	63.8±4.36	59.04±3.3	33.33±5.7	50.0±12.5	64.75±1.6	0.00±0.00
3	*Aspergillus sp. 2*	2.38±4.12	0.00±0.00	6.67±6.67	1.66±2.88	11.6±11.5	0.00±0.00	18.2±18.2
4	*Trichoderma sp.1*	22.6±4.61	42.59±3.2	44.41±7.3	20.9±5.65	42.6±2.30	36.6±14.4	46.6±3.81
5	*Trichoderma sp.2*	42.4±5.24	0.00±0.00	1.11±1.92	42.85±7.1	53.33±6.6	25.0±8.33	0.00±0.00
6	*Trichoderma sp.3*	0.00±0.00	0.00±0.00	28.89±3.8	26.6±6.67	28.89±3.8	0.00±0.00	0.00±0.00
7	*Trichoderma sp.4*	0.00±0.00	0.00±0.00	10.0±0.00	30.3±13.8	12.9±11.5	0.00±0.00	20.0±18.0
8	*Trichoderma sp.5*	4.00±4.00	0.00±0.00	0.00±0.00	18.2±15.7	0.00±0.00	0.00±0.00	0.00±0.00
9	*Penicillium sp.2*	9.06±9.09	5.71±2.85	52.09±4.7	26.66±2.8	54.3±5.65	8.55±5.35	0.83±1.44
10	*Penicillium sp.3*	46.66±2.9	25.71±2.9	28.06±3.0	43.05±8.6	1.07±1.85	24.0±21.0	22.5±2.50
11	*Trichoerma sp.6*	26.6±15.2	42.66±2.3	32.0±13.8	29.8±8.06	14.66±6.1	3.00±3.00	0.95±1.65
12	*Penicillium sp.4*	34.9±7.27	47.0±1.47	58.1±10.0	19.67±5.2	0.92±1.59	3.00±3.00	0.95±1.65
13	*Penicillium sp.5*	0.00±0.00	41.6±14.4	44.0±4.00	44.0±0.00	0.00±0.00	66.6±1.65	0.00±0.00
14	*Aspergillus sp.3*	25.0±21.7	5.88±2.94	36.4±22.8	7.23±6.62	0.85±1.47	0.00±0.00	0.00±0.00
15	*Penicillium sp.6*	10.53±2.6	11.1±3.70	0.00±0.00	12.66±5.5	1.66±2.88	13.33±3.3	4.33±7.50
16	*Penicillium sp.7*	25.9±6.41	0.00±0.00	12.5±0.00	0.00±0.00	0.00±0.00	4.00±4.00	6.66±5.77
17	*Penicillium sp.8*	21.67±5.7	49.59±5.1	45.71±4.9	3.17±2.74	4.51±4.50	11.76±0.0	0.00±0.00
18	*Aspergillus sp.4*	0.00±0.00	6.66±1.65	60.0±12.0	48.33±2.9	4.00±4.00	41.6±14.4	2.32±2.32
19	*Trichoderma sp.7*	3.08±4.07	11.09±7.2	35.7±14.3	22.79±8.0	24.4±10.2	4.44±7.69	0.95±1.65

Table 3. Contd.

19	*Trichoderma sp.7*	3.08±4.07	11.09±7.2	35.7±14.3	22.79±8.0	24.4±10.2	4.44±7.69	0.95±1.65
20	*Penicillium sp. 9*	3.07±4.06	3.96±1.37	2.66±2.30	31.1±16.8	27.0±6.17	2.22±3.85	0.00±0.00
21	*Trichoderma sp. 8*	45.0±5.00	42.6±4.61	43.33±8.8	43.93±2.6	60.0±2.50	54.44±3.8	0.00±0.00
22	*Fusarium sp.1*	8.89±3.84	0.00±0.00	40.0±6.67	25.0±0.00	9.26±5.78	6.25±0.00	0.00±0.00

*Values are mean of three replicates ± SD. (P<0.05).

Table 4. Summary results of fungal isolates showing significantly high enzyme activities and antifungal potential..

Test	Significantly high active isolates	Selected isolates for molecular characterization
Chitinase enzyme assay	*Trichoderma* sp. 1 *Penicillium* sp. 1	*Trichoderma* sp. 1
Glucanase enzyme assay	*Trichoderma* sp. 1 *Trichoderma* sp. 5 *Trichoderma* sp. 8	*Trichoderma* sp. 1 *Trichoderma* sp. 5 *Trichoderma* sp. 8
Chitinase antifungal test	*Trichoderma sp.* 1 *Penicillium sp. 4*	*Trichoderma sp.* 1
Glucanase antifungal test	*Aspergillus sp.* 1 *Penicillium sp.* 1 *Trichoderma sp.* 1 *Trichoderma sp.* 8	*Trichoderma sp.* 1 *Trichoderma sp.* 8

DISCUSSION

In this study, soil samples collected from five different locations in nine districts of Sri Lanka were explored for fungi of interest and a baited strategy was tested to enrich the soil flora for fungi of interest.

Initially, 83 pure cultures were obtained from different locations. Out of 83, 35 were isolated from chitin selective media and 48 were isolated from glucan selective media. Highest percentages (416.66%) of isolates, with prominent biodiversity were obtained from natural forests soil samples. This may be due to high content of nutrients and adequate moisture in forest soil. Among them, only seven isolates evidence biocontrol activity in dual culture plate assay. These include two *Aspergillus* species, two *Trichoderma* species, two *Penicillium* species and one *Fusarium* species. Only *Trichoderma* species showed the coiling structures around mycelia of *R. solani* while other isolates showed only the inhibition zones between the two colonies.

Compost samples are rich in organic material that provides nutrients to the soil. Second highest percentage (200%) of isolates, was obtained from compost samples. However none of the isolates had any biocontrol activity. All twelve isolates (148%) obtained from soil samples of rubber plantations (seven *Penicillium* species, three *Trichoderma* species and two *Aspergillus* species), revealed high biocontrol activity during initial screening test. Those include seven *Penicillium* species, three *Trichoderma* species and two *Aspergillus* species.

Baiting method was proved to be a productive method for enriching the surrounding soil flora, for chitinolytic and gluconolytic species (Severgnini, 2006). Only two *Trichoderma* species were isolated from having biocontrol activity. Agricultural soils are often subjected to fungicides and fertilizers and these contain very low fungal population. Hence, the market garden yielded the lowest number of isolates with one *Trichoderma* species having biocontrol activity.

The results of this dual culture test clearly suggested that out of 83 isolates, 22 showed biocontrol activity against pathogenic fungi. There are nine *Penicillium* species, eight species of *Trichoderma*, 4 species of *Aspergillus* and 1 species of *Fusarium*. Hence, these 22 isolates were selected to carry out further analysis. According to the enzyme assay results, *T. erinaceum* (*Trichoderma* sp 1) showed significantly high activities for both enzyme assays. Production of chitinase using a medium containing colloidal chitin reached a maximum after 24 h of growth for all isolates and decreased thereafter.

In contrast, synthesis of β-1,3-glucanase increased rapidly after 96 h peaking at the fourth day for most of isolates. These results are accordance with previous

studies (EL-Katatny et al., 2000; Matraudi et al., 2009; Sharaf et al., 2012).

The results of both assays and the two well diffusion tests indicated that, *T. erinaceum* (*Trichoderma* sp. 1), *T. virens* (*Trichoderma* sp. 5) and *T. asperellum* (*Trichoderma* sp. 8) can be used as an efficient biocontrol agents against all tested fungal pathogens, especially against *R. solani* and *P. meadii* which showed high mean percent inhibition values on them. In this study, some *Aspergillus* and *Penicillium* species acted as biocontrol agents while *Trichoderma* species acted best as reported by several workers earlier.

Chitinolytic and glucanolytic fungi showed great variations towards cell wall degradation, ranging from weak to strong antifungal activity depending on inherited characters. Most studies revealed that *T. harzianum* is among the most potential species of *Trichoderma* that are commonly used for phytopathogen control (Jayalakshmi et al., 2009; Muhammad and Amusa, 2003; Lorito et al., 1998; Shabir et al., 2012).

In this study, *T. erinaceum* (*Trichoderma* sp 1), *T. virens* (*Trichoderma* sp 5) and *T. asperellum* (*Trichoderma* sp. 5) were found to be highly active isolates among 83 soil isolates. It is revealed from the present study that the ability of the above selected *Trichoderma* species to control pathogenic fungi especially *R. solani* and *P. meadii* may be due to chitinase and glucanase production. These observations were in close resemblance with previous studies such as that of Lorito et al. (1998), Ramezani (2009) and Sharma et al. (2014). *Trichoderma* spp. employs several mechanisms to combat pathogens by competition for space and nutrients, secretion of cell wall degrading enzymes (chitinases and gucanases), induction of resistance etc. (Rifai, 1969).

In both well diffusion tests, boiled enzymes were used as controls. The results suggest that proteins in the suspension were likely to affect the growth of pathogens. In this study, purified enzymes were not used. If purified, enzymes were used for better inhibitions which may be obtained. Lorito et al. (1994) reported that, incubated plant pathogens such as *R. solani*, *Ustilago avenae*, *Uncinula necator* with *Trichoderma harzianum* purified endochitinase, found complete inhibition (100%) of hyphal elongation.

However understanding the antagonistic mechanisms used by *Trichoderma* species on a wide range of pathogens is important in optimizing their use as biocontrol agents. It is also important to collect information about the effect of temperature of fungal filtrate enzyme activity, elements in supernatant and incubation time on the inhibition of pathogenic fungi growth.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Computer simulation of benzene, toluene and *p*-xylene adsorption onto activated carbon

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In this work, the adsorption of benzene, toluene and *p*-xylene on activated carbon was verified through a computer simulation. A carbon model was used as the work basis. Several steps were completed from the modeling of the carbon structures and pollutants to simulations of molecular dynamics. After analyzing the results, it was found that the groups: ether, lactone and carbonyl (ketone) present in the carbon structure provided acidic character and due to this fact, and the carbon's complex network of micropores, the adsorption became viable. This study corroborates the present understanding on this type of phenomenon.

Key words: Pollutants, computational chemistry, adsorption, molecular dynamics.

INTRODUCTION

Monoaromatic hydrocarbons are among the major contaminants of groundwater. These contaminants such as benzene, toluene and xylenes (ortho-, meta-, para-), also called BTX, are powerful depressants of the central nervous system, presenting chronic toxicity, even in small concentrations. BTX compounds contaminate water and groundwater through effluents from petrochemicals and related industries. Also, fuel leakage from underground storage tanks of gasoline in urban areas and accidents in the transportation of petroleum fuels cause the release of this kind of pollutants into the environment (Torabian et al., 2010; Chakraborty and Coates, 2004).

Among the various methods of these pollutants removal, adsorption with activated carbon is one of the most widely used, since it has the ideal physicochemical properties to adsorb organic components components of low molecular weight, such as BTX. Adsorption is a separation process that has been used in the recovery,

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concentration, separation and purification of compounds that can present high value-added. It is a process of mass transfer in which one or more compounds present in a phase (liquid, vapor or gas), are adhered on the surface of a solid (Yang, 2003; Ghiaci et al., 2004; Ahmaruzzaman, 2008).

For this process, activated carbon remains one of the most important microporous adsorbents from the industrial point of view. It presents very complex surface characteristics (porosity and surface chemistry), with pore size ranging from micropores (diameter less than 2 nm) to macropores (diameter more than 50 nm). The volumes of micropores range from 0.15 to 0.6 cm^3/g and exhibits a large variety of surface groups, impurities and irregularities. The surface characteristics of activated carbon depend on the raw material used and the mode of activation (Wibowo et al., 2007; Yalçın and Sevinc, 2000).

Simulation in computational environments allows researchers to be able to understand the behavior of matter on a scale that cannot be naturally observed. Computational modeling can be used in the fields of computational chemistry, drug design and computational biology. It offers not only ways to solve mathematical calculations, but also, and above all, the creation of virtual laboratories in which studies can be carried out very close to reality. Thus, the understanding and observing of a problem can be done reliably and effectively (Meller, 2001; Rangel-Vázquez, 2015; Ganesan et al., 2017).

In this context, since the experimental process of adsorption of BTX is well known (Wu et al., 2000; Shim and Kim, 2010), the present work proposed a practical approach of molecular modeling as a tool to study and confirm the relations between the structure and activity of the activated carbon under analysis, regarding mainly the adsorption process of the compounds: benzene, toluene and *p*-xylene on the activated carbon surface.

MATERIALS AND METHODS

Design of the main structure of carbon

From the primary model created by Bourke et al. (2007), a new structure was generated by computer modeling, using the software *MarvinSketch* 6.0.0, which provided its two-dimensional structure.

This model was optimized by DFT calculation, at BELYP theory level and 6-31G base by the software Gaussian 09. The files obtained through optimization were used to generate the data of three-dimensional coordinates, topology, loads, angles, bonds and dihedral angles, using the FF99SB force field and the *antechamber* code of the package *AMBER* 13 (Lindorff-Larsen et al., 2010). The same procedure was performed for benzene, toluene and *p*-xylene structures.

From the unit cell generated, a complex formed by double layers of carbon together with the molecules of each pollutant was created. The pore size was equal to 8Å and the plate spacing was equal to 20Å, as suggested in the work of Lima et al. (2012).

Solvation, minimization, heating and molecular dynamics

In order to simulate a system that was as close as possible to the reality of an experimental adsorption test, the system was solvated with an octahedral TIP3P water box to simulate the aqueous environment (Mark and Nilsson, 2001). The package *ambertools* version 13 (Case et al., 2012) was used to construct the mesopore model and the adsorption system containing carbon and pollutants.

The system underwent a minimization process divided into 4 stages: the first one that minimizes the water molecules; the second that considers the hydrogens of the structure; the third that minimizes the hydrogens + water and the last one that minimizes all atoms (Case et al., 2005).

The heating stage simulated the temperature increase from 0 to 25°C and was divided into five stages with increment of 5°C and 500 ps (picoseconds) of simulation in each step, and its information was collected every 50 ps. The structure was then subjected to system equilibrium for 500 ps so that the dynamics process could start.

Molecular dynamics

The molecular dynamics stage simulated the state of the system in instants from 0 to 30 ns (nanoseconds) divided into five steps with increment of 2 ns in each. For this, the AMBER FF99SB force field was used. This step was performed with the module *sander* also included in the software *AMBER* 13 (Salomon-Ferrer et al., 2013), with information collected every 10 ps. The results, a three-dimensional representation of the imposed conditions, are images obtained and rendered by the software *VMD* (Humphrey et al., 1996; Stone et al., 2001). Simulations of molecular dynamics provide information on molecular mobility at an atomic level (Case et al., 2005). Information of temperature, pressure and total energy of the system were obtained.

RESULTS AND DISCUSSION

Figure 1 shows the main structure and the minimized model of the carbon obtained from the model postulated by Bourke et al. (2007). Figure 2 shows the minimized structures of benzene, toluene and *p*-xylene. Geometric optimization alters the molecular geometry to decrease the energy of the system and produces a more stable conformation. The minimization of energy before the molecular dynamics, besides taking the structure to a minimum of energy, was performed with the intention of removing any "bad contact" created by solvation (due to ionic dissociation of water molecules) (Ivone et al., 2003; Jo et al., 2009).

Regarding the systems solvation, although there are other models, TIP3P is probably the most used because of the good dynamic and thermodynamic properties that it provides for most force fields (Jorgensen et al., 1983; Ganesan et al., 2017). The solvated model simulates the adsorption conditions in aqueous medium.

In Figure 3, comparing the initial (a) and the final state (b), it is possible to observe that there was interaction between the molecules of the pollutants and the surface of the carbon. According to Boehm (2007), functional groups and delocalized electrons are factors that determine the chemical character (acidic or basic) of the

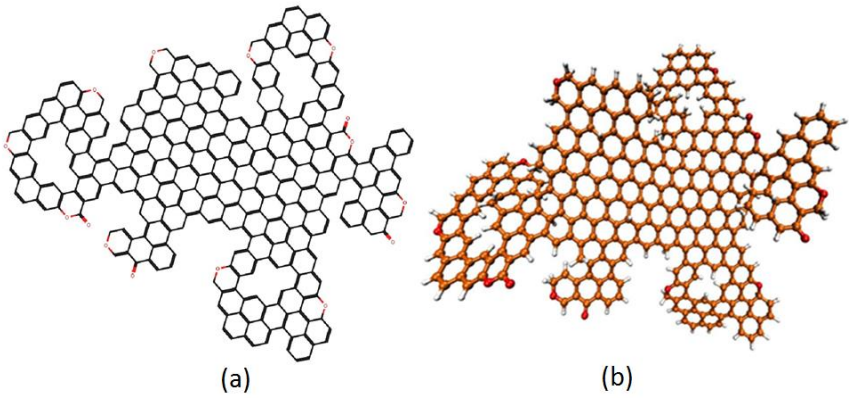

Figure 1. Two-dimensional (a) and minimized three-dimensional (b) models of activated carbon.

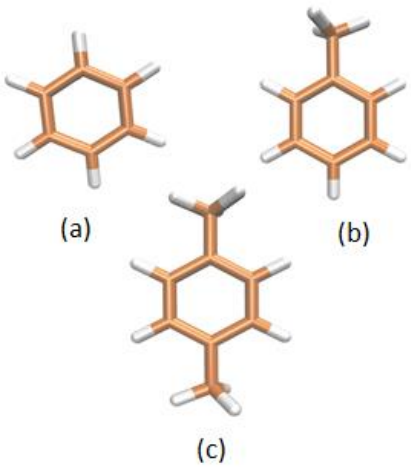

Figure 2. Structural formula of benzene (a), toluene (b) and *p*-xylene (c) compounds.

activated carbon surface, since oxygen may be present in various forms. In the case of the carbon structure under analysis, the ether, carbonyl and lactone groups (Figure 4) are present, which make the structure more acidic, apolar and consequently, it ends up exhibiting a negative surface charge. According to Wibowo et al. (2007), carbons with few oxygenated surface groups have higher adsorption capacity.

The non-polar character of the surface of the activated carbon is a preponderant factor in the adsorption of non-polar molecules, and can be increased by the appropriate modification of the chemical nature of the carbon surface (e.g. acid treatment). Due to its non-polarity and for having a larger volume of micropores, carbon adsorbs more strongly the apolar or weakly polar organic molecules, such as the pollutants benzene, toluene and *p*-xylene (Yang, 2003). With the tool *xmgrace* from *AMBER* 13 package (Zhang, 2009), the graphs of total energy vs. time, pressure vs. time and temperature vs. time were obtained from the data obtained in the dynamics files (Figures 5 and 6).

The graph energy vs. time (Figure 5) demonstrated that all adsorption processes analyzed are exothermic. This occur because when the molecule is attracted to the surface of the adsorbent, forming an intermolecular bond, there is a need to release the energy it had in the medium (Ruthven, 1984). Figure 5 also shows how the energy increases considerably during the equilibrium stage (beginning of the curve), then there is a slight decrease until its stabilization. This stabilization begins along with the dynamics process. The energy varies very little throughout this process. The mean values of energy of these processes were -57.62 x 10^3 Cal/mol for the benzene's system, -89.52 x 10^3 Cal/mol for the toluene's system and -82.35 x 10^3 Cal/mol for the *p*-xylene's system.

Just like in a real process conducted in normal conditions, the system was programmed so that the temperature and pressure remained constant throughout the dynamics process. Figure 6a and b shows the stability of these parameters.

Conclusion

Studies that focus on processes simulations are important to reduce costs, avoid problems, optimize and control changes in the system. The software used proved to be efficient at all stages of this work. Sometimes, the adsorption success is believed to be due only to the microporous structure of the activated carbon; however, this study showed that also, the ether, lactone and carbonyl groups present in the carbon structure under analysis are fundamental for adsorption of apolar compounds, as is the case of the pollutants: benzene, toluene and *p*-xylene. This study allowed a better

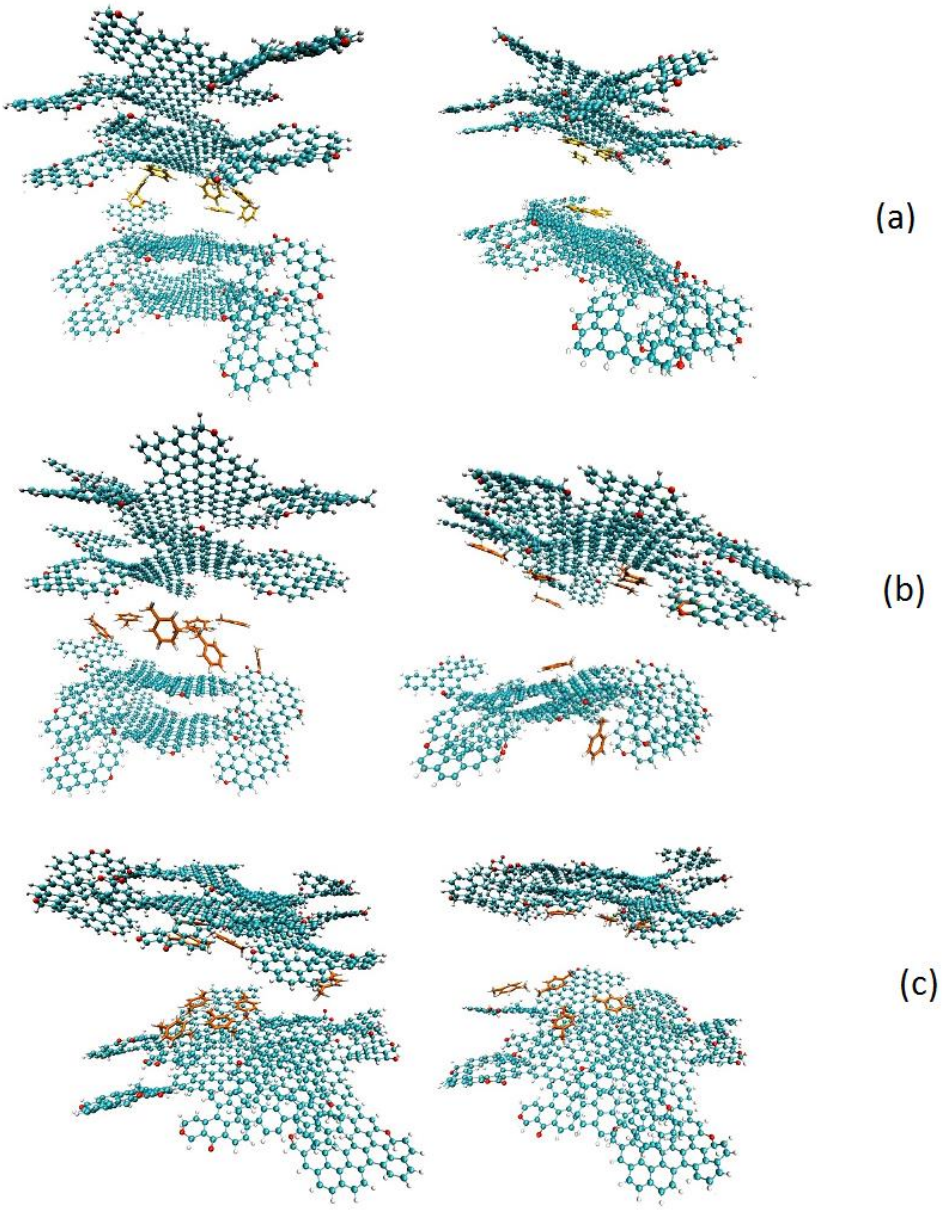

Figure 3. Molecular dynamics initial (0 ns) and final state (30 ns) of the systems containing benzene (a), toluene (b) and *p*-xylene (c).

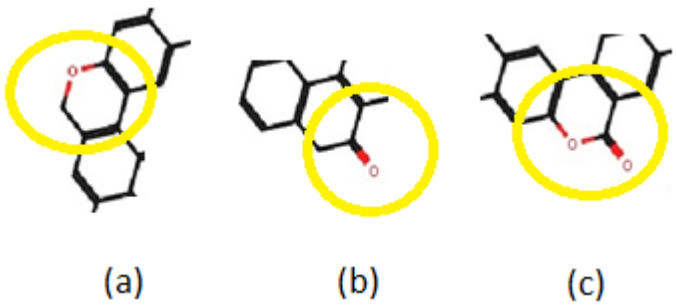

Figure 4. Functional groups found on the surface of the carbon-ether (a), carbonyl (b) and lactone (c).

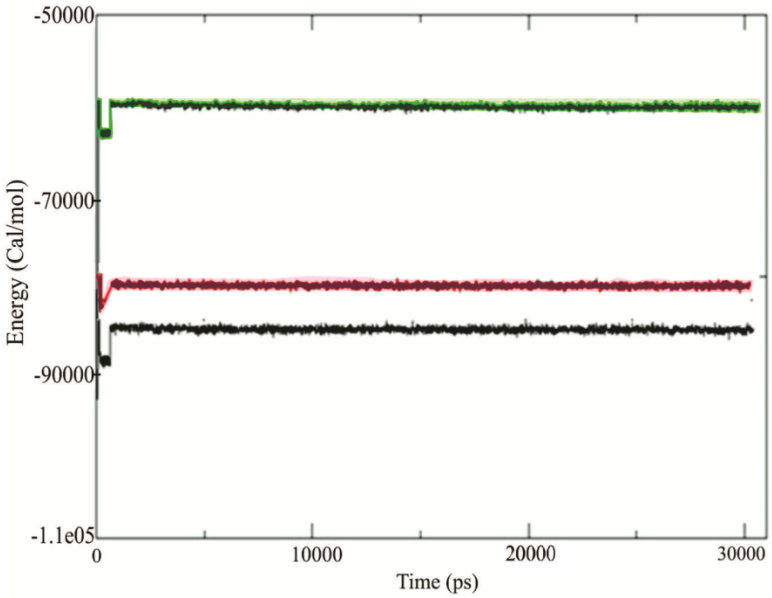

Figure 5. Representation of energy variation over time for systems containing benzene (green line), toluene (black line) and *p*-xylene (red line) from the equilibrium step to the last dynamic process.

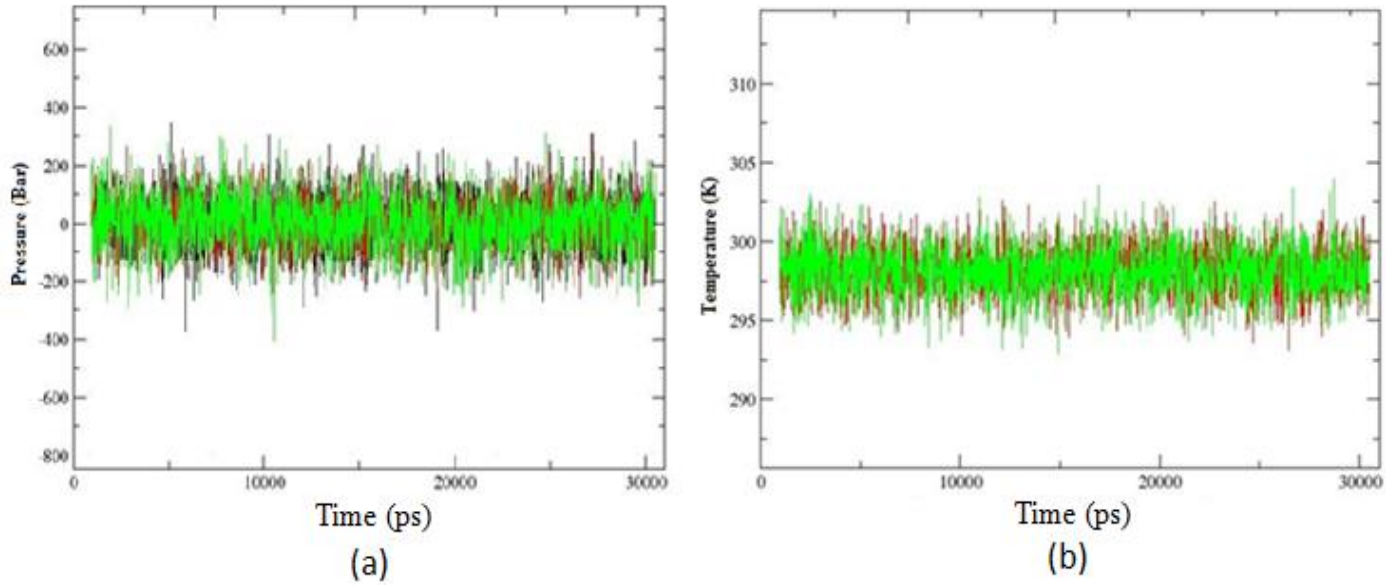

Figure 6. Representation of variation of pressure with time (a) and temperature with time (b) for all systems during the entire dynamics process. Benzene (green line), toluene (black line) and p-xylene (red line).

understanding of the phenomenon of BTX adsorption on activated carbon.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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The influence of potassium dichromate on growth and survival of the vermicomposting earthworm *Eisenia foetida*

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The present research evaluated the redox ability of Cr_6^+ in the earthworm *Eisenia foetida*, and its effects on growth and survival. An assay was conducted with an increasing doses of $K_2Cr_2O_7$ (0, 200, 400, 800, 1000, and 1200 mg $K_2Cr_2O_7$ kg^{-1} substrate) applied to a vermicomposting system which was inoculated with 30 earthworms per chamber. Earthworm growth, estimated by weight, showed a quadratic response (p = 0.03) with the lowest estimated growth at a dose of 435 mg $K_2Cr_2O_7$ kg^{-1}. Chromium (Cr) concentration increments were positively associated to mortality in adults and cocoons (p < 0.01), and total Cr accumulation in earthworms (p < 0.01). The accumulation of chromium in worms, is an indicator of the capacity of worms to resist different doses of potassium dichromate and, the effect on accumulation of Cr (VI) in vermicomposting was higher (p = 0.11). The difference of potassium dichromate in worms could indicate the oxide reduction in *E. fetidae*.

Key words: Redox chromium, growth, survival, *Eisenia foetida*.

INTRODUCTION

Chromium (Cr) is an indicator of anthropogenic heavy metal pollution in water, and its presence in effluents is associated with discharges from steel, paint, dyes, ceramic, chromate, and photography industries, tanneries, and other human activities (Kimbrough et al., 1999). The trivalent form of Cr (III) is the most stable, but the hexavalent state Cr (VI) is highly toxic to living organisms (Nriagu and Pacyna, 1988). The stronger toxicity of Cr (VI) is associated with its ability to permeate biological membranes (Rudolf and Cervinka, 2005). The chromate ions (CrO_4^{2-}) are considered more toxic than Cr (III) in the environment due to their mobility and strong oxidising capacity (Landrot, 2010). In contrast, Cr (VI) is rarely found in nature, and its presence in the environment is

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Table 1. Effect of potassium dichromate concentration on earthworm growth and mortality.

Parameter	Dose of Cr (mg $K_2Cr_2O_7$ kg^{-1})								*p*-value	
	Initial	0	200	400	800	1000	1200	Standard error of the mean	Linear	Quadratic
Length (cm)	6.49	7.16	7.17	7.42	6.72	8.15	9.14	0.264	0.12	0.10*
Weight (g)	0.68	0.74	0.65	0.71	0.69	0.78	0.89	0.022	0.12	0.03*
Mortality (%)										
Cocoon		40	21	0	46	83	96		0.06	0.03*
Larvae		70	55	0	92	97	100		0.21	0.31*
Juvenile		80	53	30	86	95	96		0.20	0.24*
Adults		21	20	12	76	93	96		0.006	0.03*

linked to anthropogenic sources (Kimbrough et al., 1999).

The earthworm *Eisenia foetida* has been used in bioassays in processes of vermicomposting to assess the dynamics of specific toxins during degradation of organic matter (OECD, 2000). Complex metabolic processes involve changes in mobility and the availability of the elements is associated with microbial activity (Sizmur and Hodson, 2009) which occur inside and outside the earthworm during the vermicomposting process. Therefore, this experiment was designed to evaluate the use of vermicomposting as a method to reduce the toxicity of chromium in the environment. Changes in growth and survival of *E. foetida* were analysed, and chromium concentrations were measured in both earthworms and vermicompost substrate.

The Lerma River has been considered by Federal Government, one of the most contaminated catchment of Mexico (Comisión Nacional de Ecología, 1988/ Consejo Lerma-Chapala, 1991-1994). Turbio River drains residual waters from leather industry of Leon city flows into the Lerma River. The problems of chromium environmental passives in soils and sediments of this river catchment, is one of the most environmental problems concern in center region of Mexico. A high total Cr content in soils and sediments of Turbio river hydrological sub basin was found by Hernández-Silva et al. (2005), with 2196.0 ppm in sediments and in soils up of 300.0 ppm.

MATERIALS AND METHODS

The glass chambers (0.30 x 0.20 x 0.15 m) were inoculated with adult *E. foetida* earthworms at the oviposition stage with a rate of 30 earthworms per bed, agree to data recorded in the OCDE, (1984). Rabbit faeces (1 kg) and fresh alfalfa (3 kg) was used as substrate in each chamber. The experiment lasted 35 days (Manyuchi and Phiri, 2013). The breeding chambers were kept in a greenhouse during the experiment. The vermicomposting pH, relative humidity, electrical conductivity, temperature and moisture were analyser and continuously monitored using a Hanna Instruments. HI 84502. The relation of C/N ratio was measure by ISO (Roht-Arriaza, 1995).

Treatments consisted of doses reference, 200, 400, 800, 1000, and 1200 mg $K_2Cr_2O_7$ kg^{-1} substrate (Sigma-Aldrich Co.) with three replicates per doses, and a total of 16 beds. The different life stages of *E. foetida* were counted (cocoon, larva, juvenile, and adult) at day 35, and the length, diameter, and weight of adult earthworms were measured by Analytical Balances and Vernier Caliper. Samples of vermicomposting and earthworm homogenates were taken from each chamber to analyse the total hexavalent Cr concentrations.

The concentration of Cr (VI) was determined by photometry with biphenyl-carbazide in acid solution (Greenberg et al., 1992). Total Cr was measured by flame atomic absorption spectrometry (Perkin Elmer, Model HGA-600) with samples treated with acid digestion (Kotz et al., 1972). The vermicomposting samples were dried at 70°C and then the acid was digested (HNO_3:HPO_4 5:1) while the earthworm homogenate was weighed, frozen at -73°C and then freeze-dried. Results were analysed as a completely randomised block (chamber) with design testing linear and quadratic effects as a function of potassium dichromate concentration (Steel et al., 1997).

RESULTS AND DISCUSSION

The length of the earthworms showed a quadratic response (p = 0.10; Table 1) with the greatest length (6.85 cm) estimated at 404 mg of potassium dichromate. According to Table 1, growth also showed quadratic response (p = 0.03) with the lowest weight (0.72 g) estimated at 435 mg dichromate. As chromium concentration increased, mortality of adults and cocoons increased (p < 0.01), but no effect was observed on larvae and juveniles. Although there was higher mortality at a higher doses of chromium, worms that survived doses of 1000 and 1200 mg $K_2Cr_2O_7$ kg^{-1} showed a tendency (p = 0.12) to increase in size and weight . As the dose of potassium dichromate increased, total Cr accumulated in the earthworms also increased (p < 0.01) without changes in the vermicomposting process (Table 2). However, Cr (VI) is tended to accumulate in the vermicomposting (p = 0.11) without changes in the earthworms. The mean temperature in the breeding chambers was 23.9°C which is close to the recommended *E. foetida* (22°C), and is within the range for survival (20 to 31°C) (Khan et al., 2007). Another parameters measured during the experiment were (relative humidity 43%, electrical conductivity 1.28 dS m^{-1}, C/N ratio 30.4 and pH 8.4) adequate for *E. foetida* growth

Table 2. Effect of potassium dichromate concentration on total chromium content and hexavalent chromium content in earthworm and substrates.

Parameter	Dose Cr (mg $K_2Cr_2O_7$ kg^{-1})							*p*-value	
	0	200	400	800	1000	1200	SEM	Linear	Quadratic
Total Cr									
Vermicompost	0.092	0.023	0.047	0.092	0.120	0.100	0.010	0.19	0.36
E. foetida	0.085	0.131	0.559	0.839	1.298	1.671	0.012	0.0003	0.002
Cr VI									
Vermicompost		0.271	0.262	0.267	0.261	0.253	0.016	0.11	0.31
E. foetida		0.037	0.068	0.051	0.063	0.094	0.018	0.15	0.39

and reproduction (Edwards and Lofty, 1972).

There are few studies on the effects of chromium on earthworm metabolism. However, considering that some metabolic pathways in earthworms are similar to those found in vertebrates (Laverack, 1963), chromium may affect DNA and protein synthesis in earthworms. Cr (VI) is reduced through short-lived Cr intermediates [Cr (V) and Cr (IV)] to ultimate stable trivalent Cr (III); this process generates reactive oxygen species which cause the DNA to damage and die (Aiyar et al., 1989). The hydroxyl radicals and hydrogen peroxide produced during the reduction of Cr (VI) could be responsible for negative effects on survival of some stages in the earthworm. Arakawa et al. (2012) demonstrated *in vitro* that Cr (III), Cr (VI) and Cr (V), the three major cellular Cr forms, induced bulky DNA adducts and oxidative DNA damage which contributed to mutagenesis of the p53 gene that leads to lung carcinogenesis.

Earthworms reach maturity between 5 and 7 cm length (Klok and De Ross, 1996), chromium concentrations did not affect this. The two stable forms of chromium are Cr (III) and Cr (VI). Chromium can be oxidised or reduced from one form to the other in the environment, depending on concentrations of the reactants, pH of the media, temperature, light intensity, and other entities involved in the reaction-surfaces/sorbents, complex agents, bacteria, oxidisers/reducers- (Rudolf and Cervinka, 2005). Results in Table 2 indicate that earthworms were able to reduce Cr (VI) to Cr (III), through the difference between Cr^{+} and Cr_6^{+}. The greatest redox activity was found at a rate of 800 mg and higher doses affected survival of *E. foetida*. It is possible that some of the chromium redox reactions occur in the gastrointestinal tract where there is significant secretion of calcium carbonate (Laverack, 1963) and significant populations of bacteria with enzymatic activity (Kim et al., 2009).

Water is one of the reactants involved in many chromium oxidation reactions, along with Cr (III) and the oxidising agent. Trivalent chromium is not very mobile in soils since it is insoluble in most environmental conditions. However, Cr (VI) is much more mobile in the environment because of its high saturation index and negative ionic form (Rudolf and Cervinka, 2005).

Sizmur and Hodson (2009) proposed that the impact of earthworms on soil metals, in terms of both metal mobility and availability, were related to changes in microbial populations, pH, dissolved organic carbon and metal speciation. Ali et al. (2015) mentioned that, earthworms have important interactions with microorganisms by stimulation of microbial populations. The presence of earthworms increases overall microbial biomass and activity and modifies the physiological profiles of microbial communities in pig manure (Aira et al., 2007).

Further studies of the changes occurring within the earthworm and its microbiota or microorganisms associated in the vermicompost are required. Bundy et al. (2002) studied the metabolism of xenobiotics by earthworms using antibiotics to inhibit gut microflora. They found that gut microflora did not influence metabolism of xenobiotics, and that some xenobiotics were not metabolised at all. This type of approach could be used in future studies to evaluate whether manipulation of Cr metabolic processes combined with other treatments in the substrate can reduce toxicity of chromium compounds.

Conclusions

Table 1 shows an ascendant increment of mortality in its different stages, from eggs to youthful, where the function of shell could act as a protective against the toxic. In the adult stage, worms show resistance in concentrations of 1000 and 1200 mg $K_2Cr_2O_7$ kg^{-1} under the process of vermicomposting, increasing its length, even at 800 mg/Kg in comparison with larvae and youth. The adaptation to concentrations of the toxic could have a relation with worm metabolism as a proof of a high resistance to chromium accumulation of earthworm *E. foetida*.

The process of vermicomposting retains most amount of $K_2Cr_2O_7$ kg^{-2} at the concentration of 400 mg $K_2Cr_2O_7$ kg^{-2}, surviving almost 100% as shown in Table 2. This table also shows the concentration of toxicant in earthworm as total chromium.

Hexavalent chromium showed a difference, which can be inferred in the process of oxide reduction in the vermicomposting (Singh et al., 2012).

Conflict of Interests

The authors have not declared any conflict of interests.

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Effect of cooking on tannin and phytate content in different bean (*Phaseolus vulgaris*) varieties grown in Tanzania

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Phytates and tannins are present in varying proportions in legume based foods. Investigation on the effect of cooking on tannins and phytates was carried out on thirty eight raw and cooked bean (*Phaseolus vulgaris*) varieties. Tannins were assayed by vanillin-hydrochloric acid method, while phytates were determined by a method developed by Haugh and Lantzsch. There was a very high significant difference (*P*≤0.00001) in the tannin concentrations between the raw and cooked bean samples. The mean tannin content for the raw and cooked samples was 1.168±0.81 and 0.563±0.503%CE, respectively. Reduction of tannins after cooking ranged from 20%CE in M'mafutala to 81%CE in GLP 2 with an average reduction of 56.3%CE. Equally, there was a very high significant difference (*P*≤0.0001) in the phytate concentrations between the raw and cooked bean samples. The mean phytate content for the raw and cooked samples were 0.0219±002 and 0.0122±003 µg/mL, respectively. Cooking reduced the anti-nutritional factors significantly (p≤0.0001). The extent of anti-nutritional factors reduction varied between bean varieties. Cooking is therefore important for mineral absorption during digestion process in humans as it makes the minerals less bound and hence physiologically available.

Key words: Beans, phytates, tannins, minerals, raw, cooking, bioavailability.

INTRODUCTION

The food that has been eaten and swallowed is mixed with saliva, gastric fluids and churned by peristaltic movements of the stomach into a creamy fluid called the chyme. The chyme is an extremely complex matrix, in

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which the physico-chemical conditions change continuously with diet (Päivi et al., 2003). The absorption of mineral elements is a very complicated system and several components in foods can form soluble and insoluble complexes with these elements under intestinal conditions (Gibson et al., 2010). Legumes, cereals and other plant related foods, which form the base of diets for most African communities, contain phytate (*myo*-inositol hexaphosphate and other inositol phosphates), a known inhibitor of iron and zinc absorption (Hurrell et al., 1992; Lönnerdal, 2000; Afify et al., 2011). Normally encountered levels of phytates in cereals and legumes can reduce protein and amino acid digestibility by up to 10% (Gilani et al., 2012). Phytate-containing foods may be a strong contributing factor for poor iron and zinc status in population that consume these diets (Gibson et al., 2010). Common beans (*Phaseolus vulgaris*) are a staple food and the major source of iron for populations in Eastern Africa and Latin America but a major constraint to beans, is low iron absorption, attributed to inhibitory compounds (Petry et al., 2015). Several attempts could be done to reduce anti-nutritional effects of phytate such as soaking, drying, germination and drying. In a study conducted in Egypt to reduce phytate content, the phytate was significantly reduced from 23.59 to 32.40% and 24.92 to 35.27% for soaking and germination treatments, respectively (Afify et al., 2011). However, absorption of mineral elements from diets is likely to be higher with an enhancer when compared with low-bioavailability diets (Collings et al., 2013). Phytic acid inhibits iron absorption in a dose-dependent manner above a molar ratio of phytate to iron of 1:7 (Hurrell, 1992). However, ascorbic acid counteracts the effect of phytate when ascorbic acid:iron ratio exceeds 4:1 (Zijp et al., 2010). In legume grains, phytate is located in the protein bodies in the endosperm and occurs as a mineral complex, which is insoluble at physiological pH of the intestine (Sandberg, 2002). In a study in Malawi, a high intake of phytate was correlated with poor iron and zinc status in pre-school children (Gibson et al., 2010).

Similarly, tannins (polyphenols) are most commonly found in dicotyledons, particularly in Leguminosae (Salunkhe et al. 1990). With regard to the legume grains, tannins are found in dry beans (*Phaseolus vulgaris* L.*),* peas *(Pisum sativum)*, chickpeas *(Cicerarietinum* L.), faba beans (syn. broad bean, field bean; *Vicia faba* L.), cowpeas (*Vigna unguiculuta* L.) and lentils *(Lens culinuris* L.). In most grain legumes, tannins are present as condensed tannins (Salunkhe et al., 1990). According to Hurrell et al. (1999) and Yuwei (2013), certain polyphenols are able to form complexes with iron, which makes the complex-bound iron unavailable for absorption. Brune et al. (1989) reported that the amount of iron binding phenolic galloyl groups in foods roughly corresponds to the degree of inhibition of iron absorption. However, Hurrell et al. (1999) concluded that all major types of food polyphenols can strongly inhibit dietary non-heme iron absorption. On the other hand, literature reveals that plant tannins play a protective role in the defence of plants against environmental influences. Increased concentrations of tannins therefore have been found in plants under environmental stress as compared to similar plants without environmental stress (Islam et al., 2002; Yuwei, 2013).

Most studies have investigated the content and effect on mineral and protein absorption of tannins and phytates in foods. However, few studies have explored the effect of cooking on the levels of these anti-nutritional factors. The aim of the study therefore was to investigate whether conventional cooking had an effect on reducing the levels of tannins and phytates in the *P. vulgaris* beans which is among the major protein and mineral source in the diet of most African communities.

MATERIALS AND METHODS

Bean samples

A total of 38 *P. vulgaris* bean seed varieties were obtained from University of Nairobi, Kenya and multiplied at Sokoine University of Agriculture, Morogoro Tanzania to obtain enough beans to be subjected to cooking and laboratory analysis for tannins and phytates. Tannin and phytate content were determined on raw and cooked bean grain samples. The analyses were done in the Department of Food Technology, Nutrition and Consumer Sciences, in collaboration with the Department of Animal Science and Production laboratories.

Preparation of raw and cooked bean grain samples

For each variety, two batches of bean grain samples were prepared. One batch was ground in its raw state and bean flour was obtained. The second batch was boiled in water as it is often done in homesteads, until cooked. The samples were mashed into thick paste and put in oven maintained at 105°C for 24 h. Tannin and phytate analysis was carried out on the raw and cooked samples.

Determination of tannin content

A quantitative value of tannin was assayed by vanillin-hydrochloric acid method. For each bean variety, about 0.25 g of milled sample was weighed and placed into Erlenmeyer flask. 10 mL of 4% HCl in methanol was pipetted in a flask. The flask was covered with para-film and shaken for 20 min on a wrist action shaker. The extract was transferred into centrifuge tubes and centrifuged for 10 min at 4500 rev/min. The supernatant aliquot was transferred into 25 mL flask and the residue from centrifuge tube was rinsed using 5 mL of 1% HCl in methanol, covered with para-film and shaken for another 20 min. The residue mixture was centrifuged again for 10 min and the aliquot were combined with the first extract.

A set of catechin standard solutions were prepared and added into test tubes with 1 mL absolute ethanol. Slowly, 5 mL of vanillin-HCl reagent was added in each sample and 1 mL sample extract. A blank sample with 5 mL of 4% HCl in absolute methanol was prepared. The absorbance of standard solutions, sample extracts and sample blanks were read on a spectrophotometer set at 500 nm exactly 20 min after adding vanillin-HCl reagent to the standard solutions and sample extracts. A standard curve absorbance (y) against catechin concentration (x) was prepared from the catechin

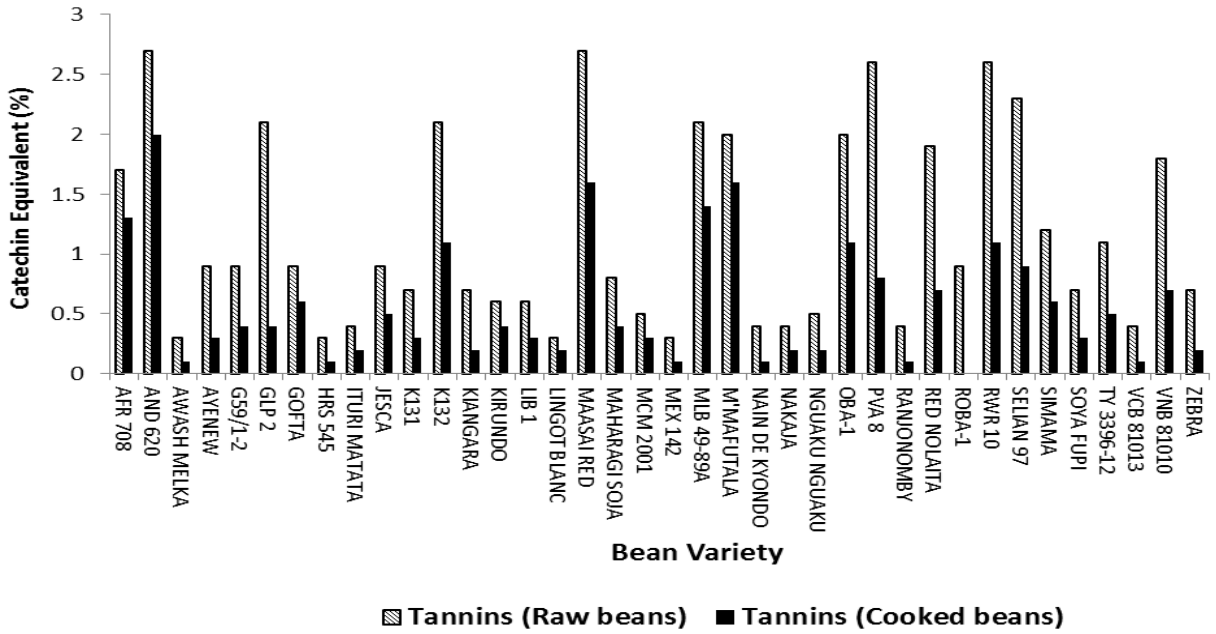

Figure 1. Effect of cooking on tannin levels in raw and cooked beans.

standard solution readings and the slope was computed and hence tannin concentration was determined from the equation of the curve.

$$CE(\%) = \frac{CC\ x\ VM}{VE\ x\ Wt}$$

Where CE = catechin equivalent; CC = catechin concentration (mg m/L); VM = volume made up (mL); VE = volume of extracts and Wt = weight of sample.

Determination of phytate content

Phytate was determined by a sensitive method developed by Haugh and Lantzsch (1983). About 1 g of bean samples was weighed and phytate was extracted with 10 mL of 0.2 NHCl. During the extraction process, the mixture was stirred for 30 min by using a magnetic stirrer. To a 0.5 mL, 1 mL of ammonium iron III sulphate in HCl (0.2 g ammonium iron (III) sulphate $12H_2O$ in 100 mL of 2N HCl and made up to 1 L) was added in the test tube and boiled for 30 min in a boiling water bath and then cooled to room temperature in ice water. The contents of the tube were mixed and centrifuged for 30 min at 3000 rev/min. 1 mL of the supernatant was transferred to another tube and 1.5 mL of 2-2′-bipyridine solution (10 g of 2-2′-bipyridine with 10 mL thioglycolic acid in 1000 mL water) was added. Absorbance of the solution was determined at 519 nm wavelength against distilled water. A standard curve was prepared using phytate-phosphorus at concentration between 3 and 30 µg/mL treated the same way but without the sample. All determinations were done in triplicate.

Statistical analysis

Data for all the determinations were analysed using excel office 2010 and statistical product and service solutions (SPSS version 16) computer software. Students t-test was used to test for significance difference between the treatment means for each variety (Fresh and cooked), while one way analysis of variance was used to test for significance differences among the varieties. Mean separation were done by Duncan multiple range test at P<0.05.

RESULTS

Effect of cooking on tannins and phytates contents of *P. vulgaris* bean varieties

The tannin and phytate levels in the *P. vulgaris* bean varieties are presented in Figures 1 and 2. A paired t-test analysis showed a very significant difference ($P \leq 0.00001$) in the tannin concentrations between the raw and cooked bean samples. The mean tannin content for the raw and cooked samples were 1.168±0.81 and 0.563±0.503%CE, respectively with 0.457-0753%CE 95% confidence interval of the mean difference. The minimum and maximum tannin values for the raw and cooked samples were 0.3 to 2.7 and 0.0 to 2.0%CE, respectively. The raw and cooked bean varieties with highest tannin concentrations were Maasai Red and (2.7 and 1.6%CE) and AND 620 (2.7 and 2.0%CE) while the lowest was Lingot Blanc (0.3 and 0.2%CE) and MEX 142 (0.2 and 0.1%CE), respectively (Figure 1).

Similarly, phytate concentration varied significantly between varieties (Figure 2). There was a very significant difference (P=0.0001) in the phytate concentrations between the raw and cooked bean samples. The mean phytate content for the raw and cooked samples were 0.0219±002 and 0.0122±003 µg/mL, respectively with

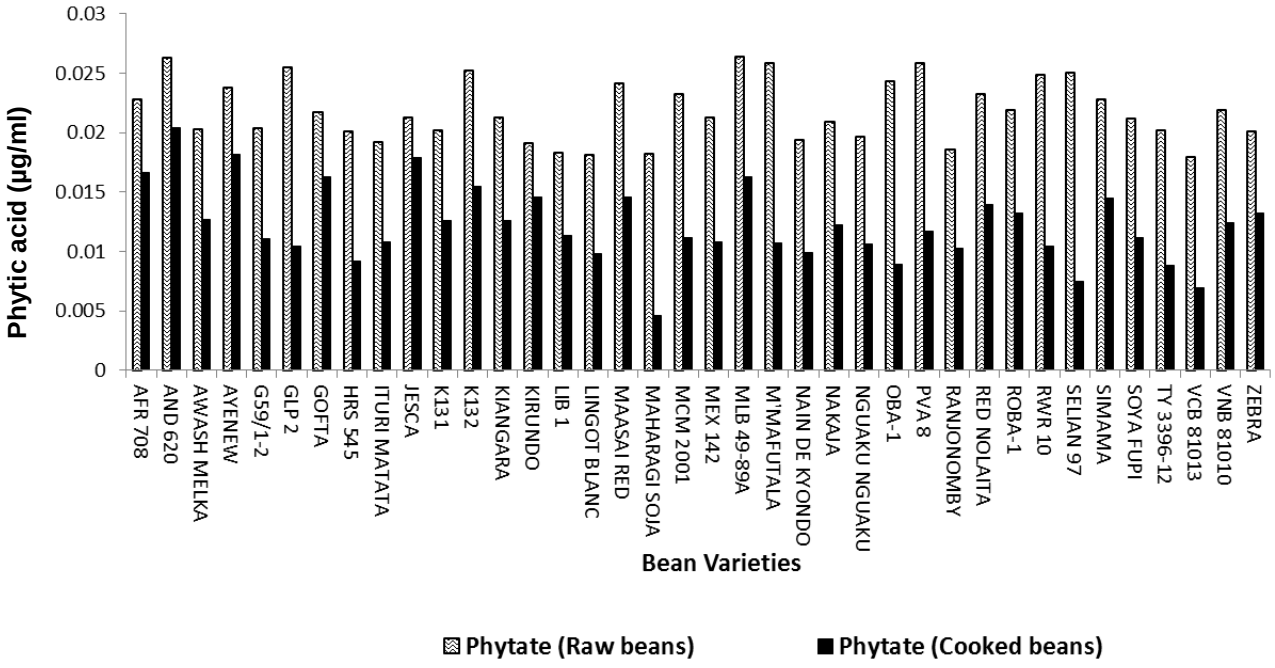

Figure 2. Effect of cooking on phytate levels in raw and cooked beans.

0.0086 to 0107 µg/mL 95% confidence interval of the mean difference. The minimum and maximum phytate values for the raw and cooked samples were 0.021 to 0.0227 and 0.0111 to 0.0133 µg/mL, respectively. The raw bean varieties with highest phytate concentrations were MLB 49-89A (0.026 µg/mL) and AND 620 (0.0263 µg/mL), while the lowest was VCB 8101 (0.018 µg/mL) and Maharagisoja (0.0182 µg/mL).

Percentage reduction of tannins and phytates after cooking of bean samples

A one way analysis of variance showed a very significant reduction (P=0.0001) in tannins from raw to cooked bean samples. Tannin reduction ranged from 20% in M'mafutala to 81% in GLP 2 with an average reduction of 56.3±15.5% of all the samples tested with 0.48 to 58.76% confidence interval of the mean (Table 1). Equally, there was a very significant reduction (P=0.0001) in phytate from raw to cooked bean samples. Phytate reduction ranged from 16% in Jesca to about 75% in Maharagisoja with an average reduction of 44.2±13.3% of all the samples tested with 39.7to 48.57% 95% confidence interval of the mean (Table 1).

DISCUSSION

The results revealed that cooking has an effect of reducing the anti-nutritional factors of *P. vulgaris* bean, although the extent of reduction varies from one bean variety to the other. With regard to mineral absorption, cooking is therefore advantageous as an amount of minerals and proteins will be less bound and hence become physiologically available. A recent review has revealed that cooking improves nutritional quality of beans as well as other vegetables which contain high amounts of anti-nutritional factors in raw state (Fabbri and Crosby, 2016). In some studies, cooking decreased significantly ($p \leq 0.05$) polyphenol content inherent in the karkade seeds, but other processing methods studied did not (El Gasim et al., 2008). In another study, cooking resulted in a high reduction of phytate and oxalates on green leafy vegetables (Ilelaboye et al., 2013). Heat degradation, leaching out effects, change in chemical reactivity and formation of insoluble complexes might be the factors that resulted in the significant reduction of these anti-nutrients by cooking (Alonso et al., 2000; Yagoub et al., 2004; Wu et al., 2016). Other studies however, gave mixed results, the phytic acid content of karkade seeds (888.33 mg/100 g) and sorghum genotypes were unaffected by soaking, sprouting and cooking (Alonso et al., 2000; Wu et al., 2016). Additionally, it was observed that most of the bean varieties, which were black and red coloured such as GLP 2, K132, PVA8 and RWR 10 were the ones with relatively highest tannins as compared to the white, yellow and light brown coloured such as Awashmelka, HRS 545 and Ranjonby; although colour was not a factor investigated in this study.

According to Manan et al. (1987), the effects of a

Table 1. Percentage tannin and phytate reduction of cooked bean samples.

Bean variety	Tannin reduction (%)	Phytate reduction (%)	Bean variety	Tannin reduction (%)	Phytate reduction (%)
MAHARAGI SOJA	50.0	74.7	VNB 81010	61.1	43.4
SELIAN 97	60.9	70.1	NAKAJA	50.0	41.6
OBA-1	45.0	63.4	KIANGARA	71.4	40.8
VCB 81013	75.0	61.7	RED NOLAITA	63.2	40.3
GLP 2	81.0	59.2	ROBA-1	55.6	39.7
M'MAFUTALA	20.0	58.7	MAASAI RED	40.7	39.7
RWR 10	57.7	58.2	K132	47.6	38.5
TY 3396-12	54.5	56.4	MLB 49-89A	33.3	38.3
PVA 8	69.2	54.8	LIB 1	50.0	38.3
HRS 545	66.7	54.2	K131	57.1	37.6
MCM 2001	40.0	51.9	AWASH MELKA	66.7	37.4
MEX 142	66.7	49.3	SIMAMA	50.0	36.4
NAIN DE KYONDO	75.0	49.0	ZEBRA	71.4	34.3
SOYA FUPI	57.1	47.2	AFR 708	23.5	27.2
NGUAKU NGUAKU	60.0	46.2	GOFTA	33.3	24.9
LINGOT BLANC	33.3	45.9	KIRUNDO	33.3	23.6
G59/1-2	55.6	45.6	AYENEW	66.7	23.5
RANJONOMBY	75.0	44.6	AND 620	25.9	22.4
ITURI MATATA	50.0	43.8	JESCA	44.4	16.0

normal cooking process on the phytic acid and the nutritional quality of three varieties of Pakistani peas and lentils, which involved steeping, followed by boiling of the seeds, resulted in a reduction of considerable amounts of phytic acid in peas and lentils by 82 and 76%. The study revealed that boiling caused the highest reduction in tannins followed by autoclaving and microwave cooking. Autoclaving and fermentation were the most effective in reducing phytic acid content. However, there was no apparent relationship observed between the loss of phytates from the seeds and the improvement of nutritional quality. A study in Nigeria by Nzewi and Cemauck (2011) reported a significant reduction of phytate from 1.54±0.04 to 0.18±0.11 mg/g, representing 88% reduction in boiled asparagus beans. Similarly, tannins were reduced from 3.72±0.12 to 1.88±0.08 mg/g or 49% reduction. Iorgyer et al. (2009) studied the effect of boiling on pigeon pea seeds and reported no significant effect on phytates but cooking resulted in 47.06% destruction of tannins in pigeon peas.

In another study, Khattab and Arntfield (2009) observed that the residual content of antinutritional factors of cowpeas, peas and kidney bean seeds after soaking, boiling, roasting, microwave cooking, autoclaving, fermentation and micronization resulted in significant decreases in tannins, phytic acid, trypsin inhibitor activity and oligosaccharides as compared to the raw seeds. Similarly, Vijayakumari et al. (1996) revealed that cooking for 3 h resulted in significant reduction of phytic acid, raffinose, stachyose and verbascose in both germplasms of the Indian tribal pulse, *Mucuna monosperma*, while autoclaving was more effective in eliminating the contents of phytic acid in the germplasms.

In several single-meal studies, phytate has been shown to significantly inhibit iron and zinc absorption, but few studies have explored the effects of long-term modification of phytate intake (Yuwei, 2013). Lind et al. (2003) assessed the longitudinal effects of the extensive reduction of the amount of phytate in infant cereals, a common complementary food, on micronutrient status. Despite a reduction in the daily phytate intake from infant cereals of 77%, they found no greater effect on haemoglobin, serum ferritin, or serum zinc than that with commercial complementary cereals, which were richer in phytate. Feeding infant formula, with its lower iron content but presumably higher bioavailability, resulted in a significantly lower haemoglobin and higher prevalence of anaemia than did feeding phytate-reduced infant cereal. Iron-fortified infant cereals are commonly used in complementary diets (Mamiro et al., 2004). Other antinutritional compounds such as phytohaemaglutinins, which are alectin present in many varieties of common bean, especially red kidney and faba beans can be deactivated by cooking beans at 100°C (212°F) for ten minutes (Yuwei, 2013).

Conclusion

Conventional cooking has an effect on reducing the

tannins and phytates present in *P. vulgaris* beans. This is beneficial in that some amount of minerals will not be bound and hence become physiologically available for metabolic activities. This is advantageous for the vegetarians and most African households with respect to mineral intake due to the fact that beans are an important food item consumed to a large extent and mostly on daily basis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Genomic DNA isolation method from fresh wheat leaf samples without liquid nitrogen

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The advent and application of molecular markers technology enabled dissection of genomic regions underling important agronomic traits in crop plants. Isolation of sufficient quantity and good quality DNA from a large number of plant samples is a major challenge in most genetic and genomic analyses. Wheat is the world's third most important food security crop on which most molecular breeding and genetic engineering researches have been undertaken. So far, several wheat DNA isolation protocols and commercial kits are available. However, in some, the cost is so prohibitive for small laboratories, and hence finding cost effective alternative makes small biotechnology laboratories of developing countries beneficial to the technology. The present study was targeted to provide alternative economical, reliable and safer genomic DNA isolation method in wheat. Accordingly, genomic DNA was isolated from 27 bread wheat recombinant inbred lines (RIL) raised in greenhouse with and without liquid nitrogen. From 100 mg, 20-day old leaf samples, averagely 1890.7 ng/µl sufficiently pure genomic DNA (A260/A280 ratio of 1.8) was extracted. In the extraction process, ammonium acetate and sodium acetate solutions were also avoided. Polymerase chain reaction (PCR) analysis of the extracted DNA using two simple sequence repeats (SSR) markers produced polymorphic bands confirming the suitability of the extracted DNA for PCR. Therefore, it is possible to deduce that we have adopted a simple, cost effective, reliable and safer DNA isolation method that can help to extract good quality and sufficiently pure genomic DNA for further molecular studies.

Key words: Wheat, genomic DNA isolation, liquid nitrogen, polymerase chain reaction (PCR) analysis, simple sequence repeats (SSR) markers.

INTRODUCTION

Nowadays, molecular markers are widely used to promote the speed and efficiency of crop improvement program. Molecular marker technology becomes a very useful tool toidentify and localize genes/Quantitative Trait

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Loci (QTLs) underling economically important agronomic traits, germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization, phylogenetic analysis, marker-assisted selection (Gupta et al., 1999). So far, several DNA marker systems have been developed and are routinely being applied in plant breeding program. Some of them are restriction fragment length polymorphsm (RFLPs), random amplified polymorphic DNAs (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR), simple sequence repeats (SSR) or microsatellite and single nucleotides polymorphism (SNPs).Although each marker system has its own pros and cones, next generation sequence derived SNPs marker has been considered to be the most powerful, automated and abundant marker system suitable for genomic studies on a wide range at genomic scales (Rafalski, 2002; Zhu et al., 2003).

DNA extraction is the preliminary step in all DNA marker assays. Marker assisted crop improvement involving scanning of large sets might be hundreds to thousands of germplams, and isolation of sufficient quantity and high quality genomic DNA from such huge plant samples is the major bottleneck in most genetic and genomic studies (Xin and Chen, 2012). Although the advent of polymerase chain reaction (PCR) enabled to amplify minute quantity (nano-gram) of DNA, production of reproducible PCR product requires sufficiently pure DNA. Wheat is the world's third most important food security crop (Green et al., 2012) on which most molecular breeding and genetic engineering researches have been undertaken.

So far, enormous wheat DNA isolation protocols are available including Stein et al. (2001), Dellaporta et al. (1983), Sharp et al. (1988), Murray et al. (1980), Chun et al. (1993), McCarthy et al. (2002), Clarke et al. (1989), Benito et al. (1993), etc. Including these, most reported plant DNA isolation protocols utilize liquid nitrogen for easily grinding of plant tissues without degradation of the intact DNA by the plant's native enzymes. However, liquid nitrogen has been complained to be hazardous, difficult for handling and costly (Saini et al*.,* 2013). In addition, although several plant genomic DNA extraction commercial kits are available (Xin and Chen, 2006); their cost is so prohibitive for small laboratories (Xin and Chen, 2012).Hence, adopting or developing cost effective DNA isolation method which can eliminate the requirement of liquid nitrogen or highly costly DNA isolation kit can be promising and economical to scientists of developing world. Therefore, the present research was initiated to develop/adopt an economical genomic DNA isolation protocol from wheat fresh leave samples without liquid nitrogen for downstream molecular studies.

MATERIALS AND METHODS

The experiment was conducted at International Center for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India in 2016. As part of the protocol, 100 mg of bread wheat leaves were collected from 20 days old seedlings raised under greenhouse condition. Collected leaves were rinsed with distilled water, blot dried, cut into1 to 1 – 2 cm length and placed in sterile mortar and pastel. After rapping with aluminum foil, the mortar and pestle (with the sample) were maintained in -70°C freezer for 12 h. Then, deep frozen leaves were quickly gridded using mortar and pestle and transferred into sterile 2.0ml Eppendorf tube using sterile spatula. Subsequently, 700 µl pre-warmed (65°C) 2% CTAB extraction buffer (composed of 1 M Tris-HCl, 5 M NaCl, 0.5 M EDTA, 2.0 g CTAB powder and 1.0 g PVP) with 0.2 vol% β-mercaptoethanol was added and then incubated in water bath at 65°C for 40 min. Then, 800 µl of chloroform/ iso-amayl alcohol (24:1) was added and shaken vigorously. After centrifugation at 12,000 rpm for 10 min, the supernatant (~ 600µl) was transferred into a new 1.5 ml tube. After adding 600µl cold 100% iso-propanol, it was carefully shaken by inversing the Eppendorf cap. Freezing for 2 h at -20°C followed by centrifugation at 12,000 rpm for 10 min resulted in DNA pellet. The DNA pellet was washed twice with 800 µl of cold 75% ethanol with subsequent centrifugation at 12,000 rpm for 10 min. The resulted DNA pellet was air-dried and re-suspended in 100 µl of 1× TE buffer for 1 h at room temperature. Finally, 1 µl of RNaseA (10 mg/ml) was added and incubated in water bath at 37°C for 30 min. The resulted DNA concentration and quality were determined using Nanodrop Spectrophotomer at wavelength of A260and A280 and in 1% agarose gel at 100V for 1 h. Nanodrop readings of the DNA isolated with and without liquid nitrogen were analyzed using SAS software 9.2 version (SAS Institute Inc., 2008).

Polymerase chain reaction (PCR) test

The DNA obtained the current protocol was tested using available and published two polymorphic SSR markers closely linked to *Septoria tritici blotch* resistance gene *stb* 6 (Figure 1A) and *stb11* (Figure 1B) in wheat. The PCR amplification was carried out in 25 µl reaction mixture that composed of 10×PCR buffer with $MgCl_2$,10 mM dNTPs, 0.4 µM each of forward and reverse primers, 1 µl dimethyl sulfoxide (DMSO), 1 µl Taq DNA polymerase (5 Units), 1 µl template DNA and 16.5 µl double distilled sterile H_2O using a thermal cycler (Applied Biosystems Veriti 96 Well Thermal Cycler). Amplification conditions involved an initial denaturation at 94°C for 3 min followed by 45 cycles with 1 min denaturation at 94°C, 1 min annealing at 55°C (xgwm369) and 52°C (xbarc137), primer extension at 72°C for 2 min followed by final extension step of 10 min at 72°C. PCR product was fractionated in 3% agarose gel electrophoresis using 1× TAE buffer at 100V for 2 h. The gel was stained with ethidium bromide and visualized under UV light and subsequently photographed. A 50 bp ladder was used to determine the amplification size.

RESULTS AND DISCUSSION

To determine the reliability and efficiency of the current extraction method, genomic DNA was isolated from 27 bread wheat RIL obtained from CIMMYT both with- and without liquid nitrogen (Table 1). As shown in Tables 1 and 2, the quality of extracted DNA estimated based on UV absorbance ratio of λ260 to λ280 is averagely 1.8458 (Table 2) which lies in the acceptance range of 1.8 to 2.0. It was confirmed that the DNA extracted in the present method without liquid nitrogen is sufficiently pure for further molecular downstream studies. Surprisingly, this

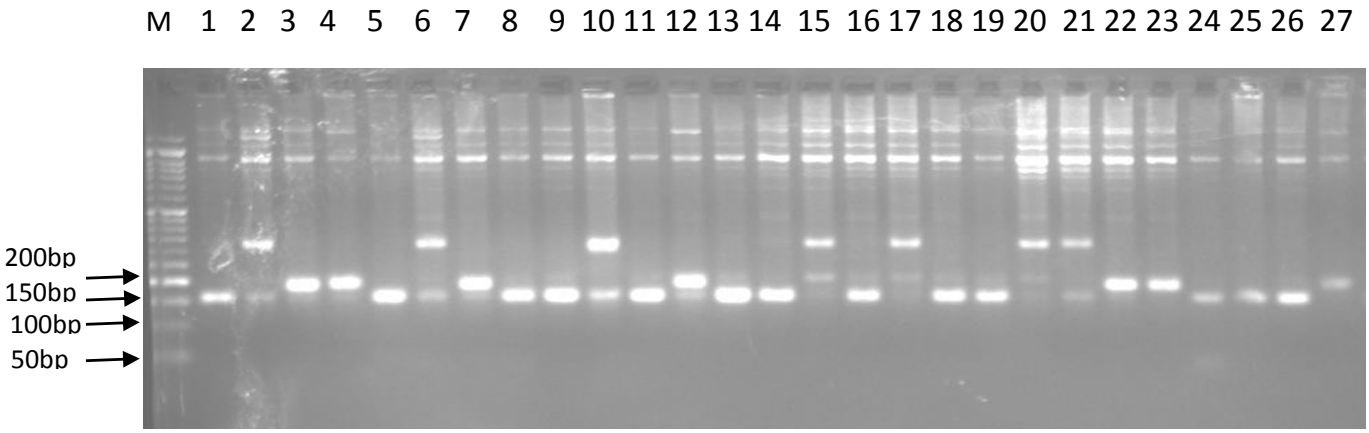

Figure 1A. PCR amplification of the SSR marker linked to *stb6* gene with *gwm369* primer. The marker produced polymorphic bands and the resistance gene is expected in those with 184bp amplification size.M:50bp ruler ladder, and 1to 27 indicate wheat lines used in this experiment.

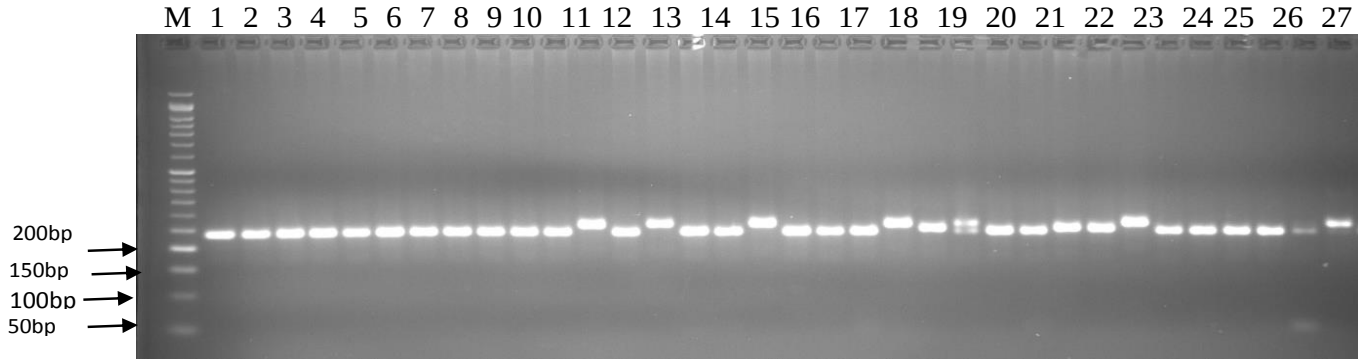

Figure 1B. PCR amplification of the SSR markers linked to *stb11* gene of wheat that conifers resistance to septoria disease resistance using *xbarc137*marker. The marker produced polymorphic bands among the samples and the resistant lines are expected to have an amplification size of 260bp.M:50bp ruler ladder, and 1to 27 indicate wheat lines used in this study.

Table 1. Nanodrop Spectrophotomer estimation of DNA quantity and quantity extracted using and without liquid Nitrogen.

Sample Code	Without liquid Nitrogen	DNA Quality λ260/λ280	DNA Concentration ng/µl	With liquid Nitrogen	DNA Quality λ260/λ280	DNA Concentration ng/µl
S1	1	1.733	1409	2	1.922	1857
S2	1	1.965	2926	2	1.959	2422
S3	1	1.938	2108	2	1.978	1610
S4	1	1.917	2030	2	1.956	1832
S5	1	1.255	2334	2	1.961	2520
S6	1	1.924	2534	2	1.971	2656
S7	1	1.284	1217	2	1.969	1837
S8	1	1.973	2688	2	1.966	2372
S9	1	1.721	2134	2	2.02	1598
S10	1	1.374	2667	2	1.943	1729
S11	1	1.983	1979	2	1.936	2435
S12	1	1.882	2796	2	2.024	1945
S13	1	1.928	2267	2	1.965	1848

Table 1. Contd.

S14	1	1.931	2603	2	1.90	2185
S15	1	2.00	1414	2	1.96	1550
S16	1	1.988	1364	2	1.978	1513
S17	1	1.98	889	2	1.964	1383
S18	1	1.915	2298	2	1.919	1869
S19	1	1.985	2140	2	1.97	2094
S20	1	1.948	1218	2	1.808	1818
S21	1	1.96	1737	2	2.023	1725
S22	1	1.977	1277	2	2.014	1398
S23	1	1.962	1389	2	1.901	1731
S24	1	1.60	2580	2	1.806	2532
S25	1	1.81	885	2	1.905	1688
S26	1	1.923	852	2	1.807	1459
S27	1	1.981	1315	2	1.975	1510

Table 2. Summary of extracted DNA quality and quantity from fresh leaf samples of wheat with- and without liquid nitrogen.

Methods	Mean	
	Quality (A260/A280)	DNA Concentration (ng/µl)
1	1.8^{b}	1890.7^{a}
2	1.9^{a}	1893.2^{a}
CV(%)	8.4	28.07

Means in a column with the same letter are not significantly different by Tukey'sStudentized Range (HSD) Test at α =5. % significant level. Method 1= without Liquid nitrogen and Method 2= with liquid nitrogen.

quality is significantly higher than the DNA extracted using liquid nitrogen. This might be due to deep freezing of the mortar and pestle which provided longer cold environment to facilitate easy crushing of the leave samples and to inhibit DNA degradation by plant native enzymes.

Regarding extracted DNA concentration, there is no statistically significant difference by Tukey's Studentized Range (HSD) Test at α = 5% significant level (Table 2). In PCR reaction, the amount of DNA concentration required per reaction is small (about 100ng). Thus, the DNA concentration produced by the present protocol is sufficiently enough for 1890 reactions. Hence, it is possible to deduce that the current protocol is sufficiently reliable in producing high quantity of genomic DNA that can be used for marker assisted selection (MAS), mutant analysis and other genomic studies that involve large sample studies. The very interesting finding of this research is not only avoiding the use of liquid nitrogen, it also does not involve the use of Proteinase K, ammonium acetate and sodium acetate which are commonly used by other DNA isolation protocols (Dellaporta et al., 1983; George, 2004; Dehestani and Tabar, 2007; Shahriar et al., 2011).

Conclusion

The advent and application of molecular marker technology enabled plant scientists to track the transfer or presence of gene/QTLs underlining useful agronomic traits in crop plants. Most genetic and genomic studies like MAS, mutant analysis in transformation and diversity analysis involve screening of large sets of samples, and preparation of good quality and sufficient amount of DNA from such huge plant samples is the major challenge. So far reported DNA isolation protocols Such as CTAB (Saghai-Maroof et al., 1984), SDS (Sodium Dodesyl Sulfate) (Edwards et al. 1991) and other protocols (Dellaporta et al., 1983; Rogers and Benedich, 1985; Doyle and Doyle, 1990; Ziegenhagen and Scholz, 1993; Lodhi et al., 1994) used liquid nitrogen for easily grinding of plant tissues and to deactivate the plant's native enzymes to avoid DNA degradation. However, liquid nitrogen is compiled to be hazardous, difficult to handle and costly (Saini et al., 2013). Hence, the present study

was targeted at designing a simple, economical and safe genomic DNA isolation method in wheat without the use of liquid nitrogen. Accordingly, 27bread wheat RILs were used in the study. Using 100 mg leaf samples, averagely 1890.7 ng/µl good qualities (A260/A280 ratio of 1.8) genomic DNA was isolated. This interesting result was obtained not only by avoiding liquid nitrogen but also other reagents like Proteinase K (the most costly); ammonium acetate and sodium acetate solutions were not used in this improved method. The isolated DNA samples were checked with PCR using two SSR markers linked to *stb* genes responsible for septoria resistance. Interestingly, the two markers amplified in all the 27 samples, and produced polymorphic bands. Therefore, it is important to deduce that we have produced a simple, reliable and economical DNA isolation method that does not depend on liquid nitrogen, Proteinase K, ammonium acetate and sodium acetate which fevers small laboratories in developing countries.

Conflict of Interests

The authors have not declared any conflict of interests.

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