

A photograph of laboratory glassware, including a large Erlenmeyer flask and several test tubes, all containing a vibrant red liquid. The glassware is wet with condensation, suggesting it has been used in a laboratory setting. The background is a soft, out-of-focus blue and white.

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Full Length Research Paper

Physicochemical and microbiological characteristics of Italian salami made of lamb and enriched with pequi (*Caryocar brasiliense*, Cambess)

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The purpose of this study was to evaluate and compare the inclusion of different levels of pequi (*Caryocar Brasiliense*, Cambess) pulp in the processing of Italian salami made of lamb for the evaluation of their physicochemical and microbiological characteristics. Six formulations of Italian salamis were processed: no pequi pulp (control - treatment 1); 4% pequi pulp (treatment 2); 8% pequi pulp (treatment 3), 12% pequi pulp (Treatment 4), 16% pequi pulp (treatment 5) and 20% pequi pulp (treatment 6). During the maturation period, all Italian salamis made of lamb enriched with pequi presented similar behaviors; and acidity (pH) and water activity (aw) results showed stable end values. For protein, the Italian salami made of lamb without pequi pump presented a higher content. For one of the lowest contents of fat, treatment 3 showed the highest content of humidity. The contents of fat and ashes did not present any significant difference when comparing the Italian salami formulations. A color tending to dark, yellowish and reddish shades was observed in the Italian salamis made of lamb and enriched with pequi. All Italian salamis were microbiologically satisfactory, showing ideal sanitary conditions for consumption. Pequi is a typical fruit from the Brazilian cerrado region, with characteristic color, odor and flavor, and it is an option for inclusion in Italian salamis, but additional studies should be conducted to inspect the sensory acceptability of these products.

Key words: Cerrado fruit, fermented stuffed meat sausages, physicochemical parameters, microbiological parameters.

INTRODUCTION

The lamb meat is a source of protein similar to other species; however, consumption is restricted due to factors involving the dark color, and the flavor and sharp odor. Lima (2009) states that lamb consumption in Brazil (0.7 kg/inhabitant/year) is still lower than farmer's expectation, justified by Brazilians, not used to consume

lamb and the supply is irregular as it costs a little higher than bovine meat.

An alternative to trade and increase lamb consumption would be through industrialization, for instance, stuffed lamb sausages (Pelegri et al., 2008). The market for fermented sausage has shown significant growth and

high competitiveness; these foods are part of the food habits of a considerable portion of Brazilian consumers (François et al., 2009).

Caryocar Brasiliense, Cambess is a fruit of high nutritional value, rich in vitamins A, E, C, polyunsaturated fatty acids, phosphorus, potassium and magnesium, which are substances responsible for the proper functioning of the body (Sousa et al., 2012), in developing new meat products are attractive to consumers as nutritional value, antioxidant activity (Roesler et al., 2007), color, flavor with unique and intense aroma. Although the consumption of fruits from the cerrado region has grown substantially in recent times, they are still not commercially exploited, either by lack of knowledge or lack of incentives for their commercialization and market consolidation (Silva et al., 2014).

According to Lima et al. (2007), the yellow pulp of pequi (*C. brasiliensis*, Cambess), due to its exotic flavor, strong and characteristic aroma, is greatly appreciated and consumed by the population of several states in the North, Northeast and Central West regions of Brazil as a condiment when cooking rice, chicken and meats, and in liqueur production.

Therefore, the development of salami flavored with pequi fresh pulp may make the product have high nutritional value and provide increased consumption of lamb meat. Thereby, the aim of this study was to develop Italian type salami lamb flavored with pequi (*Caryocar brasiliense*, Cambess), and to assess its physicochemical and microbiological characteristics.

MATERIALS AND METHODS

The research was experimentally conducted at the Food Engineering Unit at Instituto Federal Goiano –Rio Verde Campus, GO.

Ingredients and starter culture

Lamb was used in Italian salamis, containing 28.89 g/100 g protein, 2.7 g/100 g fat, 70.06 g/100 g humidity, 7.2 g/100 g ashes and 5.95 pH, and bacon with 9.03 g/100 g protein, 47.38 g/100 g fat, 36.41 g/100 g humidity, 1.5 g/100 g ashes and 7.2 pH. The pequi pulp had 4.04 g/100 g protein, 15.7 g/100 g fat, 65.86 g/100 g humidity, 6.6 g/100 g ashes and 6.3 pH, as well as salami condiment and starter culture.

Santa Inês lamb and bacon were acquired in local trade, packed vacuum with the seal of Federal Inspection Service, according to Normative Instruction nº3, of January 17, 2000, which regulates the humane methods of livestock slaughter (Brasil, 2000).

As a starter culture, the study used dehydrated Bactoform® T-SPX, comprised of *Staphylococcus xylosose* and *Lactobacillus pentosus*, provided by Chr. Hansen, in the proportion of 1.3 ml of culture for every 1 kg of meat, after hydration in the proportion of 25

Table 1. Formulation of Italian salamis made of lamb and enriched with pequi.

Treatment	Formulation (%)		
	Lamb	Pequi pulp	Bacon
1	80	-	20
2	76	4	20
3	72	8	20
4	68	12	20
5	64	16	20
6	60	20	20

g for every 100 ml of mineral water, according to the manufacturer's recommendations.

The commercial condiment, of brand Aglomax® possessed in its composition the following ingredients: refined non-iodized salt, natural dehydrated spices, maltodextrin, flavor enhancer monosodium glutamate, stabilizer sodium tripolyphosphate, antioxidant sodium erythorbate, preservers nitrite and sodium nitrate.

Pequi pulp was obtained from fruits that came from a farm located in the municipality of Itarumã in the interior of Goiás (0°19'45"S, 51°15'16"O, 863 m altitude) in native forest in the Cerrado biome. The region's climate was classified as megathermal or tropical water activity (aw) according to the Köppen climate classification and is a Tropical Savanna subtype, with dry winters and rainy summers.

The region's average temperature is 25°C, and the average rainfall is approximately 1.600 mm, with the highest rainfall occurring in January and the lowest in June, July and August (< 50 mm month⁻¹). The fruits were screened, sanitized in a sodium hypochlorite solution at 200 ppm, peeled and pulped manually with the help of previously sanitized stainless steel knives.

Italian salami processing

Six formulations of Italian salamis were produced, as indicated in Table 1. Figure 1 shows the flowchart of pequi-based Italian salami processing. Before salami processing, all devices were sanitized by immersion, for 10 min, in a sodium hypochlorite solution at 200 ppm.

Shoulder top and leg cuts were used, with superficial fat and visible connective tissues removed, followed by lamb chopping and grinding. Lamb and bacon grinding was conducted using an 8 mm disc in a Moedor Camargo®. After grinding, all ingredients were manually and aseptically mixed in the following order: meat, bacon, condiment, pequi pulp *in natura* (ground) and starter culture, on plastic trays previously sanitized, identified according to every treatment and submitted to stuffing. 50% meat of top and 50% meat of leg was used in each formulation.

One Embutideira Camargo® stuffer was used to stuff the lamb meat, as well as collagen casings gauge 40, suitable for salami, cut in pieces of around 20 cm length, totaling five strings of around 200 g per treatment. After the stuffing process, the strings were placed in a cooled incubator (BOD) at 25°C and relative humidity of 95% for maturation until they reached 0.90 aw. After the maturation

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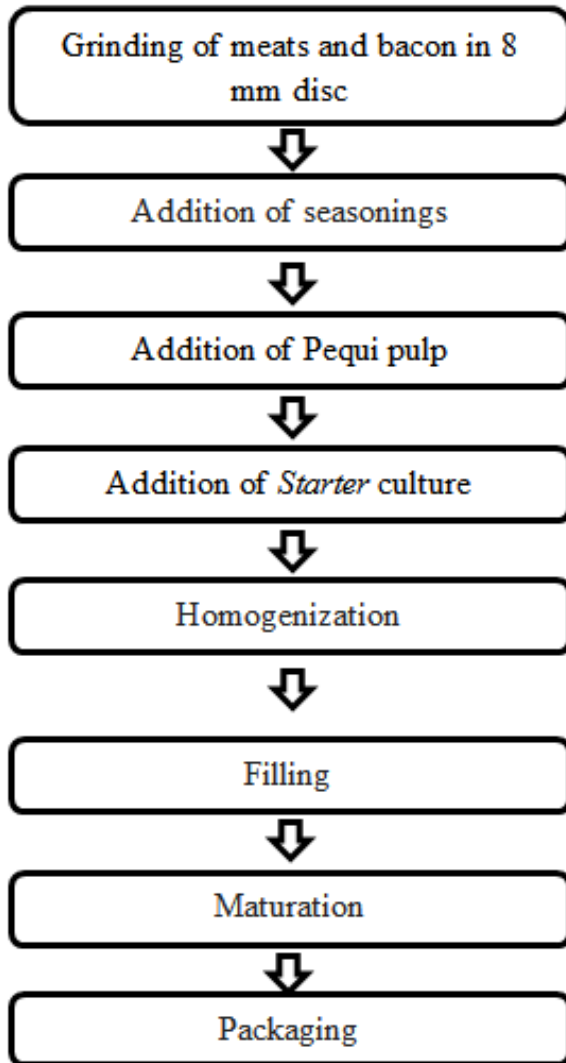


Figure 1. Flowchart of the processing of Italian salamis made of lamb and enriched with pequi.

period, they were vacuumed packed and stored at 10°C for analyses (Figure 2).

Physicochemical analyses

The characterization of raw materials (pequi pulp, Santa Inês lamb and bacon) involved the analyses of humidity, pH, fat, protein and ashes, according to the recommendations of AOAC (2000) and a color analysis that used a colorimeter (ColorQuest II, Hunter LabReston, Canada). The results were expressed in L*, a* and b*, with values of L* (luminosity or brightness) ranging from black (0) to white (100), values of chroma a* ranging from green (-60) to red (+60) and values of chroma b* ranging from blue (-60) to yellow (+60), according to Paucar-Menacho et al. (2008).

During salami maturation, the pH value and water activity were determined at days 1, 5, 9, 13, 17, 21, 25 and 29. The pH value was determined using Hanna® pH bench meter, model H1 221, and the water activity was determined using a Hygropalm, Model Aw1®.

Three samples from each treatment were randomly collected for

the analysis of Italian salamis with pequi; and they were analyzed in terms of protein, humidity, fat and ashes, following the recommendations of AOAC (2000), and color according to Paucar-Menacho et al. (2008). The treatments consisted of three replicates and analyzes in triplicate per replicate, totaling nine results by treatment.

Microbiological analyses

The microbiological analyses were conducted with the lamb, bacon, pequi pulp and Italian salamis with pequi to evaluate the quality of raw materials and Italian salamis with pequi, using the most probable number (MPN) method for total coliform and thermotolerant coliform counts, a classic cultural method of salmonella determination and direct count on plates for *Staphylococcus aureus* analysis, following the recommendations of Silva et al. (2001).

Statistical analyses

The physicochemical analyses of Italian salamis with pequi were conducted using a completely randomized design (CRD), in triplicate, with three repetitions for all analyses. The results were submitted for the analysis of variance (ANOVA) and Tukey's test, with 5% significance level, using Sisvar software (Ferreira, 2003). The pH and water activity results during the maturation period were submitted for the analysis of variance and regression analysis using Sigma Plot 10.1, Jandel Scientific (2010).

RESULTS AND DISCUSSION

Figure 3 shows the pH values of Italian salamis made of lamb and pequi during the maturation period, indicating that all treatments present the same behavior of reduced pH values on the first five days due to released lactic acid, which is created after the fermentation of hexoses by lactic bacteria, with pH increase after this period, due to reactions of decarboxylation and deamination of amino acids, releasing ammonia and increasing pH values.

The reduction in the pH values on the first days of fermentation is important for the production of quality salamis through the inhibition of pathogenic and degrading microorganisms, color conversion and stabilization, and production of compounds related to the flavor and aroma characteristics (Terra et al., 2004).

Furthermore, the pH values of Italian-type salami observed in this experiment are similar to those reported by Macedo et al. (2008) in their study of the preparation of sausages fermented by probiotic *Lactobacillus*. Similar results to this study have been reported in fermented cured products (Backes et al., 2013; Lorenzo and Franco, 2012; Cavalheiro et al., 2010; Olivares et al., 2010).

The reduction in the pH values leads to reduced water activity due to reduced water retention capability of meat proteins as pH approaches the isoelectric point, speeding up dehydration and slowing down the water activity (Chasco et al., 1996). Figure 4 shows this behavior, illustrating the water activity of Italian salamis made of lamb and enriched with pequi during the maturation period.

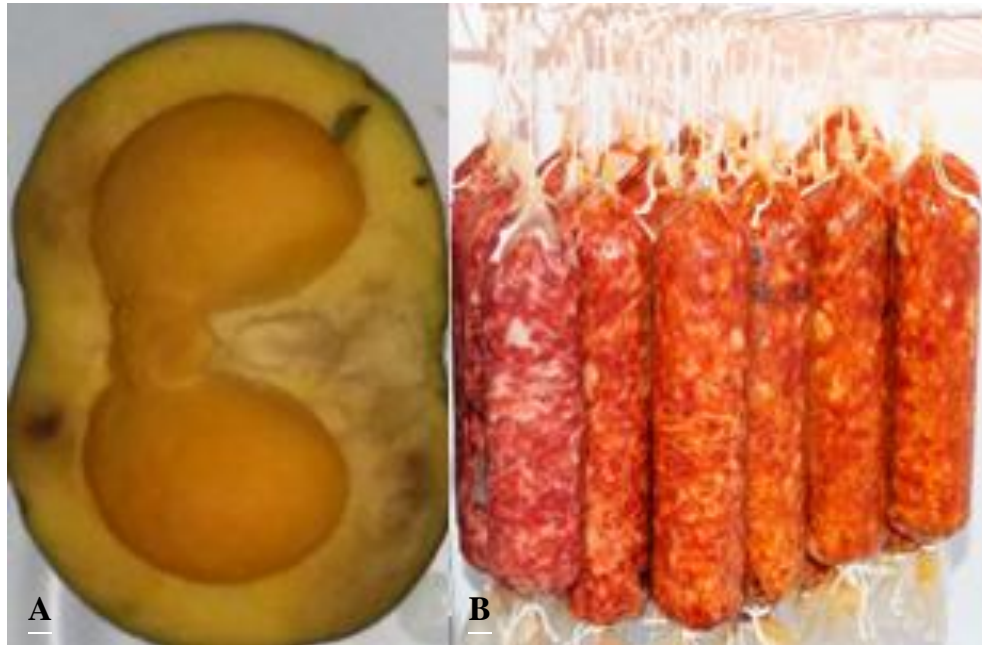


Figure 2. Unpeeled pequi (A) and Italian salami made of lamb enriched with pequi (B).

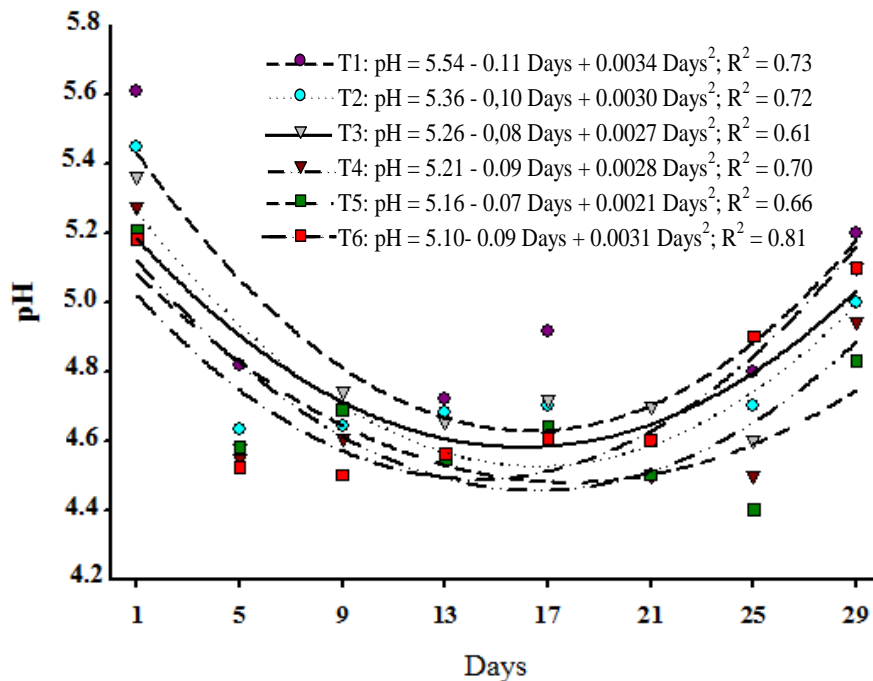


Figure 3. pH of Italian salamis made of lamb and enriched with pequi during the maturation period if Italian salamis made of lamb and enriched with pequi during the maturation period.

The reduction in the pH values and water activity of Italian salamis made of lamb and enriched with pequi during the maturation period agree with the behavior

reported by Cirolini et al. (2010) when analyzing an Italian salami produced with native starter cultures, and by Macedo et al. (2008), when studying the production of

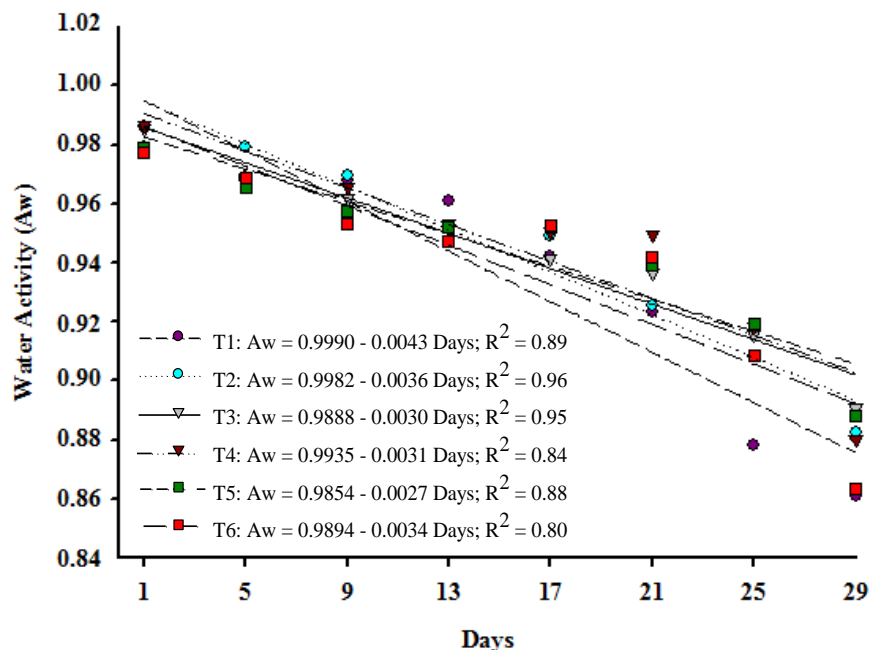


Figure 4. Water activity of Italian salamis made of lamb and enriched with pequi during the maturation period.

Table 2. Results from pH and water activity (Aw) analysis of Italian salamis made of lamb and enriched with pequi.

Parameter	Treatment						CV (%)	F value
	1	2	3	4	5	6		
pH	4.70 ^a	4.91 ^a	4.75 ^a	4.94 ^a	4.83 ^a	4.81 ^a	3.56	0.331 ^{ns}
Aw	0.87 ^a	0.88 ^a	0.88 ^a	0.88 ^a	0.89 ^a	0.87 ^a	5.55	2.09 ^{ns}

Mean values on the same line, with the same letter, do not present differences when compared with each other. ^{ns}Not significant.

stuffed meat sausages fermented with probiotic *Lactobacillus*.

Table 2 shows the end mean values of pH and aw during the maturation period of Italian salamis with pequi. The incorporation of different pequi pulp concentrations in the formulation of Italian salamis made of lamb did not influence ($p > 0.05$) the end values of pH and Aw, as the pH value of the pequi pulp is similar to the pH value of the meat, not showing pH differences as a result of meat replaced with pequi pulp. The end values of Aw reported in this study agree with the values required in the Brazilian legislation (Brasil, 2000), which establishes maximum 0.90 for Italian salamis.

The end pH values of Italian salamis in this study were similar to the values of 4.81- 4.93 mentioned by Scheid et al. (2003) in the physicochemical evaluation of Italian salamis with different concentrations of clove (*Eugenia caryophyllus*). However, lower pH values were reported by Paulsen et al. (2011) in a study that analyzed the

quality characteristics of salami made of wild boar meat, produced with different meat cuts and adipose tissue, with and without bacterial fermentation, whose end pH value was 6.05. However, Ordonez (2005) reported that meat products presenting pH < 5.0 and aw < 0.91 are considered stable.

In agreement with the end content of water activity presented by Italian salamis made of lamb and enriched with pequi, Cavalheiro et al. (2010) observed aw values ranging from 0.85 to 0.88 for cured and fermented stuffed meat sausages with the addition of ostrich meat combined with pork.

Regarding the physicochemical parameters (Table 3), the Italian salamis made of lamb presented fat contents ranging from 49.48 g/100 g to 40.16 g/100 g. The Italian salami made of lamb without pequi pulp (treatment1) presented high protein value (49.48 g/100 g), not showing significant difference ($p \leq 0.05$) when compared to treatments 2 (4% pequi pulp), 3 (8% pequi pulp) and 4

Table 3. Results of physicochemical analyses of Italian salamis made of lamb and enriched with pequi.

Parameter (g/100 g)	Treatment						CV (%)	F value
	1	2	3	4	5	6		
Protein	49.48 ^a	44.86 ^{ab}	44.31 ^{ab}	44.27 ^{ab}	41.60 ^b	40.16 ^b	11.71	3.47 ^{**}
Humidity	24.16 ^{ab}	24.15 ^{ab}	24.75 ^a	21.00 ^b	22.37 ^{ab}	20.93 ^b	10.49	4.47 ^{**}
Fat	39.38 ^a	37.47 ^a	37.98 ^a	39.04 ^a	38.82 ^a	38.10 ^a	5.42	1.10 ^{ns}
Ashes	6.19 ^a	6.40 ^a	6.28 ^a	6.35 ^a	6.49 ^a	6.33 ^a	4.41	1.263 ^{ns}

Mean values on the same line, with the same letter, do not present differences when compared with each other. ^{**}significant at 1% probability level. ^{ns}Not significant.

Table 4. Results of color (L*, a* and b*) of Italian salamis made of lamb and enriched with pequi.

Parameter	Treatments						CV (%)	F value
	1	2	3	4	5	6		
L*	43.17 ^c	45.39 ^b	46.09 ^b	46.00 ^b	46.89 ^{ab}	48.81 ^a	4.64	13.45 ^{**}
a*	10.79 ^a	6.14 ^b	6.07 ^b	4.75 ^c	4.56 ^c	4.53 ^c	13.64	147.507 ^{**}
b*	7.48 ^c	8.69 ^b	8.69 ^b	9.59 ^{ab}	9.84 ^a	9.85 ^a	11.52	13.56 ^{**}

Mean values on the same line, with the same letter, do not present differences when compared with each other. ^{**}Significant at 1% probability level. ^{ns}Not significant.

(12% pequi pulp), and which did not differ from treatments 5 (16% pequi pulp) and 6 (20% pequi pulp).

Lamb has more fat than pequi (*C. Brasiliense*, Cambess). However, due to the addition of pequi pulp in the formulations, a lower protein content was observed in Italian salamis made of lamb and enriched with pequi, both observing the values determined in the Brazilian legislation (min 25 g/100 g).

The Italian salamis made of lamb and enriched with pequi presented higher protein contents than the salamis made of Santa Inês lamb produced by Lima (2009) (34.87 g/100 g) and Lappe (2004) (30.83 to 32.50 g/100 g).

Regarding the humidity content, the results agree with the values determined in the Brazilian legislation (max. 35 g/100 g). Treatment 3 (8% pequi pulp) presented greater humidity with 24.75 g/100 g, with significant difference ($p \leq 0.05$) when compared to treatments 4 and 6. The other Italian salamis made of lamb (treatment 1, 2 and 5) did not differ significantly from the treatments above in terms of humidity. The Italian salami humidity is related to the fat content. For presenting one of the lowest fat content, treatment 3 showed the highest humidity level.

The values of salami humidity obtained in this study agree with data reported by Coelho et al. (2010) (31.5 to 28.5 g/100 g) in the production of stuffed meat sausages fermented with probiotic microorganisms.

No significant difference was observed ($p > 0.05$) in fat and ashes when comparing the treatments. Italian salamis may contain up to 32% of lipids in their composition (Brasil, 2000). The Italian salamis made of

lamb and enriched with pequi presented protein contents ranging from 38.48 to 37.47 g/100 g, contents above the values determined in the Brazilian legislation, which may have been influenced by low humidity.

In general, the Italian salamis presented dark (median values of luminosity), yellowish (positive b* component) and reddish color (slightly positive a* component) (Table 4). A higher content of luminosity (L*) and yellow color (b*) was observed in treatment 6 (20% pequi pulp), as well as reduced values of these parameters with lower pequi pulp concentrations. Thus, as pequi pulp increased in formulations, the Italian salamis presented clearer color, with more yellowish pigments, which is directly related to the yellow color of pequi pulp.

Regarding the reddish color, the Italian salamis of lamb without pequi pulp presented more reddish pigments, significantly differing from the Italian salamis of lamb with pequi pulp. The increased values of L* and b* and the reduced values of a* are consistent with the addition of pequi pulp in the formulations. Due to the addition of pequi pulp, different results of luminosity and reddish pigments from this study were reported by Matos et al. (2007) when studying the effect of fermentation type on the end quality of fermented cooked stuffed sausages made of lamb, and by Cirolini et al. (2010), when studying Italian salamis with native starter cultures.

The microbiological quality of Italian salamis made of lamb and enriched with pequi was considered satisfactory, as they presented negative results for total coliform, thermotolerant coliform and *Staphylococcus aureus*, as well as absence of Salmonella, indicating compliance with the Brazilian legislation (Brasil, 2000),

showing proper sanitary standards for consumption. These results are attributed to the quality of raw materials, the sanitization conditions of manufacture and product characteristics, which was confirmed by François et al. (2009), when studying the physicochemical and sensory properties of fermented stuffed sausages made of different proportions of pork and lamb for disposal.

Conclusion

Italian salamis made of lamb with the addition of pequi pulp presented good protein levels and, in general, all Italian salamis made of lamb presented high levels of fat. Studies using lower bacon concentrations should be conducted to meet the standards required by law. The addition of pequi pulp increased luminosity and intensity of yellow color of Italian salamis made of lamb that can be a visual attraction for consumers.

Based on pH and water activity results, the Italian salamis made of lamb and enriched with pequi are stable and can be stored at ambient temperature. Pequi is a typical fruit from the Brazilian cerrado region, with characteristic color, odor and flavor, and it is an option for inclusion in Italian salamis, but additional studies should be conducted to inspect the sensory acceptability of these products.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Grain yield and its components study and their association with normalized difference vegetation index (NDVI) under terminal water deficit and well-irrigated conditions in wheat (*Triticum durum* Desf. and *Triticum aestivum* L.)

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Six genotype of *Triticum aestivum* L. in 1991 and one genotype of *Triticum durum* Desf. and three of *T. aestivum* L. in 1992 were studied under different water regimes: full irrigation (R1), mild water stress (R3) and severe water stress (R2) at Magneraud (France). Traits evaluated were grain yield and its components, stress susceptibility index (SSI) and normalized difference vegetation index (NDVI). The analysis of variance revealed significant differences between regimes and among the cultivars for all traits except between regimes for thousand grains weight in 1991. The regime x variety interaction was significant for grain yield, thousand grains weight and NDVI in 1992 and for grain yield in 1991. For all traits, durum wheat (*T. durum* Desf.) has higher reduction in the two water stress than the common wheat (*T. aestivum* L.). Correlations studies revealed that grain yield, grains number/m², thousand grains weight and NDVI were associated with each other except for correlations between thousand grains weight on one hand and grain yield (1992) and grains number/m² (1991) on the other hand. 51.55, 27.88, 4.12% (1991) and 75, 43 and 20.2% (1992) of grain yield, grains/m² and thousand grains weight variability, respectively were explained by means NDVI variability. The grain yield and grains number/m² could be predicted using a single regression with NDVI.

Key words: Grain yield, grain yield components, NDVI, durum wheat and bread wheat.

INTRODUCTION

Yield safety can only be improved if future breeding attempts will be based on the valuable new knowledge acquired on the processes determining plant development and its responses to stress (Barnabás et al., 2008). Drought is a major factor limiting the productivity of wheat throughout the world in addition to other environmental stresses, particularly high temperature, salt and cold stresses. The average yield of wheat is quite low in the

areas which present water stress. The extent of modification depends upon the cultivar, growth stage, duration and intensity of stress (Araus et al., 2002). For Siddique et al. (2000), the best option for crop production, yield improvement and yield stability under soil moisture deficient conditions is to develop drought tolerant crop varieties. Rajaram et al. (1996) suggested that simultaneous evaluation of germplasm should be carried out

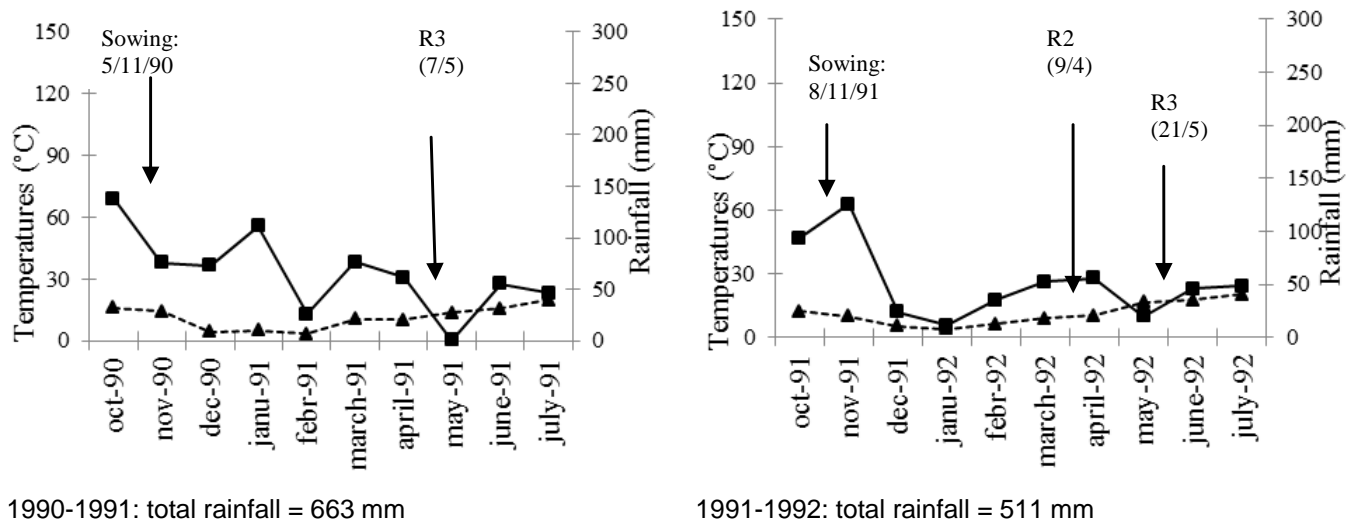


Figure 1. Rainfall and temperatures of the two years. ■ Rainfall (mm), ▲ Temperatures (°C). R2: severe water stress at meiosis, R3: Mild water stress at anthesis.

both under near optimum condition (to utilize high heritability and identify genotypes with high yield potential) and under stress conditions (to preserve alleles for drought tolerance). Several drought tolerance indices were suggested by different researchers. Among these indices, we can quote the stress susceptibility index (SSI) (Fischer and Maurer, 1978), the tolerance index (TOL) and mean productivity (MP) (Rosielle and Hamblin, 1981) and the stress tolerance index (STI) (Fernandez, 1992).

Recently, indirect assessments of agronomic and physiological traits can be also performed using spectral reflectance techniques (Araus et al., 2002). Spectral reflectance indices are non-destructive and instantaneous methods for assessing the physiological status of an entire crop or community in the field (Peñuelas et al., 1993).

Among the most widely used vegetation indices are the simple ration (SR) and the Normalized Difference Vegetation Index (NDVI) (Araus et al., 2001). The normalized difference vegetation index, based on the green vegetation absorbing solar radiation in the spectrum band by chlorophyll, and scattering in the near-infrared region, has been reported to be positively correlated with grain yield, and could serve as an indirect selection criterion to improve yield (Sharma et al., 2011).

The objective of this study was to investigate the effect of water stress on wheat (*Triticum durum* Desf. and *Triticum aestivum* L.) grain yield and its components and their relationship with NDVI, with the aim of its use in the

screening of genotypes.

MATERIALS AND METHODS

Two field experiments were carried out during 1990/1991 and 1991/1992 growing seasons at ITCF experimental station of Magneraud (Charente Maritime, France) on clay-calcareous soil and hard limestone, under movable greenhouse. The experiments laid out in a randomized complete block design with two factors (water stress and varieties) during 1990/1991 and criss cross design during 1991/1992 with three replications in each test. Each plot consisted of 30 m². Six varieties of *T. aestivum* L. in 1991 and one variety of *T. durum* Desf. and three varieties of *T. aestivum* L. in 1992 were tested at Magneraud (France). The varieties studied were Artaban, Scipion, Beauchamp, Festival, Soissons and Thesee (bread wheat) during 1990/1991 and Ambral (durum wheat), Festival, Soissons and Thesee (bread wheat) during 1991/1992. The water regimes studied were full irrigation (R1) and mild water stress (R3) during 1990/1991, while in 1991/1992 a severe water stress (R2) was added. Mild water stress was applied from anthesis to maturity, while severe water stress was applied from meiosis to maturity. Whatever is the year; December, January and February (Figure 1) are the coldest months of the year with respective average temperatures of 4.2, 5.0 and 3.4°C (1991) and 4.3, 4.0 and 6.4°C (1992). July had the highest average temperatures with 19.9°C (1991) and 20.3°C (1992). The total of rains and irrigations receipts in 1991 are 828.5 mm (R1) and 618.5 mm (R3), whereas in 1992 the total is 633 mm (R1), 368 (R2) and 428 mm (R3). Greenhouse opening and closing were controlled automatically by pluviograph and moisture plate. Greenhouse comes to cover the crop when rain reaches 0.5 mm and opening when the rain stops. Greenhouses were installed on 7/5/1991 and 9/4/1992.

The agronomical practices were the usual ones of Magneraud.

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Abbreviations: SSI, Stress susceptibility index; NDVI, normalized difference vegetation index; TOL, tolerance index; MP, mean productivity; STI, stress tolerance index; SR, simple ration

Table 1. Mean squares, degrees of freedom and coefficients of variation (CV %) of variance analysis for the traits studied (1991).

Sources of variation	df	Grain yield	Grains/m ²	Thousand grains weight	NDVI
Variety (G)	5	254.67***	69551080.00***	287.58***	0.0053***
Water regime (I)	1	399.17***	20783552.00***	0.16 ns	0.1285***
Block	3	37.65*	1882698.62 ns	3.38 ns	0.0003 ns
Interaction (G*I)	5	33.51*	1792902.38 ns	1.05 ns	0.0012 ns
Error	33	11.19	745747.38	2.14	0.0004
CV %		4.2	4.6	3.4	10.3

*, **, ***Significant at P<0.05, P<0.01 and P<0.001, respectively. NDVI: Normalized Difference Vegetation Index. ns: not significant.

Table 2. Mean squares, degrees of freedom and coefficients of variation (CV %) of variance analysis for the traits studied (1992).

Variation sources	df	Grain yield	Grains/m ²	Thousand grains weight	NDVI
Variety (G)	3	136.86** (4.2) ¹	70464048.00*** (5.1)	239.12*** (1.7)	0.0078*** (3.02)
Water regime (I)	2	2121.05** (6.2)	47489696.00* (7.4)	120.30*** (2.8)	0.1125*** (2.13)
Block	2	118.45 ns	5500640.00 ns	1.95 ns	0.0010 ns
Interaction (G*I)	6	81.93** (4.3)	447576.00 ns (3.6)	19.28*** (3.0)	0.0024*** (2.13)
Error G	6	10.62	1114192.00	0.38	0.0002
Error I	4	22.90	2362616.00	1.12	0.0001
Error (G*I)	12	11.06	571780.00	1.22	0.0001

*, **, ***Significant at P<0.05, P<0.01 and P<0.001, respectively and (4.2)¹ = CV en %. NDVI: Normalized Difference Vegetation Index. ns: not significant.

The seeding rate was 250 grains/m². Nitrogenous fertilizer was estimated by projected balance method for 100 q ha⁻¹. Each trait was measured on 4 rows 2 m in length delimited at three-leaves stage. The traits measured in each experiment were grain yield, grain number/m², thousand grains weight, stress susceptibility index (SSI) or percentage of reduction and Normalized Difference Vegetation Index (NDVI). SSI and NDVI (Araus et al., 2002) were calculated as below:

$$SSI = \frac{(Y_p - Y_s)}{Y_p} * 100$$

$$NDVI = \frac{NIR - RED}{NIR + RED}$$

Y_s : Yield of given genotype under stressed conditions; Y_p : yield of a given genotype under non-stressed conditions. NIR : Near-infrared value, RED : red reflectance value.

A simulation radiometer SPOT type CIMEL (Guyot et al., 1983) was used to measure NIR and RED. This instrument gives reflectance corresponding to spectral channels of SPOT satellite (Dembele, 1989):

i. Channel 1: λ around 500 - 600 nm (green);

ii. Channel 2: λ around 610 - 680 nm (RED);
iii. Channel 3: λ around 790 - 890 nm (Near-infrared).

Only two channels, RED and Near-infrared, have been used to calculate NDVI for wheat and barley (Royo and Villegas, 2011). The measures are realized always at the same moment of the day, at the zenith, at clear time and without wind. The NDVI means were calculated from measurements of 12 days (1991) and 14 days (1992) from 4 June to 9 July and 3 June to 15 July, respectively, (corresponding to meiosis and maturity stages) for each plot of tests. The analysis of variance, Newman-Keuls test, correlation coefficients (r) for the pair-wise comparisons of traits and coefficients of determination (r²) were calculated.

RESULTS

Analysis of variance (Tables 1 and 2) revealed significant differences for varieties in the two tests (1991 and 1992) for grain yield (p<0.001 and p<0.01, respectively, for 1991 and 1992), grains/m², thousand grains weight and NDVI (p<0.001 for the two years). Water regimes differences were significant for grain yield (p<0.001 and p<0.01, respectively, for 1991 and 1992), grains/m² (p<0.001 and p<0.05, respectively, for 1991 and 1992), thousand grains weight (p<0.001 for 1992) and NDVI

Table 3. Means of traits studied for each variety and each water regime and stress susceptibility index (SSI) (1991).

Varieties and means of water regime	Grain yield (q ha ⁻¹)			Thousand grains weight (g)	Grains number/m ²	NDVI mean
	R1	R3 (stressed)	SSI. (%)			
Artaban	76.88 ^{cd}	66.90^d	12.98	50.73^a	14166.25^e	0.580^b
Scipion	86.48 ^{ab}	83.58^a	3.35	45.35 ^b	18802.63 ^c	0.625 ^a
Thesee	80.99 ^{bc}	76.00 ^{bc}	6.16	44.48 ^b	17655.75 ^d	0.601 ^b
Beauchamp	86.06 ^{ab}	79.58 ^{ab}	7.52	43.69 ^b	18969.13 ^c	0.632 ^a
Festival	72.32 ^d	72.48 ^c	-2.21	35.66 ^c	20303.88 ^b	0.596 ^b
Soissons	87.93 ^a	77.52 ^{bc}	11.83	35.31 ^c	23090.00 ^a	0.649 ^a
Mean R1	81.78			42.48	19489.29 ^a	0.666 ^a
Mean R3	-	76.01 ^b		42,59	18173.25 ^b	0.562 ^b
SSI (%)			7.06		6.75	15.62

For thousand grains weight, grains number/m² and NDVI, the interaction variety × water regime is not significant and the classification of varieties is the same in each water regime (Table 1). Different letters mean significant differences.

Table 4. Means of grain yield (q ha⁻¹) and thousand grains weight (g) and stress susceptibility index (SSI) for each variety and each water regime (1992).

Variety	General mean of varieties	Grain yield			SSI%		Thousand grains weight			
		Means of varieties in each water regime					Means of varieties in each water regime			
		R1	R2	R3	R2	R3	R1	R2	R3	
Festival	75.98 ^a	85.18 ^a	62.89 ^a	79.87 ^{ab}	26.17	6.23	33.90 ^c	35.70 ^c	31.13 ^b	34.87 ^c
Soissons	80.07 ^a	86.34 ^a	68.11 ^a	85.75 ^a	21.11	0.68	33.35 ^c	33.88 ^d	31.03 ^b	35.13 ^c
Ambral	71.96 ^b	90.63 ^a	49.80 ^b	75.44 ^b	45.05	16.76	37.27 ^b	42.23 ^b	29.50 ^b	40.07 ^b
Thesee	80.12 ^a	88.80 ^a	67.81 ^a	83.76 ^a	23.64	5.66	44.55 ^a	44.90 ^a	42.77 ^a	45.97 ^a
Means		87.74 ^a	62.15 ^c	81.21			39.18 ^a	39.18 ^a	33.61 ^b	39.01 ^a

Different letters mean significant differences

($p < 0.001$ for the two years). Variety × water regime interaction was significant for only grain yield ($p < 0.05$ and $p < 0.01$, respectively, for 1991 and 1992), thousand grains weight and NDVI ($p < 0.001$) during 1992 indicating the presence of variability in each agronomical trait as well as the diversity of the growing conditions in each environment. The highest coefficient of variation for two years is 10.5% (NDVI for 1991) (Tables 1 and 2). The mean of all the traits under all water regimes showed that the traits under stressed conditions were always lower than under non-stressed conditions in the two tests.

In 1991, the means of the grain yield (Table 3) varied from 87.93 q ha⁻¹ (Soissons) to 72.32 q ha⁻¹ (Festival) at R1 regime and 83.58 (Scipion) to 66.90 q ha⁻¹ (Artaban) at R3 regime. Variety mean values over the two regimes varied from 50.73 g (Artaban) to 35.31 g (Soissons) for thousand grains weight; from 23090 (Soissons) to 14166.25 (Artaban) for grains/m² and from 0.649 to 0.580, for NDVI mean. The highest SSI based on the mean of the grain yield in 1991 (Table 3) are given by Artaban (12.98%) followed by Soissons (11.83%). Scipion gives the lowest SSI (3.35%), whereas Festival

has negative index (-2.21%). The average reductions of the grains number/m² and of NDVI mean are respectively 6.75 and 15.62%. The decrease of the grains number/m² caused a better grain filling.

In 1992, the means of the grain yield (Table 4) varied from 90.63 q ha⁻¹ (Ambral) to 85.18 q ha⁻¹ (Festival) at R1 regime, 68.11 q ha⁻¹ (Soissons) to 49.80 q ha⁻¹ (Ambral) at R2 regime and 85.75 q ha⁻¹ (Soissons) to 75.44 q ha⁻¹ (Ambral) at R3 regime. At R1 regime, all the varieties gave grain yields statistically equal. Whereas at R2 and R3 regimes, the varieties behave differently, indeed, the variety Ambral has the lowest grain yields. For NDVI mean (Table 5), means values over the three regimes for the four varieties varied from 0.593 (Soissons) to 0.530 (Festival) for R1, from 0.408 (Soissons) to 0.321 (Ambral) for R2 and from 0.523 (Soissons) to 0.421 (Festival) for R3. Ambral has the highest stress susceptibility index (Table 4) for grain yield, thousand grains weight and grains number/m² at the two regimes, whereas Soissons presents the lowest stress susceptibility index for all the traits. For all traits, Artaban (bread wheat) and Ambral (durum wheat), in 1991 and 1992 respectively, have the

Table 5. Means of grains/m² and Normalized Difference Vegetation Index (NDVI) for each variety and each water regime and stress susceptibility index (SSI) (1992).

varieties	Thousand grains weight		Grains/m ²			NDVI			
	SSI %		General mean of varieties	SSI %		General mean of varieties	Means of varieties in each water régime		
	R2	R3		R2	R3		R1	R2	R3
Festival	12.80	2.32	22328.71 ^b	15.39	3.99	0.443^c	0.530^c	0.377 ^a	0.421^c
Soissons	8.41	-3.69	23955.31^a	14.02	4.46	0.508^a	0.593^a	0.408^a	0.523^a
Ambral	30.14	5.11	19049.22 ^c	21.47	12.35	0.450 ^c	0.588 ^a	0.321 ^b	0.443 ^c
Thesee	4.74	-2.38	17948.41 ^c	19.94	7.75	0.475 ^b	0.560 ^b	0.391 ^a	0.473 ^b
Means R1			22660.56 ^a				0.568 ^a		
Means R2			18709.59 ^b					0.374 ^c	
Means R3			21091.09 ^a						0.465 ^b

Different letters mean significant differences.

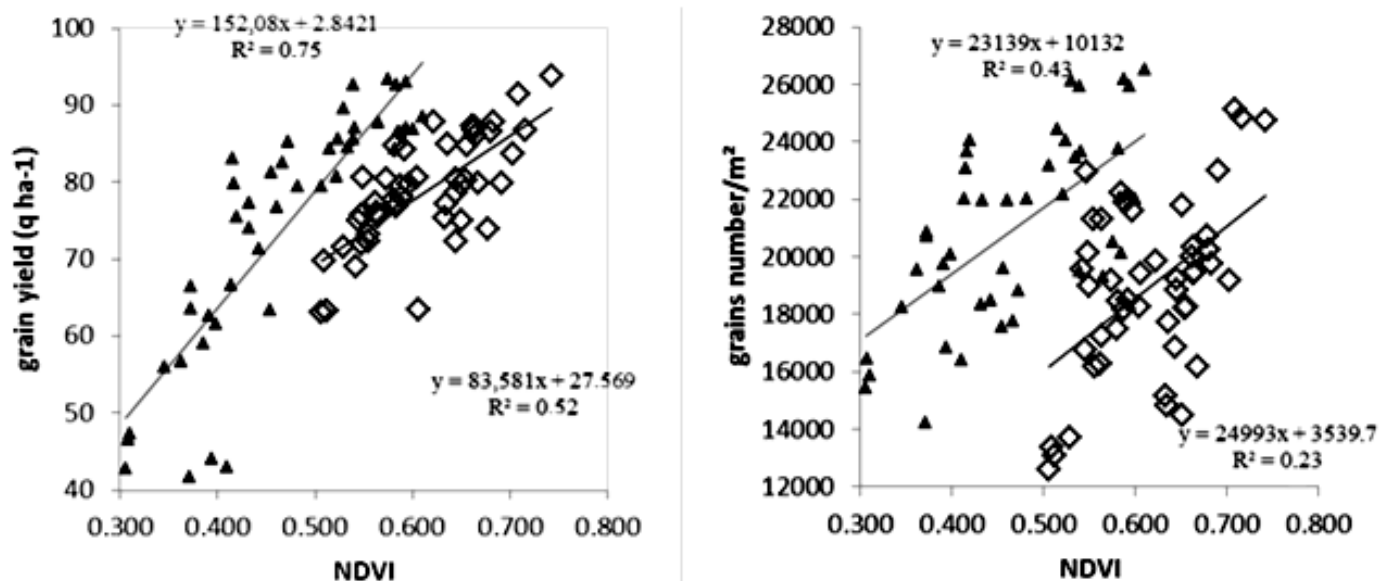


Figure 2. Relationship between grain yield, Grains number/m² and NDVI □: 1991, ▲: 1992.

highest reduction in the water stress compared with the others wheat varieties. Among the other bread wheat varieties, Thesee followed by Soissons have the lowest reduction in 1992 for grain yield but Artaban followed by Soissons have the highest reduction in 1991 (Tables 3 and 4) and Festival the highest in 1992.

The correlations between traits showed that grain yield was mainly correlated with grains number/m² ($r = 0.531^{***}$ and 0.754^{***} , *, ** and *** significant at $p < 0.05$, 0.01 and 0.001, respectively, for 1991 and 1992) and NDVI ($r = 0.718^{***}$ and 0.866^{***} , respectively, for 1991 and 1992). The correlations between NDVI and grains number/m² were also high ($r = 0.528^{***}$ and 0.656^{***} for 1991 and 1992, respectively). The correlation between NDVI and thousand grains weight is significant in 1992 (r

$= 0.449^{**}$) and non-significant in 1991 ($r = -0.203$). 51.55, 27.88, 4.12% (1991) and 75, 43 and 20.2% (1992) of grain yield, grains/m² and thousand grains weight variability, respectively, were explained by means NDVI variability (Figure 2).

DISCUSSION

Statistically significant genotypic variation for grain yield and its components have been reported previously in bread wheat (Abd El Moneim et al., 2010; Shamsi and Kobraee, 2011) and durum wheat (Elhani et al., 2007) under full irrigation and water stress conditions. In this study, we used six wheat genotypes (six genotype of *T.*

aestivum L. in 1991 and one of *T. durum* Desf. and three of *T. aestivum* L. in 1992. These genotypes expressed high variation for all the traits studied (grain yield, grains number/m², thousand grains weight and NDVI) and for the two tests in all regimes except for grain yield at R1 in 1992. The significance of genotype term suggested that genetic differences exist among the varieties studied, but also a genotype × water regime interaction was found in our study. According to Gallais (1990), the presence of genotype × environment interaction means that expression of the genes is not the same under diverse environmental conditions. On the basis of percentage reduction (SSI) calculated for yield and yield components certain varieties are more stable than others. It is the case of Festival, however this variety gives grain yield lower than the others in both regimes in 1991. In 1992, it behaves as the other bread wheat varieties. The mean of all the traits under all water regimes showed that the traits under stressed conditions were always lower than under non-stressed conditions in the two tests. At R1 regime, all the varieties gave grain yields equal statistically in 1992. Whereas at R2 and R3 regimes, the varieties behave differently, indeed, the variety Ambral gave the lowest grain yields. According to Bányai et al. (2012), in the case of the water deficit there was an increase in the number of sterile basal and apical spikelets, but the grain loss affected all the spikelets in the ear.

Artaban (1991) and Ambral (1992) have the highest SSI for grain yield, but percentage reduction for thousand grains weight and grains number /m² is the highest at the two regimes for Ambral. Among the bread wheat varieties, Soissons has the lowest stress susceptibility index for the majority of traits in 1992, but it has the highest in 1991 and Festival the highest in 1992. In 1991, it is Scipion who has the lowest SSI, whereas Soissons has a SSI almost equal to that of Artaban for grain yield. Therefore, the tolerance to water stress depends on varieties within the same species and the environment in which they are cultivated. For all traits, the bread wheat Artaban (in 1991) has the highest reduction in the water stress compared with Thesee, Festival, Soissons, Scipion, and Beauchamp. The durum wheat Ambral (in 1992) has the highest reduction in the water stress compared to Thesee, Festival Soissons (Table 4). Our results in 1992 are similar to those of Marty and Slafer (2007). According to these authors, averaged yield was similar for both wheat, but bread wheat out yielded durum wheat in severely stressed environments while durum wheat possessed a higher yield potential. It is likely that the tolerance to the water stress of the common wheat compared with the durum wheat is due to the D genome in the bread wheat. The D genome originated from *Aegilops tauschii* (Coss.) Schmalh. (*Aegilops squarrosa* auct. non L.). The genus *Aegilops* L. represents an important natural source of useful genes for wheat breeding with particular emphasis on biotic and abiotic

stress resistance (Belkadi et al., 2003). Bread and durum wheat genotypes were characterized by different physiological reactions to the applied drought stress and by clearly different molecular responses (Aprile et al., 2009). According to these authors, the genome organization accounted for differences in the expression level of hundreds of genes located on D genome or controlled by regulators located on the D genome.

The correlations between traits showed that grain yield was mainly correlated with grains number/m² and NDVI. The correlations between NDVI and grains number/m² were also high. Grain yield, grains/m² and thousand grains weight variability were explained by means NDVI variability (Figure 1). Our results confirm those reported by Gutiérrez-Rodríguez et al. (2004) who showed that NDVI has stronger association with yield under drought conditions. Grain yield can be predicted using single regression with NDVI (Lobos et al., 2014). In the irrigated treatments, plants remained green longer than water stressed treatments. Our results are in agreement with those of Bányai et al. (2012). Indeed, the normalized difference vegetation index (NDVI) based on the green vegetation absorbing solar radiation in the spectrum band by chlorophyll. The water stress causes a decrease of the photosynthetic surface and thus a decrease of NDVI.

Conclusion

Results obtained in these experiments indicate that drought stress significantly decreased the grain yield, grains number/m², thousand grains weight and Normalized Difference Vegetation Index (NDVI). For all traits durum wheat (*T. durum* Desf.) has the highest reduction in the two water stress than the bread wheat (*T. aestivum* L.). The treatments x variety interactions were significant for grain yield, thousand grains weight and NDVI in 1992 and for grain yield in 1991. Correlations studies revealed that grain yield, grains number/m², thousand grains weight and NDVI were associated with each other except for correlations between thousand grains weight on one hand and grain yield (1992) and grains number/m² (1991) on the other hand. 51.55, 27.88, 4.12% (1991) and 75, 43 and 20.2% (1992) of grain yield, grains/m² and thousand grains weight variability, respectively, were explained by means NDVI variability. The grain yield and grains number/m² could be predicted using a single regression, with NDVI.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Sequence analysis of mitochondrial DNA hypervariable region III of 400 Iraqi volunteers

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The aims of this research were to study mitochondrial DNA hypervariable region III and establish the degree of variation characteristic of a fragment. The mitochondrial DNA (mtDNA) is a small circular genome located within the mitochondria in the cytoplasm of the cell and a smaller 1.2 kb pair fragment, called the control region (D-loop). DNA was extracted from blood following a standard phenol-chloroform method. Polymerase chain reaction (PCR) products were purified by EZ-10 spin column then sequenced and detected by using the ABI 3130xL DNA analyzer. New polymorphic positions detected as T460A, C426G, T471A, T482A, A493T and C518G may in future be suitable sources for identification purpose.

Key words: Frequency, HVIII, mitochondrial DNA, polymorphic positions.

INTRODUCTION

Unlike the double-helix form of the nuclear DNA, the mtDNA is a circular molecule with only 16,569 base pairs, and is present in the cytoplasm of unfertilized ovum during the reproduction cycle. Mitochondrial DNA does not recombine and thus there is no change between parent and child, unlike nuclear DNA. mtDNA is only passed on from mother to child and this is an important fact (Ingman and Gyllensten, 2003; Ukhee et al., 2005). There is more sequence divergence in mitochondrial than in nuclear DNA (Brown et al., 1993; Giulietta et al., 2000). Mitochondrial DNA is a useful genetic marker for answering evolutionary questions due to its high copy

number, maternal mode of inheritance, and its high rate of evolution (Stoneking and Soodyall, 1996). In modern population genetics research, studies based on mitochondrial DNA (mtDNA) and Y-chromosome DNA are an excellent way of illustrating population structure while tracing uni-parental inheritance and ancestry-mtDNA is maternally inherited while the Y-chromosome is paternally inherited.

Reasons for using mitochondrial DNA rather than DNA within the nucleus includes; first, multiple copies: each mitochondrion contains its own DNA, with many copies of the circular mitochondrial DNA in every cell. It is thought

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Table 1. Primer sequence, Region amplified, Fragment size (bp) and PCR Product length for HVIII.

Primer name	Primer sequence (5' - 3') (Forward; F, Reverse; R)	Region amplified	Fragment size (bp)	PCR Product length
HVIII-1	F: 5'- TCATCAATACAACCCCGCC -3' R: 5'- TTGGTTGGTTCGGGGTATGG -3'	482-501 555-536	20 20	74
HVIII-2	F: 5'- TCTCATCAATACAACCCCGC -3' R: 5'- GGTTCCGGGTATGGGGTTAG-3'	480-500 549-530	21 20	70
HVIII-3	F: 5'- TATTTTCCCCTCCCCTCCCA -3' R: 5'- TTCGGGGTATGGGGTTAGCA -3'	450-470 547-528	21 20	98
HVIII-4	F: 5'- CAATACAACCCCGCCCAT -3' R: 5'- TTTGGGGTTTGGTTGGTTCG -3'	48-504 563-544	19 20	78
HVIII-5	F: 5'- CAATACAACCCCGCCCATC -3' R: 5'- TTGGGGTTTGGTTGGTTCGG -3'	486-505 562-543	20 20	77

that each mitochondrion contains between 1 and 15, with an average of 4 to 5, copies of the DNA (Reynolds, 2000) and there are hundreds, sometimes thousands, of mitochondria per cell. The result is that there are many thousands of copies of the mitochondrial DNA in every cell. This compares with only two copies of nuclear DNA; second, better protection: the mitochondrion also has a strong protein coat that protects the mitochondrial DNA from degradation by bacterial enzymes. This compares to the nuclear envelope that is relatively weak and liable to degradation; third, higher rate of evolution: DNA alterations (mutations) occur in a number of ways. One of the most common ways by which mutations occur is during DNA replication. An incorrect DNA base may be added; for example, a C is added instead of a G. This creates a single base change, or polymorphism, resulting in a new form. These single base mutations are rare but occur once every 1,200 bases in the human genome. The result is that the rate of change, or evolutionary rate, of mitochondrial DNA is about five times greater than nuclear DNA (Bar, 2000). This is important in species testing, as even species thought to be closely related may in time accumulate differences in the mitochondrial DNA but show little difference in the nuclear DNA and finally, maternal inheritance: A further reason for the use of mitochondrial DNA in species testing, and in forensic science, is its mode of inheritance. Mitochondria exist within the cytoplasm of cells, including the egg cells. Spermatozoa do not normally pass on mitochondria and only pass on their nuclear DNA. The resulting embryo inherits all its mitochondria from its mother (Brown, 2000; Brown, 2002; Tully, 2004).

This polymorphism allows scientists to compare mtDNA from crime scenes to mtDNA from given individuals to ascertain whether the tested individuals are within the maternal line (or another coincidentally matching maternal line) of people who could have been the source of the trace evidence.

MATERIALS AND METHODS

Sample collection

Blood samples were collected from 400 healthy unrelated volunteer donors, recruited from Iraq.

Mitochondrial DNA extraction and amplification

DNA was extracted from blood following a standard phenol-chloroform method. Amplification of HVIII region was carried out using five sets of primers encompassing the three hypervariable regions, respectively (Table 1). A portion of a noncoding region encompasses positions from 438 to 574 for HVIII amplified in accordance with the Anderson reference sequence (Anderson et al., 1981). 20 µL of Master Mix was added to a PCR tube. The pipette tip was changed and 20 µL of Primer Mix added to the PCR tube. 10 µL of extracting DNA was added to the PCR tube after changing the pipette tip again. The whole liquid was allowed to settle at the bottom of the tube, and not elsewhere. The volume in the PCR tube was checked using the PCR tube with 50 µL in it; the location of the tube on the grid after putting the mixture in the thermal cycler. 95°C hold for 10 min, 30 cycles of: 94°C for 30 s, 52.5°C for 30 s, 65°C for 1 min, 72°C hold for 10 min, 4°C hold, ∞ infinity is the cycling protocol for amplification of mtDNA PCR.

Purification, cycle sequencing and sequence analysis of mitochondrial DNA

Purification of mitochondrial DNA was by EZ10-spin column DNA cleanup kit 100 prep. The DNA Sequencing of the PCR products was done using the BigDye™ Terminator (Applied Biosystems). The separation of the cycle sequencing products was carried out. Detection was by using the ABI 3730xL DNA Analyzer, cap array size 96 and cap array length 50. The reference sequence described by Anderson et al. (1981) was compared to the data observed. Within the coding region Mitochondrial DNA, sequencing results are studied from a consensus sequence derived from multiple sequence results. Data were analysed by Sequencher™ (SEQUENCHER™ 4.7 User Manual for Windows © 1991-2007) and aligned with the Anderson sequence (Anderson et al., 1981) using sequence Navigator software. They were accepted by stating the nucleotide position followed by the code for the polymorphic base (for example, 263G).

Statistical analysis

Genetic diversity for the analyzed DNA fragment was calculated according to the formula:

$$h = (1 - \sum x_i^2) / (n - 1)$$

Where n is sample size and xi is the frequency of i-th mtDNA type (Gu, 2001).

The probability of two randomly selected individuals from a population having identical mtDNA types is:

$$(P = \sum x_i^2)$$

Where, p is the frequencies of the observed haplotypes (Jones, 1972).

RESULTS AND DISCUSSION

Haplotypes and variable positions detected in mitochondrial DNA HVIII noncoding region

The study enabled identification of 87 different haplotypes and 29 polymorphic nucleotide positions in HVIII (Table 1). The most frequent variant (H1) was consistent with the Anderson sequence. Substitutions determined during the study are transitions and transversion. This fact is consistent with abundant literature data revealing significant domination of transitions over transversions (Brown et al., 1982; Yang and Yoder, 1999; Mohammed and Imad, 2013., Imad et al., 2014a). Eleven (11) polymorphic positions, T453A, T460A, C462G, T471A, T482A, A493T, G513C, C518G, T523A, G526C and C527G have transverse substitution (Table 2). All the other substitutions determined during the analysis are transitions. The most frequent variant differed in the single position A464G, according to the CRS sequence. The number of analyzed markers has been increased to compensate for the increasing number of profiles in the databases in order to minimize accidental matches between unrelated individuals. Progression of new technology is therefore very slow and the use of SNPs has sometimes met a reluctant reception.

Mitochondrial DNA genetic diversity

Genetic diversity for the analysed DNA fragment was calculated according to the formula: $D = 1 - \sum p^2$ and recorded 0.94%. The calculated value of the genetic diversity should be understood as high in the context of noncoding function of the analysed DNA fragment. The relatively high gene diversity and a relatively low random match probability were observed in this study.

Walsh et al. (1991) and Tang (2002) show that the polymorphism of mtDNA coding area is less than that of mtDNA control region. Therefore, more efficient poly-

morphic sites should be used to provide an improved discrimination power for forensic mtDNA testing (Homer et al., 2008; Holland et al., 2011; Imad et al., 2014a). As forensic markers, they should be phenotypic neutral to avoid landing investigators into serious situations of medical genetic privacy and ethnics, especially for mtDNA coding area whose mutation often correlated with an increased risk of some diseases. With the whole mtGenome sequences being researched, we are optimistic that the polymorphism sites within mtDNA coding area will be useful in combination with control region SNPs so as to increase the discrimination power of mtDNA.

There is a simple program called Mito Analyzer attached to the database which enables convenient access to information concerning polymorphic positions. In cases where there is an abundance in the sample, for example mass graves in mass disasters, there are newly discovered forensically validated methods such as ESI-MS (David et al., 2013).

Significant assistance for the research was provided by Mitomap computer database, which contains information concerning human mtDNA (Mitomap: A Human Mitochondrial Genome Database, <http://www.mitomap.org>) (Table 3). This database includes data about currently known variable positions, their possible association with genetic diseases, and references to the literature. There is also a simple program called Mito Analyzer attached to the database which enables convenient access to information concerning polymorphic positions.

Conclusion

Sequence analysis of the noncoding region of mtDNA (HVIII) conducted on a population of 400 unrelated individuals enabled identification of 87 different haplotypes in HVIII. New polymorphic position detected as T460A, C426G, T471A, T482A, A493T and C518G described, may be in future suitable sources for identification purpose.

Conflict of interests

The authors did not declare any conflict of interest.

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Table 2. Variable positions and haplotypes for HVIII.

Anderson	453	456	460	462	464	471	477	482	485	489	493	497	499	504	507	508	511	513	514	516	518	523	526	527	533	538	545	545	553	No. of Individual
	T	C	T	C	A	T	T	T	T	T	A	C	G	T	T	A	C	G	C	C	C	A	G	C	A	A	G	C	C	
H1*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	253
H2	-	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	T	-	1
H3	-	-	C	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	3
H4	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1
H5	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	G	G	-	-	-	1
H6	-	-	-	-	-	-	-	C	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	1
H7	-	-	-	-	-	-	-	-	-	-	-	T	A	-	-	-	-	-	-	-	T	-	-	T	-	-	-	-	-	1
H8	C	-	-	-	-	-	-	-	-	-	-	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H9	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	1
H10	-	-	-	-	G	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H11	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	-	3
H12	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	2
H13	-	-	-	-	-	C	-	-	-	-	G	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	T	2
H14	A	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-	T	-	-	-	T	-	2
H15	-	-	-	-	-	-	C	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H16	-	-	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
H17	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	1
H18	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	2
H19	-	-	-	-	-	-	-	-	-	C	-	T	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H20	-	-	C	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H21	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H22	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H23	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	T	1
H24	-	-	-	T	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H25	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	T	-	-	-	-	-	-	-	-	-	-	1
H26	-	-	A	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H27	-	-	-	-	-	-	-	C	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H28	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	T	-	-	-	-	-	1
H29	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	1
H30	-	-	-	-	-	C	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H31	-	-	-	-	-	-	-	-	-	G	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H32	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	1
H33	-	-	-	-	G	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H34	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	1
H35	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	A	-	-	-	-	-	-	1

Table 3. Contd.

489	Transition	T-C	Presence	0.048	4.8
493	Transition	A-G	Presence		
493	Transversion	A-T	New	0.0375	3.75
497	Transition	C-T	Presence	0.0525	5.25
499	Transition	G-A	Presence	0.035	3.5
504	Transition	T-C	Presence	0.035	3.5
507	Transition	T-C	Presence	0.040	4
508	Transition	A-G	Presence	0.0175	1.75
511	Transition	C-T	Presence	0.0175	1.75
513	Transition	G-A	Presence		
513	Transversion	G-C	Presence	0.0325	3.25
514	Transition	C-T	Presence	0.030	3
516	Transition	C-T	Presence	0.015	1.5
518	Transition	C-T	Presence		
518	Transversion	C-G	New	0.0175	1.75
523	Transition	A-G	Presence		
523	Transversion	A-T	Presence	0.0125	1.25
526	Transition	G-A	Presence		
526	Transversion	G-C	Presence	0.0225	2.25
527	Transition	C-T	Presence		
527	Transversion	C-G	Presence	0.035	3.5
533	Transition	A-G	Presence	0.0225	2.25
538	Transition	A-G	Presence	0.015	1.5
545	Transition	G-A	Presence	0.0175	1.75
548	Transition	C-T	Presence	0.0175	1.75
553	Transition	C-T	Presence	0.020	2

New*, new polymorphic positions ; genetic diversity* , genetic diversity for the analysed DNA fragment was calculated according to the formula: $D = 1 - \sum p^2$. Genetic diversity* $D = 1 - \sum p^2 = 0.94 = 94\%$.

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Full Length Research Paper

Antimicrobial activities of grape (*Vitis vinifera* L.) pomace polyphenols as a source of naturally occurring bioactive components

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Grape pomace is a potential source of winery by-products having useful bioactive components. Antimicrobial activities of enzyme-assisted grape pomace polyphenols (GPP) were assessed against *Escherichia coli* IFO 3301 and *Staphylococcus aureus* IFO 12732 using plate count and spectrophotometry assays. GPP have shown credential growth inhibition against *E. coli* and *S. aureus*, respectively. The higher growth inhibition was mediated by the higher GPP concentrations against both *E. coli* and *S. aureus*, which implies dose dependency. GPP also exhibited bactericidal effects against both the Gram-positive and Gram-negative bacteria, whereas, Gram-positive bacteria have shown more susceptibility than Gram-negative bacteria. It is revealed that GPP is a potential source of natural antimicrobial agents.

Key words: Grape pomace, polyphenols, antimicrobial activity.

INTRODUCTION

Grape pomace contains naturally occurring bioactive components. Phenolic compounds, as secondary plant metabolites, play a critical role in human health. Bioactive components of fruits and vegetables have been shown to be beneficial for human health (Liu, 2003; Georgiev et al., 2014), including flavonoids, a major class of phytochemicals commonly found in fruits and vegetables

(Vinson et al., 2001; Altameme et al., 2015). Considerable attention has been paid to polyphenols because of their diverse biological functions (Gulluce et al., 2003; Almeida et al., 2006). Grape pomace is generally underutilized and thrown away by the wine factory as waste products. Grape skins are the rich sources of anthocyanins, hydroxycinnamic acids,

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Abbreviations: LB, Luria broth; GPP, grape pomace polyphenols; DMSO, dimethyl sulfoxide; PBS, phosphate buffer saline.

flavanols and flavonol glycosides; flavanols were mainly present in the seeds (Kammerer et al., 2004). Anthocyanins, catechins, flavonol glycosides, phenolic acids and alcohols, and stilbenes are the principal phenolic compounds of grape pomace (Schieber et al., 2001). Epidemiological studies indicate that fruits, vegetables and plant-based phenolic metabolites are beneficial to human health because of their potent antioxidant activity and wide range of pharmacologic properties such as antioxidant, anticancer, and platelet aggregation inhibition activities (Waterhouse and Walzem, 1998; Teixeira et al., 2014). Antibacterial activities shown by phenolic compounds may be because of iron deprivation or hydrogen bonding with vital proteins, such as microbial enzymes (Field and Lettinga, 1992). There is no systematic way to use grape pomace.

Finding an effective way of using grape pomace is needed, as it is a good source of natural bioactive components, which could be used as a functional food component. In this study, we made an attempt for the utilization of underutilized grape pomace and to assess the antimicrobial activities of grape pomace polyphenols as a source of naturally occurring bioactive components.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO) was purchased from WAKO Pure Chemical Industries Ltd. (Tokyo, Japan). Luria broth (LB) medium was purchased from SIGMA-ALDRICH Inc. (Tokyo, Japan). MacConkey agar was purchased from Nippon Seiyaku Co. Ltd. (Tokyo, Japan), and Mannitol salt agar was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). *Escherichia coli* IFO 3301 and *Staphylococcus aureus* IFO 12732 were collected from the Institute for Fermentation (Osaka, Japan). Other chemicals used were of biochemistry grade.

Preparation of sample

Red grape pomace was collected from Nagano prefecture, Japan. 1:9 samples with PBS (phosphate buffer saline, pH 7.3) were homogenized using blending machine. Cellulase enzyme (0.25 mg/mL) was added to the homogenized samples and then incubated at 55°C for 24 h. After enzymatic digestion the samples were centrifuged at $7500 \times g$ for 20 min and supernatant samples were collected. The extracted supernatant was filtered through filter paper (Whatman no. 4) to remove unutilized residues. Supernatant samples were vacuum drying to remove organic solvent and then lyophilized for collection of grape pomace polyphenols (GPP). Dried lyophilized GPP was dissolved in 1% dimethyl sulfoxide (DMSO) using distilled water to make stock solution which was then sterilized by micro-filtration through 0.45 μm Millipore filter and kept at 4°C until use.

Microorganisms and culture conditions

Gram-positive bacterium *S. aureus* IFO 12732, and Gram-negative bacterium *E. coli* IFO 3301 were used in this study. Each bacterial strain was incubated in LB medium at 37°C for overnight. After incubation the bacterial solution was centrifuged two times and

washed by using PBS (phosphate buffer saline, pH 7.0), and the test bacterial solution was prepared with PBS to give a concentration of 10^7 CFUs/mL by using a haematometer (Neubauer, LO-Laboroptik GmbH, Friedrichsdorf, Germany).

Determination of microbial growth inhibition

A 500 μL of mid-logarithmic phase bacterial cultures (10^7 CFUs/mL) was inoculated in 4.5 mL LB medium to make the final concentration of 10^6 CFUs/mL. A 2.0 mg/mL of GPP stock solution was prepared and then serial two-fold diluted in a 1.0 and 0.5 mg/mL using LB medium and also control (without samples) were taken to measure the growth inhibition of *E. coli* and *S. aureus*. The GPP was diluted using 1.0% DMSO. The cultures were incubated in a rotary shaker at 37°C and growth inhibition was determined by measuring the absorbance at OD 600 nm using a UV-VIS spectrophotometer SHIMADZU-1700 (Tokyo, Japan). Absorbance readings were taken for 360 min, followed by 60, 120, 180, 240, 300 and 360 min intervals. A growth curve was plotted with the obtained absorbance readings (Farouk et al., 2007). All the measurements were done in triplicate.

Determination of antimicrobial activities as killing effect

The GPP was added to 5.0 ml of PBS (phosphate buffer saline, pH 7.0) containing 500 μL of mid-logarithmic phase bacterial culture (10^7 CFUs/mL) to prepare the final concentration 10.0 mg/mL and 10^6 CFUs/mL. The tested bacterial cultures with samples were incubated at 37°C for 60, 120, 180, 240, 300 and 360 min intervals to determine the log survival ratio. The visible colony forming units (CFUs/mL) was measured with the above time intervals using MacConkey and Mannitol salt agar plate, incubated at 37°C for 24 h to measure the log reduction of *E. coli* and *S. aureus*. In addition, dose-dependent killing effect was also assessed using mid-logarithmic phase bacterial cultures (10^7 CFUs/mL) that were inoculated in a 500 mL PBS (phosphate buffer saline, pH 7.0) to make the final concentration of 10^6 CFUs/mL. In the 500 ml of PBS the final concentration of GPP was 3.0, 6.0, 9.0 and 12.0 mg/mL to measure the killing effect of *E. coli* and *S. aureus* incubated at 37°C for 6 h. After incubation to measure the killing effect of bacteria a serial 10-fold dilution were prepared using PBS (pH 7.0) and plated onto MacConkey and Mannitol salt agar plates and incubated at 37°C for 24 h. And then log survivals were enumerated using visible colonies on agar plates, while the count detection limit was maintained between 5 and 50 CFUs. The bacterial killing effect was calculated using the following formula: killing effect (Log CFUs/mL) = $\log_{10}^{nc} - \log_{10}^{np}$, where nc and np were CFUs/mL of mock and treated cells (Hoq et al., 2008). All the data represents the mean of triplicate tests.

Statistical analysis

Statistical analysis was performed using student's *t*-test. Paired tests were done to assess the differences between groups. All data were evaluated as mean \pm SD. The differences between means were assessed using analysis of variance (ANOVA) followed by Duncan's new multiple range test. Statistical probability $p < 0.05$ were considered significant. All the tests were done in triplicate.

RESULTS AND DISCUSSION

Microbial growth inhibition

Grape pomace polyphenols have shown specific

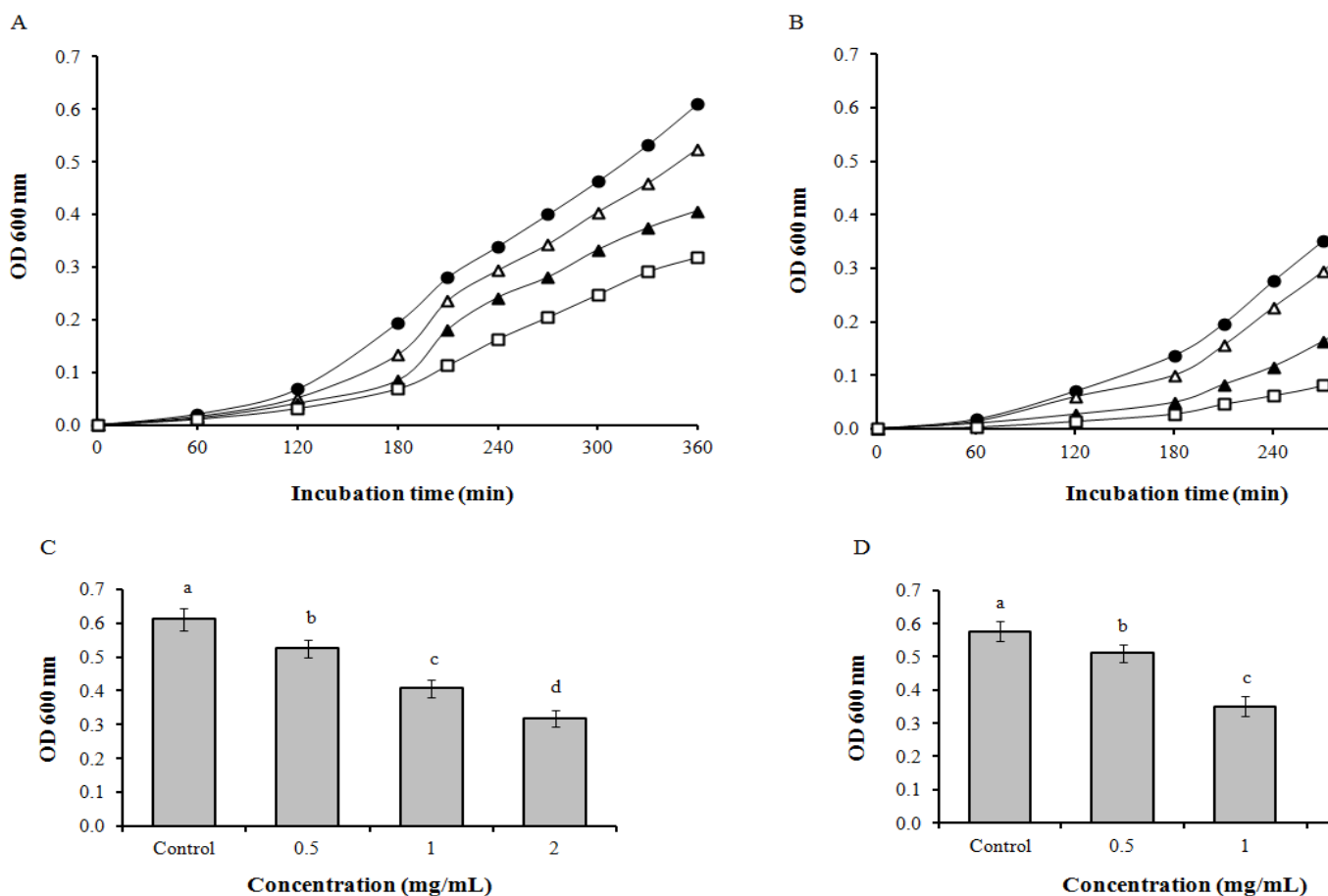


Figure 1. Growth inhibitory activities of GPP against *E. coli* (A) and *S. aureus* (B) under neutral pH condition incubated at 37°C for 360 min. Absorbance readings were measured at OD 600 nm. Data shown in Figure A and B is the representative of three independent experiments. Figure C and D indicate the growth inhibitory effects of GPP after 360 min of incubation against *E. coli* and *S. aureus*, respectively. ●, control; ▲, 0.5 mg/mL; ▲, 1.0 mg/mL; □, 2.0 mg/mL. All data represents mean \pm SD. Different superscripts with uncommon alphabet differ significantly ($p < 0.05$).

antimicrobial activities and corresponding bacteriostatic effects. Figure 1 shows the growth inhibitory activities of GPP against *E. coli* and *S. aureus* at OD 600 nm. As shown in Figure 1A, GPP was indicating gradually increasing growth inhibition against *E. coli* depending on exposure time. In contrast, higher tendency of growth suppression was observed on the higher concentration of GPP, which implies the dose dependency of growth inhibition. Figure 1B shows the growth inhibition of GPP against *S. aureus*. It was observed that growth inhibitory activity was gradually increased depending on exposure time, whereas higher concentrations have shown the higher growth suppression activity. Figure 1A and 1B show the growth inhibitory activity of GPP against *E. coli* and *S. aureus* after 6 h of incubation. It was found that significant ($p < 0.05$) growth inhibition was observed against both the Gram-positive and Gram-negative bacteria as compared with control; in addition, the higher concentration indicated significant growth inhibition than against lower concentrations. It was observed that growth

inhibitory activities of GPP against both the Gram-positive and Gram-negative bacteria was mediated by exposure time and concentrations, in which GPP have shown higher tendency of growth inhibition against Gram-positive bacteria as compared with Gram-negative bacteria. Antibacterial activities shown by phenolic compounds may be because of iron deprivation or hydrogen bonding with vital proteins (Field and Lettinga, 1992). Polyphenols have shown microbial growth inhibitory activities (Bong-Jeun et al., 2004; Sanhueza et al., 2014) in addition, polyphenols were usually more active against Gram-positive bacteria than Gram-negative bacteria (Lin et al., 1999; Oliveira et al., 2013). It was confirmed that GPP have shown potent microbial growth inhibitory activities.

Antimicrobial activities as killing effect

GPP have shown bactericidal effects under neutral pH at

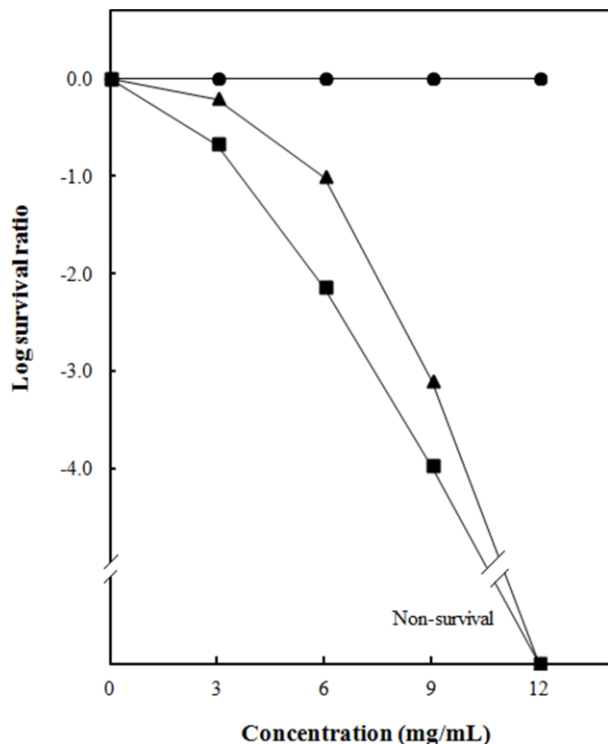


Figure 2. Effect of doses on bactericidal activities of GPP under neutral pH condition at 37°C for 6 h incubation. ●, control; ▲, *E. coli*; ■, *S. aureus*. Log survival ratio was calculated by enumerating viable cells. Showing data are the representative of three independent experiments.

37°C for 6 h of incubation with different sample concentrations. Figure 2 shows the log survival ratio of GPP against *E. coli* and *S. aureus*. It was observed that GPP have shown weak killing effect at lower concentration (3.0 mg/mL) against both the Gram-positive and Gram-negative bacteria, although Gram-positive bacteria have shown higher tendency of log reduction, whereas a moderate and strong killing effect was observed in higher doses of GPP (6.0 and 9.0 mg/mL).

In contrast, no survivability was detected in the concentration of 12.0 mg/mL against both the Gram-positive and Gram-negative bacteria. It was revealed that GPP possess strong bactericidal effects irrespective of Gram-positive and Gram-negative bacteria, which was mediated by doses and implies the dose dependency. It was reported that bacterial killing effect was dose dependent (Kao et al., 2010). Polyphenols exhibited antibacterial activities with protein-related polyamide polymers (Haslam, 1996). In contrast, polyphenols showed higher antibacterial effect on Gram-positive bacteria than Gram-negative bacteria (Viskelis et al., 2009). It was observed that GPP exhibited dose dependent bactericidal effects.

GPP exhibited bacterial killing effect against both *E.*

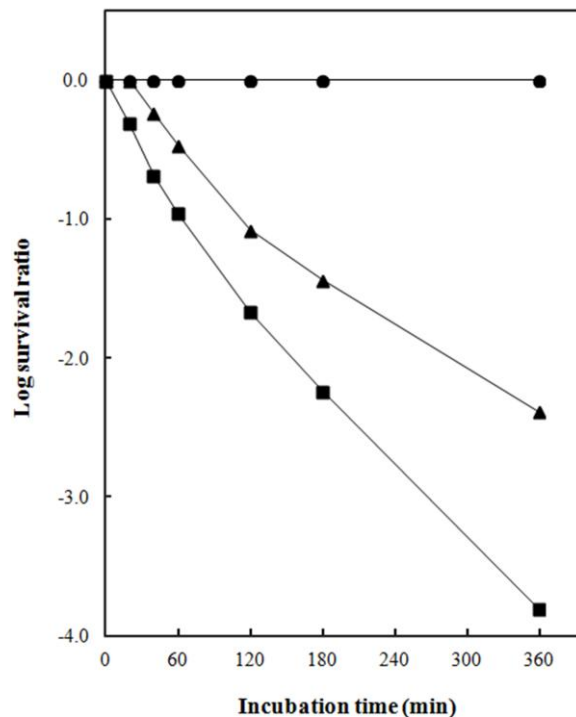


Figure 3. Effect of exposure time on the lethal effects of GPP under neutral pH, incubated at 37°C for 6 h. ●, control; ▲, *E. coli*; ■, *S. aureus*. Log survival ratio was calculated by enumerating viable cells. Data are the representative of at least three independent experiments.

coli and *S. aureus*. As shown in Figure 3, a quick and drastic log reduction was observed against both the Gram-positive and Gram-negative bacteria depending on the exposure time.

A weak killing effect was detected after 1 h of exposure, whereas a rapid and strong killing effect was observed after 2 to 6 h of exposure. In addition, Gram-positive bacteria have shown potent bactericidal effects as compared with Gram-negative bacteria. Polyphenols revealed the higher antimicrobial properties against Gram-positive bacteria than against Gram-negative bacteria (Burdulis et al., 2009). It was also observed that bacterial killing effect was associated with exposure time (Paulo et al., 2010).

Conclusion

It was demonstrated that bactericidal effects of GPP was mediated by the exposure time. Thus, it was revealed that grape pomace polyphenols exhibited bacteriostatic as well as bactericidal activities against both the Gram-positive and Gram-negative bacteria and that these underutilized GPP could be a good source of antimicrobials for further utilization in the food manufacturing industry to control or prevent food-borne pathogens.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Functional and catalytic active sites prediction and docking analysis of azoreductase enzyme in *Pseudomonas putida* with a variety of commercially available azodyes

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The initial critical step of reduction of azo bond during the metabolism of azo dyes is catalysed by a group of NADH and FAD dependant enzyme called azoreductases. Although several azoreductases have been identified from microorganisms and partially characterized, very little is known about the structural basis of the substrate specificity and the nature of catalysis. Azoreductase enzyme of *Pseudomonas putida* has a wider broad spectrum of substrate specificity and capable of degrading a wide variety of azo dyes. In the present study, the crystal structure of the enzyme from PDB and 10 azo dyes from NCBI PubChem compound were retrieved and their interactions were studied. These azo dyes were then docked with the FMN-dependent NADH-azoreductase enzyme to analyze the binding affinity of the azo dyes with the enzyme and predict the catalytic sites. Consequently, the catalytic residues of FMN-dependent and NADH dependent enzyme were then analysed in terms of properties including function, hydrogen bonding and flexibility. The results suggest that Ala-114, Phe-172 and Glu-174 play a predominant role as catalytic site residues in the enzyme. Furthermore, the approach emphasis on predicting the active sites of this enzyme where substrates can bind in order to give a better understanding of the biodegradation of some of the commercially important azodyes mediated by azoreductase. These results will pave way for further increase in azoreductase activity and for better understanding of the dye degradation pathway.

Key words: Azoreductase, NADH, FMN, chemical properties, docking, active sites.

INTRODUCTION

Azo dyes are known to be widely used class of dyes that are highly toxic and contain carcinogenic compounds. Although lot of research has been carried out for their removal from industrial effluents, very little attention is

given to changes in their toxicity and mutagenicity during the treatment processes (Bafana et al., 2008). Azo dyes represents almost 70% of the textile dyestuffs produced and the effluents released into the water system disturbs

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the ecological parameters of mostly water bodies (Knackmuss, 1996). Due to enormous modernization the most problematic environment pollution in the wastewater are the effluents released from production of dyes and the dyeing industries. These residual dyes in industrial effluents are a threat to public health because of its high toxicity and carcinogenicity (Bisschops and Spanjers, 2003; Weisburger, 2002). The uncontrolled release of these compounds in the environment causes severe problems by decreasing light absorption which significantly affect photosynthetic activity of aquatic life and may be toxic due to the presence of aromatics or heavy metals (Banat et al., 1996). Release of such colored compounds in the environment is undesirable not only because of their aesthetic appearance, which may drastically affect photosynthesis in the aquatic ecosystems, but also because many of the dyes and/or their breakdown products are mutagenic to life (Chung and Cerniglia, 1992). Unfortunately, azo dyes present in the wastewater are normally unaffected by conventional treatment processes. Their persistence is mainly due to the sulfo and azo groups, which do not occur naturally, making the azo dyes xenobiotic and recalcitrant to oxidative biodegradation (Kulla et al., 1983). The persistence of azo dyes as reported could be further decolorized which consequently requires a putative agent for cleavage of azo bonds, after which the resulting aromatic amines can be biodegraded readily under aerobic conditions (Van der Zee and Villaverde, 2005). The cleavage of azo bonds is catalyzed by azoreductase enzyme with the aid of an electron donor. Several bacteria capable of decolorizing azo dyes have been identified, and azoreductase enzyme has been isolated and characterized from some of them (Chen, 2006).

In the recent years, bioremediation of azodyes polluted areas using bacteria as a potent organism has gained momentum in context to dye effluents and consequently it seems to play a pivotal role in bioremediation activities. Azobenzene reductases, also known as Azoreductase (EC 1.7.1.6) are a family of NAD(P)H and Flavin – dependent enzymes that have been identified from a large number of bacterial species. These enzymes are able to reduce a wide range of substrates including azo dyes/drugs, quinones, metal ions and nitro compounds. They have been found in a number of species including *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas putida* etc. Although various azoreductases may be present in the same organism but the physiological roles of most of these enzymes is generally poorly understood. It has been found that many microorganisms including cyanobacteria can transform these azo dyes into colourless products. Generally, in bacterial system the degradation of azodyes is often initiated by an enzymatic step which involves a cleavage of azo linkages with the aid of an azoreductase and an electron donor (Hong and Gu, 2010). Several workers have reported on the azo dye degradation in bacterial

system (Yeh et al., 2005; Asgher et al., 2007; Delee et al., 1998; Levine, 1991; Walker, 1970). Similarly, azoreductase activities of cyanobacterial species which are known for their ubiquitous occurrence in nature, in response to various mono and diazo compounds have also been reported (Jadhav et al., 2008; Omar, 2008). These azoreductases catalyze the reduction of the azo bond (-N=N-) in both azo pro drugs (for example, balsalazide) and azo dyes (for example, methyl red). They have been shown to reduce azo compounds via a ping pong mechanism (Chan-Ju et al., 2010). In context to the present study, the azoreductase from the *P. putida* has been taken into consideration as a model organism to understand the interaction of several toxic dyes with the enzyme using bioinformatics tools which in turn might probably throw a limelight on the prediction of active sites which could be further exploited for development of effective bioremediation process.

METHODOLOGY

Data set

The FASTA sequence of FMN dependent NADH azoreductase of *P. putida* was retrieved from the PDB database with the PDB ID 4C0W having 203 amino acids (Bernstein et al., 1977). The structures of the commercially important dyes were retrieved from the PubChem compound database namely Azobenzene, p-Aminoazobenzene, Amarant dye/F.D&C No.2, Methyl Orange, Sudan IV, Amidoschwarz, Congo Red, C.I. Food Yellow 3, Azepan-1-yl [(3S)-[2(chlorophenyl)methyl]piperidine-3-yl] methanone and Solvent Red 23 with CID numbers 2272, 6051, 5359521, 23673835, 5876571, 54599778, 11313, 6850717, 1530900 and 5809667, respectively, from PubChem compound database (Wang et al., 2009).

Homology model building and evaluation

Homology modeling of the azoreductase enzyme was performed using the CPH model server 3.2 (Nielsen et al., 2010) and the structure was further validated using Ramachandran Plot. The template generated was 4C0W which had highest similarity percentage and viewed using UCSF Chimera 1.10.1 viewer (Pettersen et al., 2004).

Chemical properties

The various chemical properties of the commercially available and industrially important azo dyes such as the molecular weight, molecular formula, hydrogen bonds (donors and acceptors), rotatable bonds, exact mass, topological polar surface area (A^2), heavy atom count, complexity and covalently bonded unit count were retrieved using PubChem Compound database (Table 2).

Active site analysis

Ligand binding site prediction of the azoreductase enzyme was carried out using DoGsite scorer (Volkamer et al., 2010). The software possesses structure-based method to predict active sites in proteins based on a Difference of Gaussian (DoG) approach

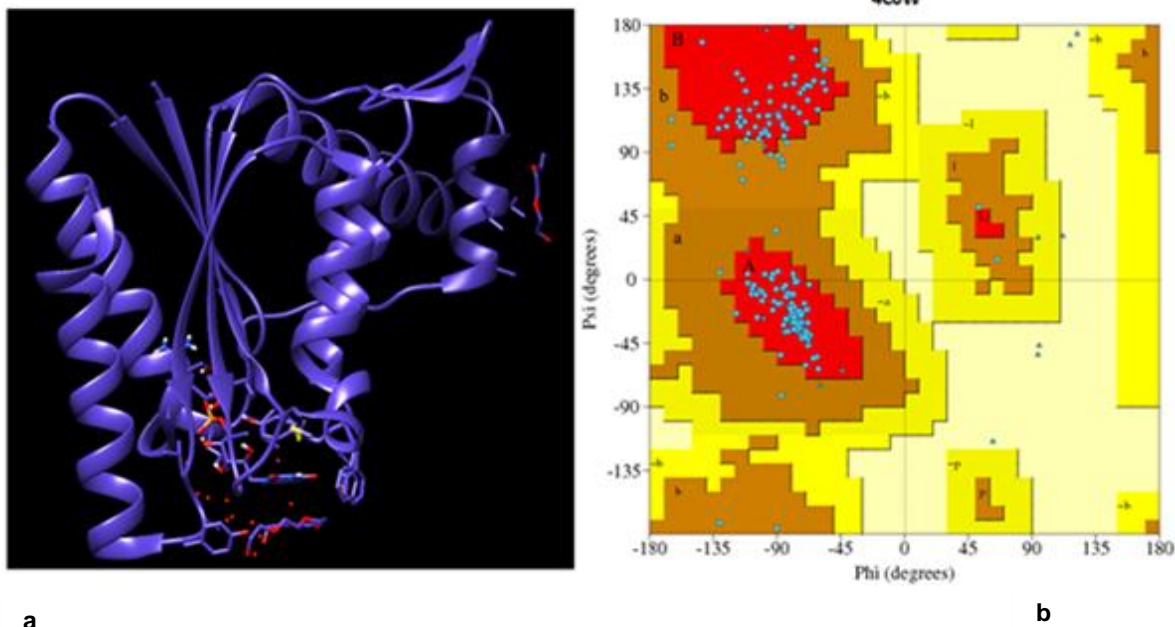


Figure 1. (a) Three dimensional structure of azoreductase enzyme from *Pseudomonas putida* (4COW) obtained from PDB with its natural ligands. (b) Ramachandran Plot of 4COW.

which originates from image processing. In contrast to existing methods, DoGSite splits predicted pockets into sub-pockets, revealing a refined description of the topology of active sites. DoGSite correctly predicts binding pockets for over 92% of the PDBBind and the scPDB data set, being in line with the best-performing methods available.

Docking studies

Docking study of the azoreductase enzyme and the commercially important dyes were carried out using Swiss Dock server (Grosdidier et al., 2011), and energy minimization was performed before and after docking by the same in an automated manner. The interpretation of docking results and their integration into existing research pipelines is greatly facilitated by the seamless visualization of docking predictions in the UCSF Chimera molecular viewer which could be launched directly from the web browser.

RESULTS

Structure retrieval

The enzyme, FMN-dependent NADH-azoreductase of *P. putida* has a crystallized structure characterized by three ligands FMN 1201, 12P 1202 and 12P 1203. The structure was obtained from PDB with the PDB ID-4COW.

Homology modeling and validation

In the present work, the azoreductase enzyme was initially explored for the best homology modeling which

basically determines the overall 3D structure of the aminoacids present in the enzyme molecule. The homology modeling using the CPH server depicted the best model of the enzyme molecule which requisite number of strands, helices and loops (Figure 1a). This structure was further validated by the corresponding Ramachandran Plot (Figure 1b) which depicted fewer numbers of amino acids in the disallowed region with maximum number of amino acids molecules in the favorable region. Most favorable regions showing 85.6% having 166 residues, additional allowed region showing 13.9% having 27 residues and generously allowed region showing 0.5% having only 1 residue. There was no residue found in the disallowed region. The Ramachandran Plot analysis further showed 226 as total number of residues, the number of Glycine (Gly) and Proline (Pro) are 16 and 14, respectively, and the number of end residues (excluding Gly and Pro) were 2.

Computation of docking score between the ligands and the enzyme

Protein and other chemical molecule interaction outputs compute putative data which could be an advantage to understand the mechanism. The cumbersome syntax of the docking engine is hidden behind a clean web interface providing reasonable alternative sets of parameters as well as sample input files. All calculations are performed on the server side, so that docking runs do not require any computational power from the user.

Table 1. Detailed docking analysis result showing of azo dyes and azoreductase enzyme. Listed above are the binding affinities, number of hydrogen bond formation of each azo dye with azoreductase. The amino acid residue position of azoreductase interacting each azo dye is also provided along with the azo dye information.

Azo dyes	Common names	Affinity (kcal/mol)	No. of H-bonds	Positions (residues)
2272	Azobenzene	-5.35	0	
5359521	Amaranth	-6.66	2	Glu-174, Phe-172
6051	p-aminoazobenzene	-5.46	2	Gly-141, Phe-98
23673835	Methyl Orange	-5.58	1	Ala-114
1530900	Azepan-1-yl[(3S)-[2(chlorophenyl)methyl]piperidin-3-yl]methanone	-3.40	1	Ala-114
587651	Sudan IV	-6.30	0	
54599778	Amiboschwarz	-6.22	1	Ala-114
11313	Congo Red	-7.22	2	Gly-180, Thr-102
6850717	C.I. Food Yellow 3	-6.48	0	
5809667	Solvent Red 23	-6.38	1	Ala-114

Docking study showed the binding affinity, number of hydrogen bonds and the binding residues. It has been noted that the binding affinities have negative values as shown in Table 1 which reveals the high feasibility of this reaction. The docked complexes were analyzed with the molecular visualization tools, Chimera 1.10.1 (Volkamer et al., 2010) as shown in Figure 2. The docking analysis showed that seven dyes viz. Amaranth, p-aminoazobenzene, methyl orange, Azepan-1-yl[(3S)-[2(chlorophenyl)methyl]piperidin-3-yl]methanone and amidoschwarz formed H-bonds with the enzyme residues Glu-174, Phe-172, Gly-141 and Ala-114. The variation in the docking score indirectly gives the idea about the rate of decolorization. This Comparing the results from DoGsite scorer and docking studies, it indicates that the amino acid residues ALA, ASP, LEU, LYS, PHE and VAL play an important role as catalytic site residues in the azoreductase enzyme of *P. putida*. This docking study also provides information on the binding affinity of the ligands with azoreductase enzyme. The rate of color removal for congo red is higher than any other azo dye and it could be assumed probably that this information would provide a better understanding of the molecular mechanisms involved in catalysis and a heuristic basis for predicting the catalytic residues in enzymes of unknown function. The natural ligands (FMN, 12P) were also found to interact with some of the ligands. In this work, the catalytic residues are reported as well as the binding affinities for some commercially important azodyes. The study made in this project would facilitate researchers a better understanding of enzyme mechanisms and also used to improve the designing strategies of less harmful azodyes

Functional site location

The catalytic or functionally important residues of a protein

are known to exist in evolutionary constrained regions. However, the patterns of residue conservation alone are sometimes not very informative, depending on the homologous sequences available for a given query protein. Hence, the prediction of functional sites in newly solved protein structures is a challenge for computational structural biology. Most methods for functional site identification utilize measures of amino acid sequence conservation in homologous sequences, based on the assumption that functional sites are relatively conserved during evolution. Protein structural information has also been used to help identify protein functional sites. Active sites of the target protein were predicted using DoGsite scorer and the output file was viewed under Chimera. Seven active sites were obtained from the study along with the corresponding amino acid residues present in each active site. Each of the sites were analyzed and compared with the amino acids interacting with the ligands in the docking study. The docking result shows that the amino acids such as ALA, ASP, LEU, LYS, PHE and VAL are very much repeated in the interaction with more than one ligand. This reveals that these amino acids are catalytic residues. The active site variations suggest that the enzyme can decolorize a wide range of azo dyes (Figure 3 and Table 3).

Molecular docking interactions showing hydrogen bonding

The hydrogen bonding in the crystal structures were used as a measure of residue flexibility. Analysis shows that the amino acids interacting with the ligands are involved in hydrogen interaction; it can be as a donor or as an acceptor. This shows that catalytic residues have a limited conformational freedom. The docking result shows that the ligands have hydrogen bonding with amino acids and it is illustrated in the docking analysis table.

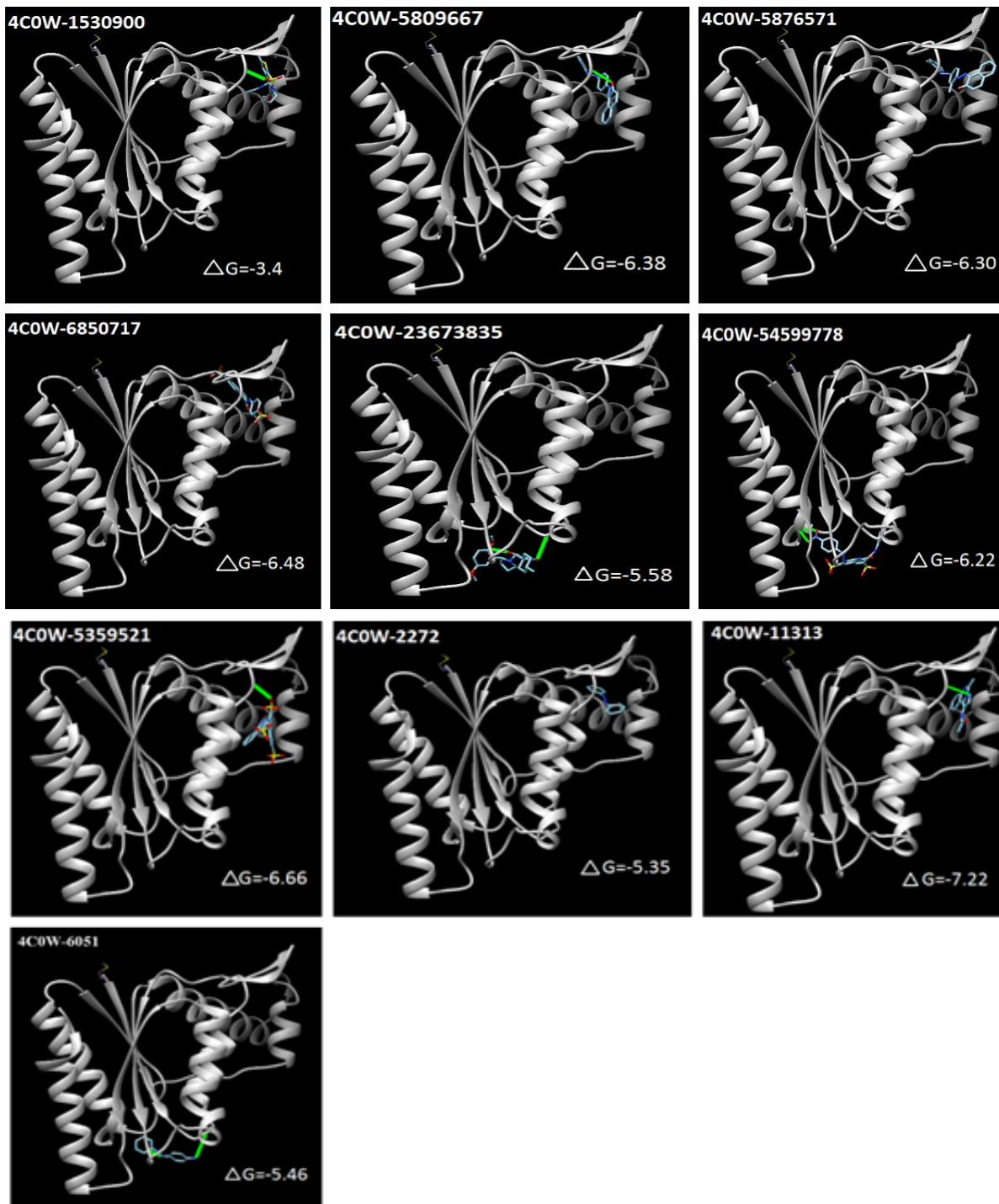


Figure 2. Docked images of the 10 industrially important azo dyes with azoreductase enzyme (PDB ID 4C0W). Figure also shows the respective binding affinities denoted by ΔG . 1530900 indicates Azepan-1-yl[(3S)-[2(chlorophenyl)methyl]piperidin-3-yl]methanone, 5809667 indicates Solvent Red 23, 587651 indicates Sudan IV, 6850717 indicates C.I. Food Yellow 3, 23673835 indicates Methyl Orange, 54599778 indicates Amiboschwarz, 5359521 indicates Amaranth, 2272 indicates Azobenzene, 11313 indicates Congo Red, 6051 indicates p-aminoazobenzene.

DISCUSSION

Due to enormous modernization, the most problematic

environment pollution is the wastewater where the effluents are being released from production of dyes and the dyeing industries. These residual dyes in industrial

Table 2. Chemical properties of the 10 industrially important dyes from PubChemCompound, showing the Molecular weight, Molecular formula, Hydrogen bond donor count, Hydrogen bond acceptor count, Rotatable bond count, Exact mass, Topological polar surface (A²), Heavy atom count, Complexity and Covalently bonded unit count of the dyes.

Dye	Molecular wt. (g/mol)	Molecular formula	Hydrogen bond donor count	Hydrogen bond acceptor count	Rotatable bond count	Exact mass (g/mol)	Topological polar surface (A ²)	Heavy atom count	Complexity	Covalently bonded unit count
Azobenzene	182.221	C ₁₂ H ₁₀ N ₂	0	2	2	182.084396	25.0	14	157	1
Amaranth	604.473	C ₂₀ H ₁₁ N ₂ Na ₃ O ₁₀ S ₃	1	5	4	603.92688	65.3	38	1200	4
p-aminoazobenzene	197.236	C ₁₂ H ₁₂ N ₂	2	3	2	197.095291	51.0	15	201	1
methyl orange	327.334	C ₁₂ H ₁₄ NNaO ₃ S	0	6	4	327.065357	93.5	22	448	2
Azepan-1-yl[(3S)-[2(chlorophenyl)methyl]piperidin-3-yl]methanone	334.88348	C ₁₉ H ₂₇ ClN ₂ O	0	2	3	334.181191	23.6	23	382	1
Sudan IV	380.441	C ₂₄ H ₂₀ N ₄ O	1	5	4	380.163711	66.2	29	670	1
Amiboschwarz	595.517	C ₂₂ H ₁₆ N ₆ NaO ₉ S ₂	4	14	7	595.031787	264.0	40	1260	2
Congo red	696.663	C ₃₂ H ₂₂ N ₆ Na ₂ O ₈ S ₂	2	12	5	696.083763	233.0	48	1180	3
C.I. Food yellow 3	452.369	C ₁₆ H ₁₀ N ₂ Na ₂ O ₇ S ₂	1	9	2	451.972481	173.0	29	818	3
Solvent red 23	352.388	C ₂₂ H ₁₆ N ₄ O	1	5	4	352.132411	66.2	27	597	1



Figure 3. Active sites displayed in three dimensional structure of the azoreductase enzyme predicted with DoGsite scorer and viewed in Chimera.

effluents are a threat to public health because of its high toxicity and carcinogenicity (Bisschops

and Spanjers, 2003; Weisburger, 2002). Unfortunately, azo dyes present in the wastewater are

normally unaffected by conventional treatment processes. Their persistence is mainly due to the

Table 3. Showing the amino acid composition of the different active sites predicted. The amino acid positions of the sites are listed above.

Active site	Residue
Site 1	SER (15,17), ALA(16,140,145), PRO 94, MET 95, TYR 96, ASN 97, PHE(98, 156), THR(139,149), GLY (141,142,146,176), LEU9143, 157), HIS(144, 153), GLU 154, ASP 155, ARG 158
Site 2	MET 1, LYS (2, 133), LEU 3, VAL 134
Site 3	ALA (70, 73), GLU 72, LYS 74
Site 4	HIS 5, ASP(7, 13), GLY 12, ASN 14, SER 15, ARG (18, 22), GLN 19, VAL (25,37), GLU 26,LYS 29
Site 5	VAL 172, ARG 173, ALA 174, HIS 175, GLY 176
Site 6	ALA (46,48, 106), ILE 47, HIS 49, PHE 50, THR 102, GLN 103, ASP 109
Site 7	ILE (6,108,164,166), PHE (83, 160), VAL (89,111,134), ALA 93, LEU(104,136,157), TRP 107, LYS 132, LEU 161

sulfo and azo groups, which do not occur naturally, making the azo dyes xenobiotic and recalcitrant to oxidative biodegradation (Kulla et al., 1983). Although lot of research has been carried out for their removal from industrial effluents, very little attention is given to changes in their toxicity and mutagenicity during the treatment processes (Bafana et al., 2008). Generally, in bacterial systems the degradation of azodyes is often initiated by an enzymatic step involving a cleavage of azo linkages with the aid of an azoreductase and an electron donor (Asgher et al., 2007). These azoreductases catalyze the reduction of the azo bond (-N=N-) in both azo pro drugs (e.g. balsalazide) and azo dyes (e.g. methyl red). They have been shown to reduce azo compounds via a ping pong mechanism (Wang et al., 2009). In recent years, we have seen a number of spectacular discoveries on surprisingly similar structures of proteins whose evolutionary kinship cannot be recognized based on primary sequence analysis alone (Gibrat et al., 1996). Hence, secondary structures allow a simple and intuitive description of 3D structures, which are widely employed in a number of structural studies. Therefore in context to the present the azoreductase enzyme has been subjected for emulating a suitable protein structure for the

evaluation of the docking parameters which would be further analyzed with the 10 commercially available dyes as shown in Table 1. The 3D structure that we have predicted using CPH model server 3.2 shows Ramachandran Plot in the favorable regions and it indicates that the structure is applicable to various applications like predicting the active sites and the amino acids involved. Thus, the modeled enzyme structure would provide an insight to the different type of azodyes which are degraded by the azoreductase enzymes and the conformational changes that take place in the enzyme structure which has been further analyzed by biodegradable systems models and docking studies (Nakanishi et al., 2001).

Furthermore, before the adjunct of the docking reactions these modeled structure has been explored for identifying the location of the ligand binding sites and this will help us in comparing the different functional sites of the azoreductase and the aspect of broad substrate specificity. The active sites which are predicted in this study revealed that the azoreductase could bind to any of the sites as shown in Figure 3, but the flexibility of effective binding sites has to be further studied using molecular modeled simulation. Although, the docking interaction predicted the number of

stable hydrogen bonds which itself indirectly indicates the binding of these dyes to the active sites of the enzyme molecule. Therefore, it could be predicted that the functional/active site of this enzyme from *P. putida* could probably be explored in further for developing and engineering effective bioremediation tools for detoxification of dyes contaminated areas.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Biosynthesis of silver nanoparticles by *Aspergillus niger*, *Fusarium oxysporum* and *Alternaria solani*

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Recently, biosynthesis of nanoparticles has attracted scientist's attention because of the use of environmentally friendly nanoparticles that do not produce toxic wastes in their process of synthesis. In this study we investigated the biosynthesis of silver nanoparticles using three fungi: *Aspergillus niger*, *Fusarium oxysporum* and *Alternaria solani*. These silver nanoparticles were characterized by means of UV-vis spectroscopy, scanning electron microscope (SEM). Results indicate the synthesis of silver nanoparticles in the reaction mixture. The synthesis of nanoparticles would be suitable for developing a microbial nanotechnology biosynthesis process for mass scale production.

Key words: Silver nanoparticles, biosynthesis, fungi, *Aspergillus*.

INTRODUCTION

Nanotechnology has recently become one of the most active research fields in Biology, Chemistry, Physics, Mathematics, Technology and Engineering which are integrated to explore benefits of the nano-world towards the betterment of the society (Koopmans and Amalia, 2010). The dimension of matter important in nanoscience and nanotechnology is typically on the 0.2 to 100 nm scale (nanoscale). The properties of materials change as their size approaches the nanoscale. Further, the percentage of atoms at the surface of material becomes more significant (Eustis, 2006). At present, different types of metal nanomaterials are being produced using silver, magnesium, oxide, copper oxide, aluminum, titanium dioxide, zinc oxide, gold and alginate (Ravishankar and Jamuna, 2011). These nanomaterials are used in various fields such as optical devices (Anderson and Moskovits,

2006), catalytic (Zhong et al., 2005), bactericidal, electronic, sensor technology, biological labelling, and treatment of some cancers and biomedical applications (Sarkar et al., 2007).

In recent years, the application of bio nanotechnology has been investigated as an alternative to chemical and physical ones. Research in bio-nanotechnology has shown to provide reliable, eco-friendly processes for synthesis of noble nanomaterials. Biological synthesis of nanoparticles using various biological systems such as yeast, bacteria, fungi, algae and plant extract have been reported (Yen and Mashitah, 2012). Metal nanoparticles have various functions that are not observed in bulk phase (Sosa et al., 2003; Sun et al., 2003) and have been studied extensively because of their exclusive catalytic, optical, electronic, magnetic and antimicrobial.

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Green synthesis is a process of synthesis and assembly of nanoparticles and has been used for a series of special production processes.

This process benefits from the development of clean, non-toxic and environmentally acceptable procedures which involve organisms ranging from bacteria to fungi and even plants (Mohanpuria et al., 2008). The microorganisms take target ions from their environment and by the cell activities through enzymes generated turn the metal ions into the element metal. Thus, it can be classified into intracellular and extracellular synthesis according to the location where nanoparticles are formed. In this paper we report the extracellular biosynthesis of silver nanoparticles (AgNPs) by using *Alternaria solani*, *Fusarium oxysporum* and *Aspergillus niger*.

MATERIALS AND METHODS

Isolation and identification of microorganisms

The fungi *Alt. solania*, *F. oxysporum* and *Asp. niger* were isolated from soil; soil samples were collected from different locations in Dammam, at the East of Saudi Arabia. Soil samples were taken from approximately 1 dm depth. One gram (1 g) of each soil sample was suspended in 9 ml water. One milliliter (1 ml) from 10-12 or 10-13 dilutions of soil suspension of the five different samples were placed on different nutrient agar plates. The plates were incubated for seven days at room temperature until colonies appeared. Isolates were purified by reinoculation of hyphen tips or cell colonies. When the microorganism appeared, it was reinoculated for three times onto new plates. The isolates were considered pure, and confirmed in the medical laboratory, King Faisal Specialist Hospital, Riyadh, Saudi Arabia. The fungi were inoculated in liquid media containing (g/l). KH_2PO_4 : 7.0; K_2HPO_4 : 2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.1%; $(\text{NH}_4)_2\text{SO}_4$: 1.0; yeast extracts, 0.6; and glucose, 10.0. The flasks were incubated at 25°C for 3 days in rotary orbital shaker at a speed of 150 rpm. The biomass was harvested after 72 h of growth by serving through a plastic sieve. The biomass was washed with sterilized distilled water to remove any medium component. 20 g of biomass (fresh weight) was mixed with 200 ml of deionized water in 500 ml Erlenmeyer flask and agitated in same condition for 72 h at 25°C after the incubation. The cell filtrate was obtained by passing it through whatman filter paper number 1. Filtrate was collected and used further for nanoparticles synthesis. For the synthesis of silver nanoparticles, 50 ml of 1 mM AgNO_3 solution was mixed with 50 ml of cell filtrate in 250 ml Erlenmeyer flask and agitated at 25°C in dark. Control (without the silver ion, only biomass) was also run along with the experimental flask (Basavaraja et al., 2008).

Evaluation of nanoparticles

The reduction of silver ion was confirmed by UV-visible spectrophotometer, 1 ml of sample was withdrawn after 24 h. The silver nanoparticles were evaluated for their surface and shape characteristics by scanning electron microscope (SEM) (Inspect s 50 FEI).

RESULTS

A bottle of the fungal show cell after removal from the culture medium and before immersion in AgNO_3 solution.

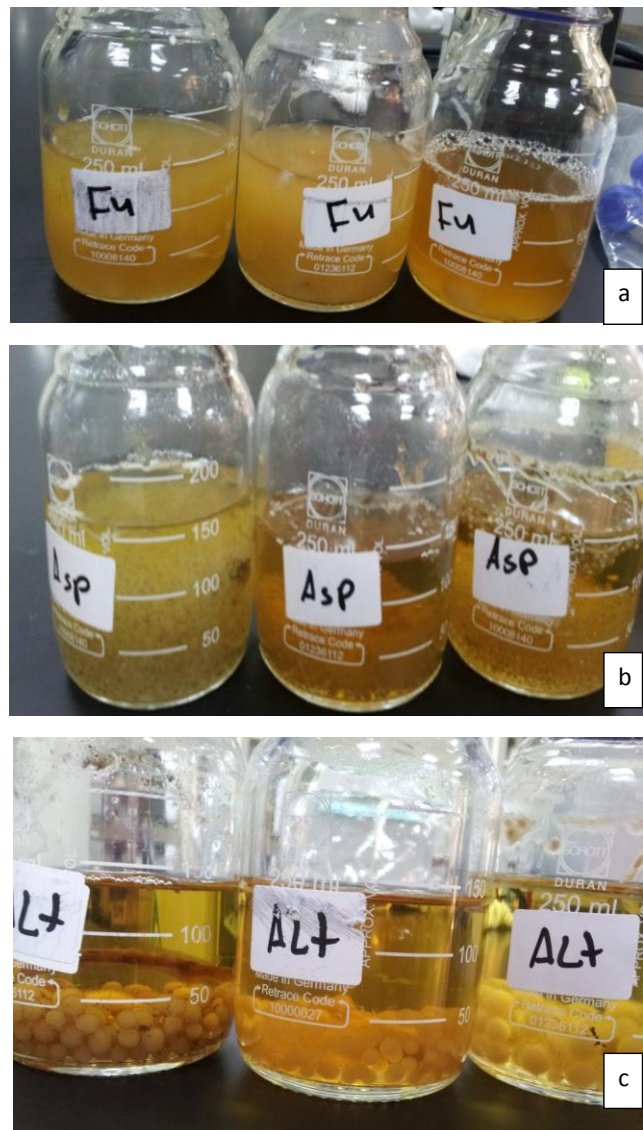


Figure 1. Fungus cultures in media (a) *Fusarium oxysporum* (b) *Aspergillus niger* (c) *Alternaria solani*.

The yellow colour of the fungal cell can clearly be observed in the bottle before immersion in AgNO_3 . The colour of the fungus filtrate changed from its natural colour to yellowish brown (Figure 1). Three different fungal species was screened for biological synthesis of silver nanoparticles and was clear (Figure 2).

The fungi were able to synthesizing silver nanoparticles with high stability. Optical spectroscopy is widely used for the characterization of nanomaterials. For three fungus, the UV-vis spectrum exhibited absorption band around 435 nm for *Asp. niger*, 445 nm for *Alt. solani* and for *F. oxysporum* it was 440 nm (Figure 3) scanning electron microscopy (SEM) image of AgNPs synthesized by fungus *Alt. solani*, *F. oxysporum* and *Asp. niger* shown in Figure 4. The morphology of the nanoparticles was spherical in nature.

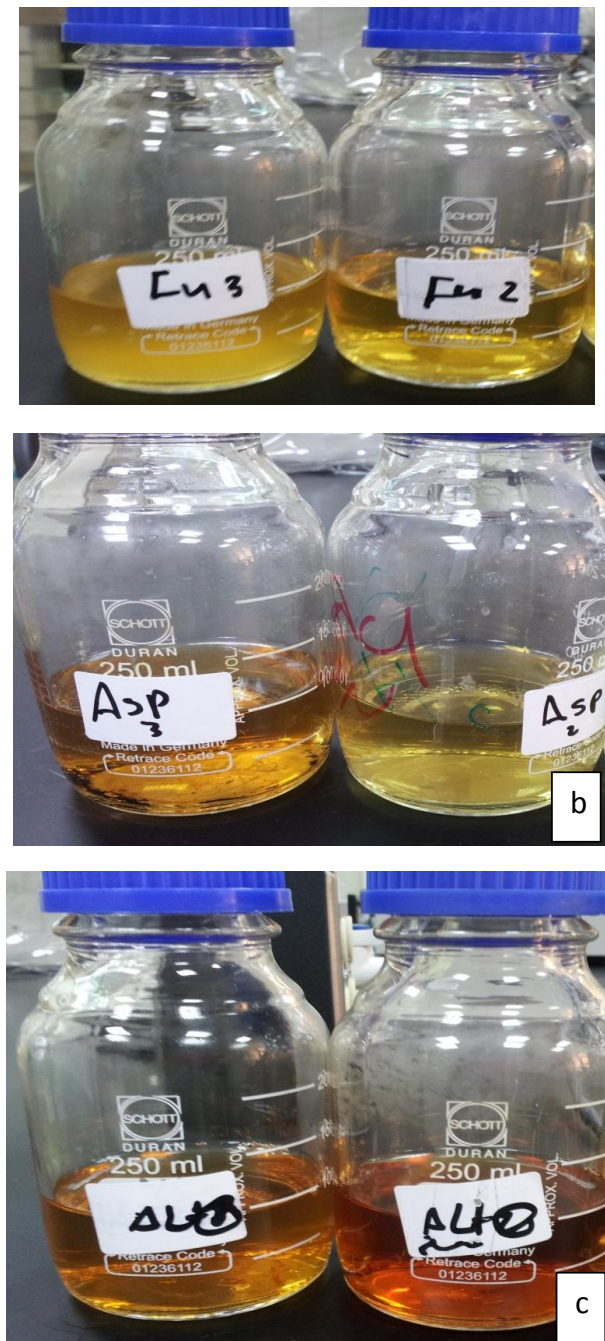


Figure 2. Biosynthesis of silver nanoparticles – colour change reaction after 24 h. **(a)** *Fusarium oxysporum*. **(b)** *Aspergillus niger*. **(c)** *Alternaria solani*.

DISCUSSION

The filtrate showed changes in colour from almost yellow to brown; this is a clear indicator of the formation of silver nanoparticles in the reaction mixture. Formation of dark brown is due to the surface plasmon resonance property of silver nanoparticles (Yen and Mashitah, 2010;

Ravishankar and Jamuna, 2011; Hemath et al., 2010; Sangeetha et al., 2012; Soheyla et al., 2013).

We used UV-vis spectroscopy to record the formation of AgNPs by reduction of AgNO_3 by fungi. The results show strong surface plasmon resonance centered at 445, 435, 440 nm for *Alt. solani*, *Asp. niger* and *F. oxysporum*, respectively which indicates the formation of silver nanoparticles, suggesting that the absorption band at the range 435-445 nm is due to electronic excitation in tryptophan and tyrosine residues in protein. Control without silver ions showed no change in colour when incubated under the same conditions. Many metals can be treated as free-electron system. These metals, called plasma, contain equal numbers of positive ions and conduction electrons (which are free and highly mobile). Under the irradiation of an electromagnetic wave, the free electrons are driven by the electric field to oscillate coherently. These collective oscillations of the free electrons are called plasmons. These plasmons can interact, under certain conditions, with visible light in phenomenon called surface plasmon resonance (SPR) (Ahmad et al., 2003; Duran et al., 2005). SPR plays a major role in the determination of optical absorption spectra of metal nanoparticles, which shifts to a longer wavelength as the particle size increases (Zhao et al., 2006). The shape and size of the result particles were elucidated with the SEM. Nanoparticles observed are spherical with a small percentage of elongated particles. It is a variation in particle size, and the average size was 20 nm for *Asp. niger* and it was 5 nm for *F. oxysporum* and *Alt. solani* 25 nm. The obtained nanoparticles are in the range of size approximately 1-50 nm and few particles are agglomerated (Narasimha et al., 2013).

In the biosynthesis of metal nanoparticles by a fungus, the fungus mycelium is exposed to the metal salt solution. That prompts the fungus to produce enzymes and metabolites for its own survival. In this process, the toxic metal ions are reduced to the non-toxic metallic solid nanoparticles through the catalytic effect of the extracellular enzyme and metabolites of the fungus (Khabat et al., 2011). This biosynthesis technique can be a promising method for the preparation of metal nanoparticles and can be valuable in environmental and biotechnological applications.

Conflict of interests

The authors did not declare any conflict of interest.

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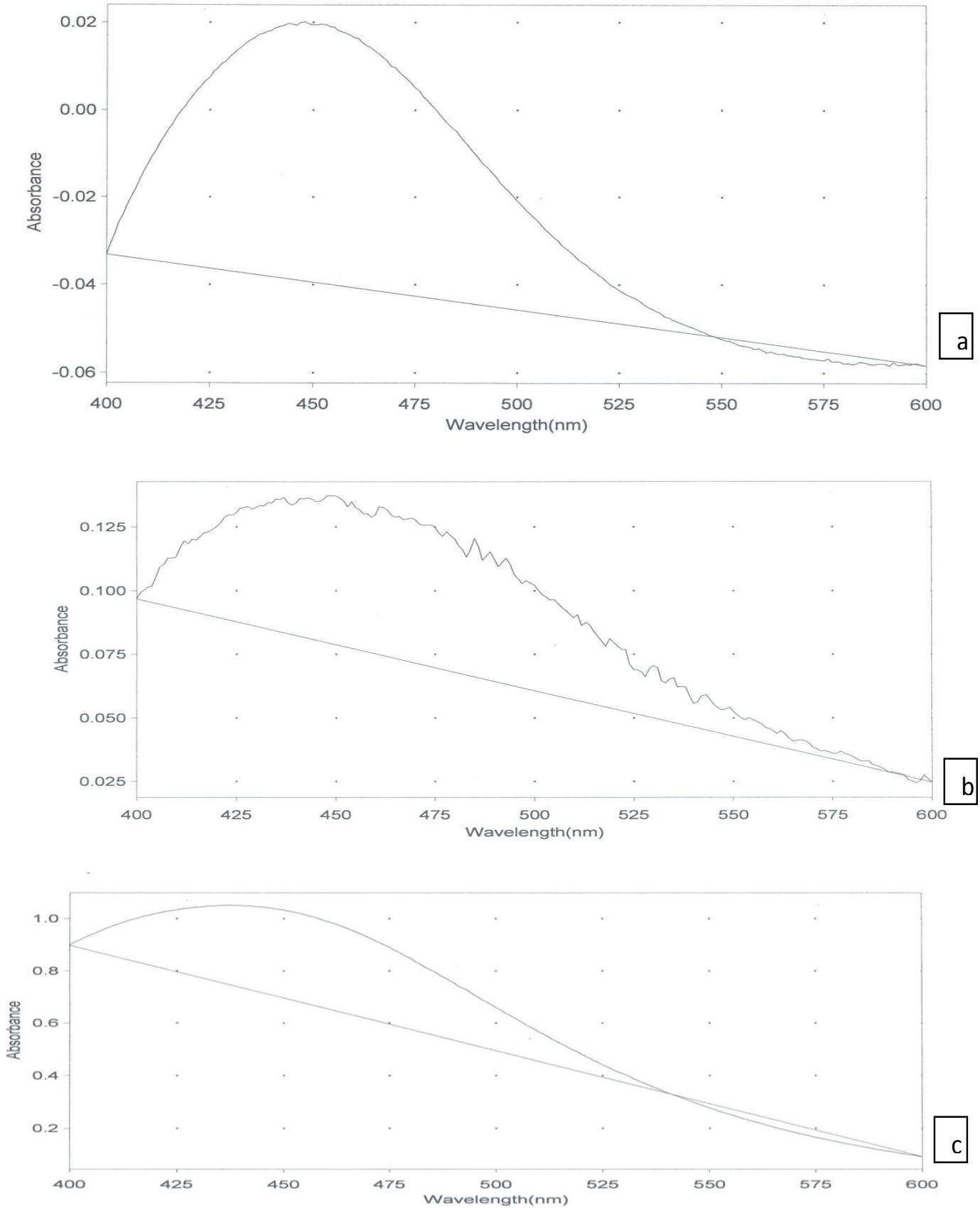


Figure 3. UV-visible absorption spectrum of AgNPs produced by (a) *Fusarium oxysporum* (b) *Aspergillus niger* (c) *Alternaria solani*.

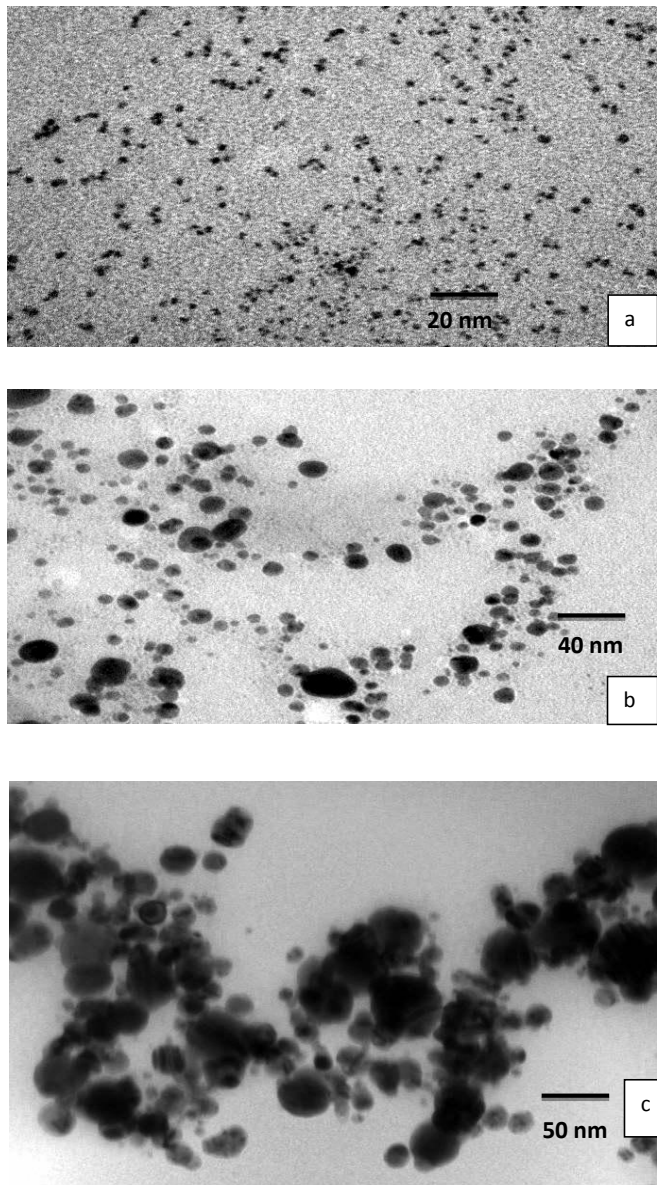


Figure 4. TEM image of AgNPs synthesized by: (a) *Fusarium oxysporum* (b) *Aspergillus niger* and (c) *Alternaria solani*.

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