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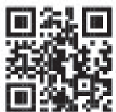

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Transcriptomic Analysis of *Yarrowia lipolytica YlNTH1* and *YlTPS1* Genes Under Different Carbon Sources

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Abstract
The biosynthesis of trehalose is catalyzed by synthase enzyme complex and degraded by neutral trehalase enzyme. *Yarrowia lipolytica* is a dimorphic yeast and used as a model organism for understanding the lipid metabolism of higher eukaryotes. *YlTPS1* and *YlNTH1* genes are responsible from the recycling of trehalose in *Y. lipolytica*. The aim of this work is to determine the expression patterns of *YlTPS1* and *YlNTH1* genes under different carbon sources by using RNAseq study. The files used in this analysis were obtained from the EMBL-EBI data bank and Study ID is "PRJEB2863". The substrates used in the work are Alkane, Glucose, Glycerol, Oleic Acid, Tributyrin and Triolein. The run accession numbers of substrates are ERR073010, ERR073011, ERR073009, ERR073008, ERR073012 and ERR073007, respectively. "Trimmomatic" tool was used for croping the readings in the next generation sequencing files. Reference genome of *Y. lipolytica* were obtained from Ensembl genome data base. The files were converted to "ERR0730xx_tophat2.bam" files and used in the "FeatureCounts" tool. We found that the transcript level of *YlTPS1* gene was 2 to 4 times higher than *YlNTH1* gene. The rate of trehalose synthesis is greater than the rate of breakdown in *Y. lipolytica* yeast cells.

Keywords: *YlTPS1, YlNTH1,* RNAseq*, Yarrowia lipolytica*

INTRODUCTION

Trehalose is a non-reducing disaccharide composed of two glucose units and an essential component of the yeast metabolism. The one of the most important function of trehalose is to protect cell membrane components, proteins and lipids, from different types of stress factors such as heat stress [1, 2]. The cytoplasmic level of trehalose is strictly controlled by recycling of trehalose continouslyin yeast cells. This recycling process involves the actions of trehalose sythesis and breakdown enzymes. The biosynthesis of trehalose is catalyzed by TPS complex, composed of Tps1p, Tps2p, Tps3p and Tsl1p subunits. The breakdown of stress-accumulated trehalose is catalyzed by neutral trehalase enzyme (Nth1p) in *Saccharomyces cerevisiae* [3].

Yarrowia lipolytica is a non-pathogenic dimorphic yeast that can shift from yeast to hyphal form, or vice versa. *Y. lipolytica* is also used as a model organism for understanding of lipid metabolism in higher eukaryotes because of its ability to utilize and accumulate high levels of lipid [4, 5]. *Y. lipolytica* stores triacylglycerol (TAG), glycogen and trehalose as a source of carbon. Yeast cells can adapt themselves to environmental fluctuations by making some metabolic and genetic switches. The accumulation of trehalose is triggered as a response to nutrient starvation such as carbon-limited condition. *YlTPS1* (YALI0E14685) and *YlNTH1* (YALI0D15598) genes are responsible from the recycling of trehalose in *Y. lipolytica* [6]. In the absence of environmental stresses, the rate of trehalose synthesis and breakdown must be enzymatically balanced in exponentially growing *S. cerevisiae* yeast cells. Therefore, the main objective of this work was to determine the expression patterns of *YlTPS1* and *YlNTH1* genes under different carbon sources by using RNA sequencing study presented in the EMBL-EBI (European Molecular Biology Laboratory- European Bioinformatics Institute) data bank The exponentially growing *Y. lipolytica* yeast cells in different carbon sources (Alkane, Glucose, Glycerol, Oleic Acid, Tributyrin and Triolein) were used for callculating mRNA levels of *YlTPS1* and *YlNTH1* genes [7]. The transcript level of *YlTPS1* gene was 2 to 4 times higher than *YlNTH1* gene in *Y. lipolytica* yeast cells.

MATERIALS AND METHODS

The files used in this analysis were obtained from the EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute; http://www.ebi.ac.uk) data bank. The name of the work being used is "Comparative transcriptomics of *Yarrowia lipolytica* on different substrates" and Study ID is "PRJEB2863". The substrates used in the work are Alkane, Glucose, Glycerol, Oleic Acid, Tributyrin and Triolein, and the full protocols were given in the web page [7]. The run accession numbers of substrates are ERR073010, ERR073011, ERR073009, ERR073008, ERR073012 and ERR073007, respectively. They were saved as "fastq.qz" files in our computer, and all "ERR0730xx.fastq.gz" compressed files extracted to uncompressed files such as "ERR0730xx.fastq". All the uncompressed files were converted to "ERR0730xx.fastqsanger" files via "fastqGroomer" tool. "Trimmomatic" tool was used for croping the readings in the next generation sequencing files that is*.fastqsanger files. The order of "Trimmomatic" tool parameters are TruSeq2-PE-fa:2:30:10 (Illuminaclip adapters), Crop: 36, Leading: 3, Trailling: 3 and Slidingwindow: 4:20. The cropped files obtained from "Trimmomatic" tool (i.e.: "ERR0730xx_trimmed.fastqsanger" files) were used in "Tophat2" mapping tool which is developed for RNAseq reads. Reference genome of *Y. lipolytica* were obtained from Ensembl genome data base [7]. As a result of "Tophat2" tool, the mapping rates are 92.3%, 66.1%, 74.8%, 81.9%, 69.8, and 67.2% for ERR073010, ERR073011, ERR073009, ERR073008, ERR073012 and ERR073007, respectively. And all the "ERR0730xx_trimmed.fastqsanger" files, obtained from "Trimmomatic" tool, are converted to "ER-

R0730xx_tophat2.bam" files by means of "Tophat2" tool. All the "*.bam" files were used in the "FeatureCounts" tool for counting the mapped readings for genomic regions. The gene annotations used in "FeatureCounts" tool were obtained from Ensembl genome data base [9]. The output files of the "FeatureCounts" software ("ERR0730xx.tabular") were opened with the application of "Openoffice - Calc" tool.

RESULTS AND DISCUSSION

The oleaginous yeast *Y. lipolytica* can use different types of hydrophobic substrates as a carbon source in order to growth and TAG accumulation [10]. The ability of *Y. lipolytica* to grow on alkanes, oleic acid, tributyrin and triolein make this yeast a good model for investigating lipid metabolism. In addition, the growth of *Y. lipolytica* on these hydrophobic carbon sources may effect the accumulation of other carbon sources such as glycogen and trehalose. From this point of view, we tried to determine expressions of *Y. lipolytica* genes involved in trehalose metabolism. RNA sequencing technology is used for monitoring mRNA levels, and thereby the gene expression levels. That's why we used RNA sequencing data base to monitoring the *YlTPS1* and *YlNTH1* gene expression patterns in different carbon sources including hydrophobic carbon sources also.

When *Y. lipolytica* yeast cells were grown in hydrophilic carbon sources such as glucose and glycerol, mRNA levels (given as read counts) were calculated as 185 read counts in glucose and 155 read counts in glycerol for *YlNTH1* gene; and 479 read counts in glucose and 480 read counts in glycerol for *YlTPS1* gene (Figure 1). As shown, *YlTPS1* and *YlNTH1* gene expressions in glucose and glycerol were nearly same, but the mRNA level of *YlTPS1* gene was 2-3 fold greater than that of *YlNTH1* gene.

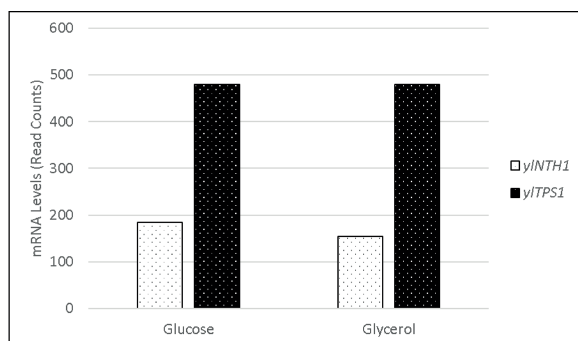

Figure 1. The mRNA levels of *YlTPS1* and *YlNTH1* genes in glucose and glycerol.

When *Y. lipolytica* yeast cells were grown in hydrophobic carbon sources such as alkane and oleic acid, *YlNTH1* and *YlTPS1* gene expression levels were given in figure 2. The gene expression levels of *YlNTH1* gene was calculated as 176 read counts in alkane and 306 read counts in oleic acid. The transcript level *YlTPS1* gene was calculated as 360 read counts in alkane and 557 read counts in oleic acid. As you can see the *YlTPS1* gene expression level was nearly 2 fold greater than *YlNTH1* gene expression. Also *YlNTH1* and *YlTPS1* gene expression levels increased when yeast cells were grown in oleic acid.

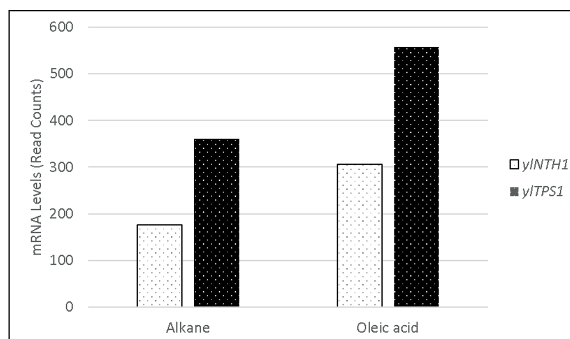

Figure 2. The mRNA levels of *YlTPS1* and *YlNTH1* genes in alkane and oleic acid.

Additionally, when yeast cells were grown in tributyrin and triolein hydrophilic carbon sources, the mRNA levels were calculated as 176 read counts in tributyrin and 158 read counts in triolein for *YlNTH1* gene; and 559 read counts in tributyrin and 525 read counts in triolein for *YlTPS1* gene (Figure 3). *YlTPS1* and *YlNTH1* gene expressions in tributyrin and triolein were nearly same, but the mRNA level of *YlTPS1* gene was nearly 4 times higher than *YlNTH1* mRNA level.

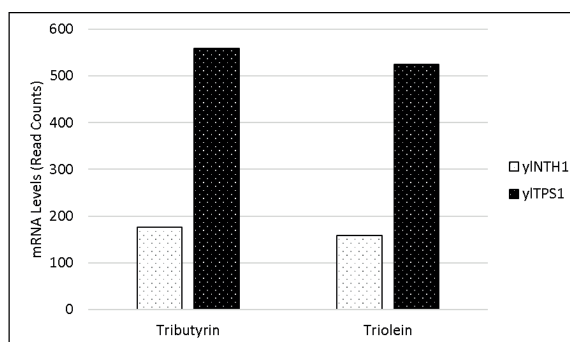

Figure 3. The mRNA levels of *YlTPS1* and *YlNTH1* genes in tributyrin and triolein.

Generally the transcript level of *YlTPS1* gene was 2 to 4 times higher than *YlNTH1* transcript level. There was no considerable effect of carbon sources -hydrophobic or hydrophilic- on *YlTPS1* and *YlNTH1* gene expressions. *YlNTH1* gene expression level of yeast cells grown in oleic acid was higher than other carbon sources. As a result in *Y. lipolytica*, the rate of trehalose synthesis is greater than the rate of trehalose breakdown.

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The Impact of Ascorbic Acid and Some Additives in the Rheological Properties of Doughs in Pasta from Agimi, Apache and Anchor Wheat

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Abstract

This study is a report about the latest researches in dough technology. Results are valid for Balkan countries. In recent years, a high temperature drying system has been established in high quality pasta production. Continuous increase in pasta production has brought an increase in demand for processed materials such as flour from wheat processing. The production with only flour is not enough to recover the major part of pasta consumption. For this reason new methods to improve the quality of pasta production with *Tricitum aestivum* flour are being investigated. We have been studying the usage of L-Ascorbic acid in the industrial level experimented in 2 types of pasta where in the first type is used only the wheat with high "glassness" and in the second type is used the flour by soft wheats with extra additives. Before the experiment in the industrial level, prior researches were made in the laboratory of "Miell Tirana" Company in Albania. Here a mixture of flour by soft wheats with extra ascorbic acid and wheats only with high "glassness" are used for the pasta production. These systems facilitate the use of processing techniques using high temperature drying systems. Pasta quality depends on too many factors like water temperature, dough, product itself, added methods of LAA, the first material and working conditions.

Keywords: dough, pasta, high, glassness, extra additive

INTRODUCTION

The purpose of our researches is to determine the ascorbic acid impact in the pasta quality, produced in the industries,which use semola and different semolats by high "glassness" and soft wheats with different drying systems, generally the method with high temperature [7,15].

In cereal chemistry and technology, ascorbic acid is used for many years as an addition to improve physical properties and technological mixture of different flour products. In this study,was used the ascorbic acid in different quantities in semola and semolats with wheat flour. In some countries like Italy, Germany, France, Switzerland etc, ascorbic acid is the only additive that helps the drying process in rototherm. Its added quantity varies from 150-180 ppm. In our study the used quantity varies from 75-200 ppm. Pasta quality has had changes and adding ascorbic acid in this technology is carefully studied. This study, which is a result of an experimental work and analysis, opens new fields of ascorbic acid applications in the actual pasta production. All experiments are done in factory. From this experiment clearly results that adding mixture of different quantities of ascorbic acid has improved the quality of cooking pasta which requires the high temperature drying system technology.

MATERIALS AND METHODS

For the production of pasta, there are used flours produced by Russian, French and Albanian wheats in a certain percentage by each cultivar.

The first major material for industrial production of pasta is exclusively the wheat with high "glassness" or its flours, semola and semolats.

The usage of ascorbic acid and its high temperatures have been object of this study. As a result, production of pasta is different in comparison with traditional technologies. The usage of high temperatures in the end of pre-drying phase, makes the pasta incur a special hydrothermic treatment in ambiental environment with temperature 73-85 grade Celsius and relative moisture around 82-83 %. The dough for long pasta with an extra 200 ppm goes into rototherm with no more than 19% of moisture and in this way the freezing of starch is avoided, before the proteinic mesh, that covers amidon particles.

Also, during staying in rototherm for 10 minutes, the long pasta, especially the softer formats of pasta (diameter 1.7-1.8 mm), incured a thermic deactivation of enzyme. In this thermic level some oxidases and beta amylases are active in temperature 60 degree Celsius and above: like alpha-amylase, lipoxygenase, peroxidase. In our case, the usage of high temperature heating pasta in rototherm, in 65 grade Celsius and above, deactivated especially the complex enzyme of phenol-oxidase. If it is not deactivated, this enzyme has negative influences. In temperatures lower than 60 degree Celsius, it is transformed between effluent reactions without enzymatic catalyst.

In this point, it can be highlighted that kinons are reput in phenols, adding reductive substances such as L-Ascorbic acid. These reductive substances exert an antioxidant synergistic action that does not allow the formation of melanin. L-Ascorbic acid also prevents the lipids oxidation, a very important result in the production of pasta with eggs [17].

So the application of high and very high temperatures and adding ascorbic acid have fundamentally changed pasta technology, making sure that SH links are oxygenated in very powerful SS links. So we have not only “pastification” of the flour of soft wheat, but also of the other flours [7].

For this study we have taken cultivars from French, Russian and Albanian wheat. After the production and evaluation of the acquired flour, there are made pasta. First it is done the production from semola to granulometric 320 to 420 with and without extras. The physical-chemical and rheological analysis are done in accordance with standard methods ICC (impurity ICC stand, 102/1, moisture ICC stand. 110/1, ash ICC stand. 104/1, wet gluten ICC stand. 106/2). The determination of rheological qualities, is made with Farinograph and Extensograph Brabender, Alveography “Chopin” and organoleptics qualities of pasta are made in accordance with the physical-chemical methods rule for the cereals, products of grinding and furnace, pasta and frozen doughs (ICC-Standard No102/1, Revised 1972) [8.9].

RESULTS AND ANALYSIS

Characteristics of wheat

In order to investigate the effect of ascorbic acid in the flour, the wheats originally from France (Apache), Russia (Anchor), and Albania (Agimi) was taken in the study.

Table 1. The qualitative indicators for studying of wheat [8]

Cultivar of wheat	Wheat qualities						
	Hectolitre Weight *(kg/hL)*	Humidity (%)	Protein (%)	F.N (sek)	Amilase AU	Gluten (%)	W (P/L)
Apache	78.3	12.6	11.8	310	600	26	200
Anchor	78	13.1	12.5	290	400	29	240
Agimi	79.2	13.2	12.8	320	700	30	245

According to Table 1 the highest content of protein and the highest W is in Agimi wheat.

From every cultivar there are taken 20 tons each for grinding and are conditioned for 18-24 hours to achieve moisture 16.5% for grinding, where we have won 2 semola fractions with granulometrics 420 micron and 530 micron [5,6].

Table 2. The quantity of produced semolas from wheat cultivars grinding

Cultivar of wheat	Semola 420 µ
Anchor	67 %
Apache	65 %
Agimi	64 %

From the data of table 2 it is observed that Anchor cultivar gives higher semola quantity than other wheats.

Table 3. The physical-chemicals and rheological qualities of acquired flours, by each cultivar after grinding.

Wheat qualities	Cultivar of wheat					
	Anchor		Apache		Agimi	
	Semola µ		Semola µ		Semola µ	
	420	530	430	520	440	530
Humidity (%)	13.8	13.5	14.1	13.8	13.7	13.8
Ash (%)	0.67	0.76	0.68	0.81	0.69	0.74
Sedimentation	58	49	40	33	51	41
Wet Gluten (%)	30	30	27.5	28	31	31
Protein (%)	13.1	12.9	12.3	12.2	13.1	13
Carbohydrate (%)	56.5	-	56.7	-	55.4	-
Water absorbtion (%)	64	65	62	63	65	66

In this table there are presented the acquired results for the physical-chemical and rheological qualities of acquired flours by each cultivar after grinding.

There are given all physical-chemical qualities of above listed cultivar semolas in table 3 [13]. The moisture of all cultivars is in normal values and it varies from 13.5% to 14.1%.

Table 4. The granulometric values of wheat semolas given for pasta production.

Sieves standards	Diameter (μ) Anchor	Diameter (μ) Apache	Diameter (μ) Agimi
About 35-40	450	470	440
About 45	410	380	340
About 60	280	240	320

The produced pasta with finely semolas (between 315 and 225 micron) has got a beautiful yellow color, without white points. The yellow color is noted more if pasta is dryed with the very high temperature technology [13].

Table 5. Semolas Characteristics

Designation	Humidity (%)	Ash (%)		Cellulose		Nitrogenated substances
		Min.	Max.	Min.	Max.	
SEMOLO	14,2	0,65	0,80	0,20	0,45	12,50
SEMOLATI	14,3	0,90	0,95	–	0,85	12,90

The first most finely materials are easier to be transformed in homogenious dough, which is an optimal base for homogenious products. They require shorter time for dough cooking and on going, they require smaller tubs for dough, since they are easier to be cleaned [4].

Rheological analysis of cultivars taken for study

The evaluation of rheological qualities of wheat cultivars is really important for the determination of the improvement of quantities in flours and in doughs that produce pasta.

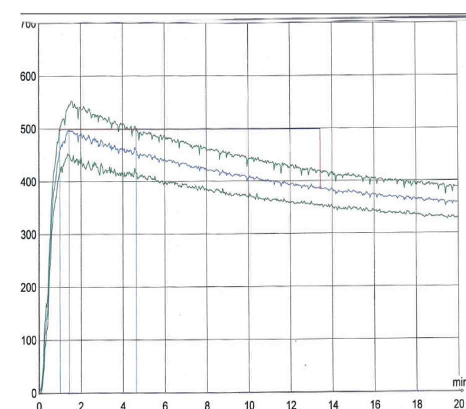
Analysis in Farinograph

Farinograph measures the dough consistency, mixing it with a constant speed and absorbs water, which enables the achievement of this consistence [8].

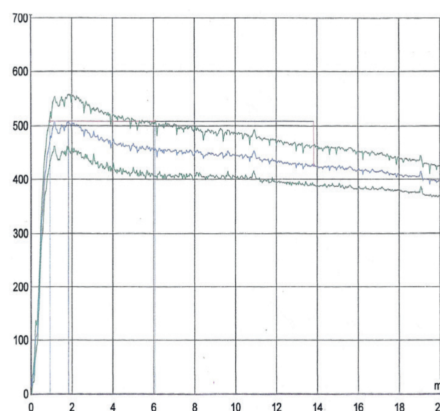
Table 6. Qualitative indicators with Farinograph

Cultivar of wheat	Farinographic characteristics			
	Water absorption (%)	Development time (min)	Stability (min)	Softness (PE)
Apache	59.0	1.7	7.2	54
Anchor	60	1.9	7.4	55
Agimi	58	2.6	6.9	77

From the data observed in table 6, clearly is seen that Anchor cultivar has a bigger absorption of water than Agim and Apache. The development time of Agim is the longest, while its stability is smaller than Anchor and Apache cultivar. Also the softening scale of Apache cultivar is smaller than that of the Anchor. So it's important using redox additives (ascorbic acid) in these kinds of wheats. [7].



Reference flour



Reference + 200 ppm ascorbic acid

Analysis in Extensograph

Extensograph measures dough opening and resistance during resting time.

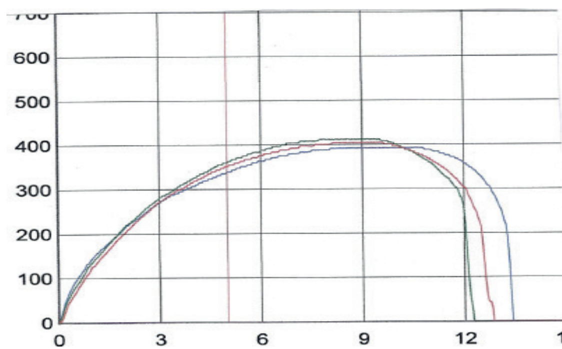
From analysis datum with extensograph we can see that it hasn't only prononuned changes in "opening" of dough,in its resistance impacted, or in self qualities of flours (that depend from cultivars we analyse, their radius), but also the ascorbic acid has got a pronounced impact upon them.

Table 7. Qualitative indicators in Extensograph

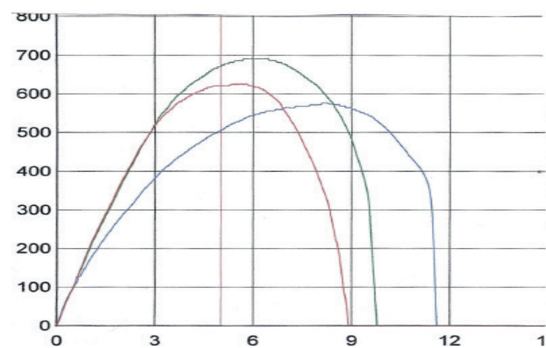
Flour Cultivars	**Extensograph**			
	Resistance in tow (EU)	**Elasticity (mm)**	**R/E**	**Energy (cm^2)**
Apache	275	159	1.69	78
Anchor	298	172	1.82	92
Agimi	289	168	1.67	89

Note: ascorbic acid 200 ppm

From the comparison of data with extensograph we can see, we have a pronounced increasement of dough resistance when we add 200 ppm ascorbic acid.



Flour free additives (Reference)



Reference + 200 ppm ascorbic acid

Addition of L-Ascorbic Acid

For this proofs are done: [13.15]

In the first one frizzy pasta reproduced with first materials in respective quantities:

-200 kg flour
-54 litres water
-200 ppm L-Ascorbic

Table 8 .Values of extensograph of Apache, Anchor, Agimi wheats flour used in the production of long pasta

After the break	**Dough of resistance**						**Dough of extension (E) (mm)**			**R5:E**			**Surface(S) (cm^2)**		
	5 min.			Max											
T	R5			R max											
45	550	520	540	580	570	580	102	103	102	5.4	5.3	5.2	78.6	78.5	78.8
90	460	430	470	525	530	535	123	125	126	3.6	3.5	3.7	79.1	78.4	78.6
135	360	370	360	400	390	395	136	137	138	2.6	2.7	2.6	79.4	78.6	78.9

The best value of extensograph is represented after 45 minutes of dough resting from the extensograph. The second proof is similar with the first one, with the only difference, that in this case, there are added 200 mg (200 ppm) melted L-Ascorbic Acid in a water part used for the dough. For all of these it is known, especially in the high and very high temperature drying systems (respectively around 74-76 degrees Celsius and 85-96 degrees Celsius), while in the traditional technologies case of drying (from 45 to 54 degrees Celsius), the necessities are lower.

It's preferable for L-Ascorbic acid to be melted in water, for the most homogenious digestion. It is outputted a long

pasta with good quality, applying these drying methods immediately after the pre-drying phase, or when water content in pasta is around 11.5%. The usage of THT systems gave pasta a bigger plasticity, in comparison to traditional systems, guaranteeing a higher resistance towards phenomena like breakings or bursts. Pasta in 95 degree Celsius is covered with a very poor mixture of air and is rich with overheated steam in atmospheric pressure, which theoretically is unappropriate to accelerate the drying. The plant for the industrial production of pasta is made of an automatic continuous press that deals with different operations, dosage of different ingredients, dough formation, which is done in two phases, with a pre-mixture of components and the next phase of dough perpetration (compression and) for the acquisition of different formats desired by the manufacturer. The good functioning of press and ongoing, of compressor, are the major factors of producing a clean product, with good commercial and cooking characteristics. The dosages are interdependent and in some modern presses, there are pre-mixture mechanism that mix too fast (turbospray) the flours and water (or also other ingredients if they have been asked to, without causing any damage to semolo or flour) [14].

The formula calculated in 100 parts is this:

100 kg flour (with 14-14.5 % water);

30.1 l water, 5200 mg L-Ascorbic acid (200 ppm). It is obtained a long pasta with good quality applying these drying methods with a water percentage in pasta around 11.5%.

We made the evaluation of dryed and boiled pasta

The criterias in which the tests about the quality of pasta products are based, include control of organoleptical, biochemical, commercial and nutritional features. In commercial features a great importance is noted in the color and absence of fractures, crackings, white and black points in the pasta surface. Pasta are produced only with semolas of Agim, Russian and French.

They deal with a color yellow to gray. The quality of pasta boiling is absolutely the most imporant aspect to verify the control [1.2].

- water absorption;
- increasement of mass after boiling;
- residue in the mixture water.

Characteristics in boiling, are shown in table 9, referring to pasta samples, produced only with semolas of Agim, Anchor and Apache wheats.

Table 9. The quality of different formats boiling of pasta with wheat base Agim, Apache, Ankor

Formati	Time for cooking (min.)(1)			Water of absortion from pasta (%)(2)			Total weight After boiling (gr)(3)			Remaining in water boiling (%)(4)		
Strow like	10	9	11	155	150	140	63	62	59	8	9	8
Star	10	9	11	190	180	170	72	71	70	9	10	11
spaghetti (diam.1,8 mm)	12	12	12.3	180	180	160	72	71	68	4	5	6
Ribbon-like	9	9	10	167	162	160	69	68	67	6	7	8
with holes	10	10	11	173	170	160	69	68	67	5	6	7
Semolina noodles pasta	12	11	12	190	185	160	77	76	75	4	5	6

(1) The time is calculated from the moment of water boil;

(2) It is calculated in 100 g pasta after boiling;

(3) It is the final weight of 25 g pasta, after boiling;

(4) The loss of total solids in boiling water in the actual moisture content.

From data in table 9, it is seen that pasta produced by semolas of Agim wheat has the lowest residue of all, in the boiling water. This shows that SS links in proteins are stronger. Also the highest absorption is noted in Agim wheat semolas pasta.

CONCLUSIONS

The mixture for dough preparation has got a moisture of 29-31%. In these conditions of hydration also the LAA effect is visible. LAA quantity must be at least 200 ppm, especially when we use flour with above indicator. Water purity is very important and its temperature has got an impact in the LAA action. When LAA is digested, the water temperature must not pass 50-55 degree celsius, while the mixture temperature with egg adding and LAA must not be higher than 38-48 degree celsius. The addition of LAA is recommended for nutritional dough with flour or mixture. In the case of low quality flour, the moisture compound is lower than 24% and it is necessary to add 200-300 ppm of LAA and eggs knowing that albumina has got a positive impact in the compound characteristics. Usage of HT drying system in rototherm, the short drying time (around 10 hours). We must note that dough enters in rototherm having max 18.5% H_2O, and in opposite case the HT effect may be negative.

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Downregulation of Tetraspanin 8 and Carbonic Anhydrase 9 Gene Expression Sensitized Pancreatic Cancer Cells to Cisplatin.

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Abstract
In this context, we used the combined siRNA and chemotherapeutic drug treatment strategy for minimizing the side effects of chemotherapy agents in pancreatic cancers.We used the gene silencing approach with siRNA to target CA9 and TSPAN8 genes, which are overexpressed in pancreatic cancer.Then expression of the targeted CA9 and TSPAN8 geneswere determined by western blot analysis. Effect gene silencing on the proliferation of Panc-1 and MiaPaca-2 cells were detected by MTT assay after transfection with ControlsiRNA, CA9siRNA and TSPAN8 siRNA. MTT cell proliferation assay was also performed to determine the effect the combined siRNA and chemotherapeutic drug treatment as to evaluate cisplatin and epirubicinsensitivity.The results demonstrated that targeted CA9siRNA and TSPAN8 siRNA have increased the sensitivity of pancreatic cells to cisplatin and epirubicin that can conclude;CA9 and TSPAN8 silencing may be a suitable candidate for therapeutic applications.

Keywords: CA9, TSPAN8, siRNA, pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the 4thmajor reason for cancer-related death in Europe [1].Pancreatic cancer is an aggressive disease and has a highly poor prognosis [2].Chemotherapy is still the first approach in the treatment of metastatic pancreatic cancer although it has insufficient effects on patients' survival[3].Development of new chemotherapy strategies specifically in combination with molecular targeted therapies are increasing progressively[4].TSPAN8, member of the tetraspanin superfamily, is a tumor-associated gene and overexpressed in several types of cancersincluding pancreatic cancers[5-7]. TSPAN8 has a pivotal role in many cancer cell vital functions, such as cancer cell migration, metastasis and tumor angiogenesis [7-12]. CA9 is hypoxic tumor marker protein that catalyzes the reversible hydration of carbon dioxide to bicarbonate and proton on the cell membrane[13]. CA9 is responsible for the cancer cell survival, as well as to several other biological processes, such as the maintenance of migration and invasion. Targeting of CA9 using therapeutic drugs is a current approach for the treatment of hypoxic solid tumors and clinically useful biomarker of the broad range of hypoxic tumors[14].

In the present study, weinvestigated the cytotoxic effects of cisplatin and epirubicin treatment with siRNA mediated gene silencing of TSPAN8 and CA9 genes in pancreatic cancer cells.

MATERIALS AND METHODS

ICell culture and transfection of siRNA

Panc-1 and MiaPaca-2 were cultured in Dulbecco's modified essential medium (Biological Industries) supplemented with 10% fetal bovine serum (Biological industries) at 37˚C in 5% carbon dioxide incubated (Nuaire). Cells were subcultured when a confluence of 80-90% was grown in T-75 flask. MiaPaca-2 cells were transfected withfinal concentrationof 100-50-10-5 nM of CA9siRNA and TSPAN8 siRNA (Santa Cruz). siLentFect™ Lipid Reagent (Biorad) is used for transfections according to the manufacturer's instructions.

Cell proliferation assay (MTT)

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Panc-1 cells or MiaPaca-2 cells(10000 cells/well) seeded in 96 wellplates and transfected with 100 nM siRNA as described above. 24h and 48hafter transfection,MTT added to cells at a final concentration of 0.5 mg/ml and 4 h incubated at 37°C, the medium was discarded and dissolve the cells in 100 µl in 2-propanol containing0.004M HCl and the absorbance was measured with aspectrophotometer at 550 nm.

Combinational treatment of cells with siRNA and chemotherapy agents

Cells were seeded (5,000 cells/well) in a 96 well plate of O/N and transfected with 50 nM siRNA and incubated 24 hours. Different concentrations of cisplatin (0.5-5-25-50-100 µg/ml) or epirubicin (0.1-1-10-100-500 µg/ml) were added and incubated for 24 hours. Non-transfected cells were also treated withthesame concentration of cisplatin and epirubicin. Cell proliferation wasmeasured by MTT assay as described above.

Western blot

Panc-1 and MiaPaca-2 cells cultured in 6 well plates 5x105cells/well. After 48-hourtransfection, medium was discarded and cells were washed with PBS. Cells were scraped with RIPA buffer, transferred to eppendorf tubes and incubated on ice for 30 minutes. The supernatant was collected by centrifugation at 12000 g,for10minat + 4oC. The amount of total protein was determined using Qubit system. 50 µg total proteinswas loaded to 12.5%SDS-PAGE and separated by electrophoresis. Separated protein was transferred to a PVDF membrane.Membrane blocked with 5% non-fat milk powder in 1xTBS-Tween 20 buffer

for1 hour at room temperature. Then membrane labeled with specific antibodies.CA9 antibody (Abcam), TSPAN8 antibody (Abcam), β-actin antibody (Sigma), horseradish peroxidase-conjugated secondary antibody (Abcam) was used in specific protein labeling. The protein bands were then visualized using the Fusion FX Vilber Lourmat imaging system. Densitometric analyses were performed with the ImageJ software program.

RESULT

Optimization of CA9 and TSPAN8 siRNA dose in MiaPaca-2 cells

MiaPaca-2 cells were transfected with CA9, TSPAN8 and control siRNAs (siCA9, siTSPAN8andsiCont) at a concentration of 50-10-5 nM. MiaPaca-2 cells were harvested 48 hours after the transfection.Western blot analysis was performed as described in the material method section and normalizedresults using β-actin bands are given in Figure 1.A for CA9 and Figure 1.Bfor TSPAN8. Protein expression level is decreased relative to the amount administered siRNA shown in Figure 1 B, D.

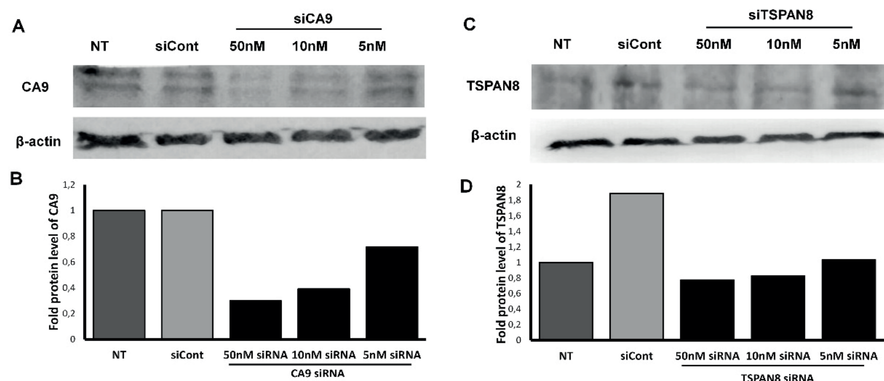

Figure 1. The dose-dependent effect of silencing CA9 and TSPAN8 siRNAs in MiaPaca-2 cells.
The effect of CA9 and TSPAN8 suppression on different concertation was examined in MiaPaca-2 cells. (A) MiaPaca-2 cells were transfected with control siRNA (siCont) concentration of 50 nM then CA9 and TSPAN8 siRNAs (siCA9 and siTSPAN8) and at a concentration of 50-10-5 nM. Cells were harvested 48 hours after the transfection, and the silencing effect at the CA9 (A) and TSPAN8 (C) protein level was determined using western blot in MiaPaca-2 cells. Western blot was performed 50 µg whole cell lysate run on 12.5% SDS-PAGE after transferred to PVDF membrane on ice for 2 h at 120V. CA9 antibody (Abcam), TSPAN8 antibody (Abcam) was 1/500 diluted by 10% BSA in PBS. Protein level CA9 (B) and TSPAN8 (D) showed bands densitometric values were calculated with ImageJ software.

Silencing of CA9 and TSPAN8 genes decreased the cell proliferation of Panc-1 and MiaPaca-2 cells.

MTT assay was performed to evaluate the effect of gene silencing on Panc-1 and MiaPaca-2cell proliferation. Cells were transfected with Control siRNA, CA9siRNA and TSPAN8 siRNA for 24 and 48 h. As seen in the Figure2 decrease in the absorbance of 550 nm for CA9 and TSPAN8 siRNA treated groups indicates the reduction of the cell viability compared to control siRNA treated cells and non-treated cells. Control siRNA (siCont) treatment didn't show statistically significant effect on cell proliferation compared non- treated cells (NT) at all-time points (Figure 2).

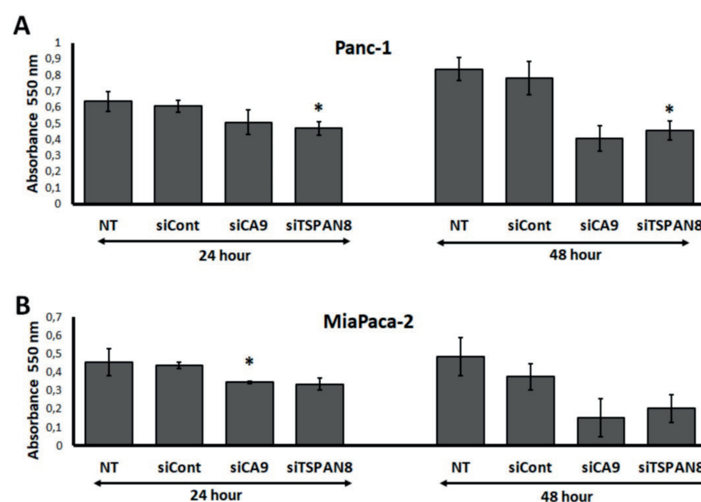

Figure 2. Effect of CA9 and TSPAN8 gene silencing on MTT cell viability assay in Panc-1 and MiaPaca-2 cells.
The time-dependent proliferation of Panc-1 and MiaPaca-2 cells was determined by MTT assay after transfection with control, CA9 and TSPAN8 siRNA. (A) Panc-1 and (B) MiaPaca-2 cells were transfected withCA9 and TSPAN8 siRNAs (siCA9 and siTSPAN8) and control siRNA (siCont) at a concentration of 50 nM. Cell proliferation was detected 24 and 48 hours after transfection with MTT. 24 and 48 hours later, MTT solution was added for 96 wells, followed by incubation at 37C° for 4 hours. Absorbance was then measured at 550 nm using a spectrophotometer

The cumulative effect of targeted gene silencing and chemotherapeutic drugs on the proliferation of pancreatic cells.

Here in we investigated the response of pancreatic cancer cells to cisplatin and epirubicin after silencing of CA9 and TSPAN8 genes. Panc-1 cells and MiaPaca-2 cells were transfected with 50 nM CA9 or TSPAN8 siRNA or control siRNA for 24 h, followed by incubation with different concentrations of cisplatin or epirubicin. After 24 hours of incubation, cell viability was measured by the MTT assay. Silencing of CA9 gene enhanced the cytotoxic effect of cisplatin on Panc-1 and MiaPaca-2 cells. Furthermore, downregulation of TSPAN8 gene dramatically sensitized the pancreatic cancer cells to cisplatin compared to CA9 downregulation (Figure 3A). The cumulative cytotoxic effect of TSPAN8 gene silencing with epirubicin treatment was only observed in Panc-1 cells (Figure 3). The effect of CA9 silencing with epirubicin was found similar in Panc-1 and MiaPaca-2 cells (Figure 3).

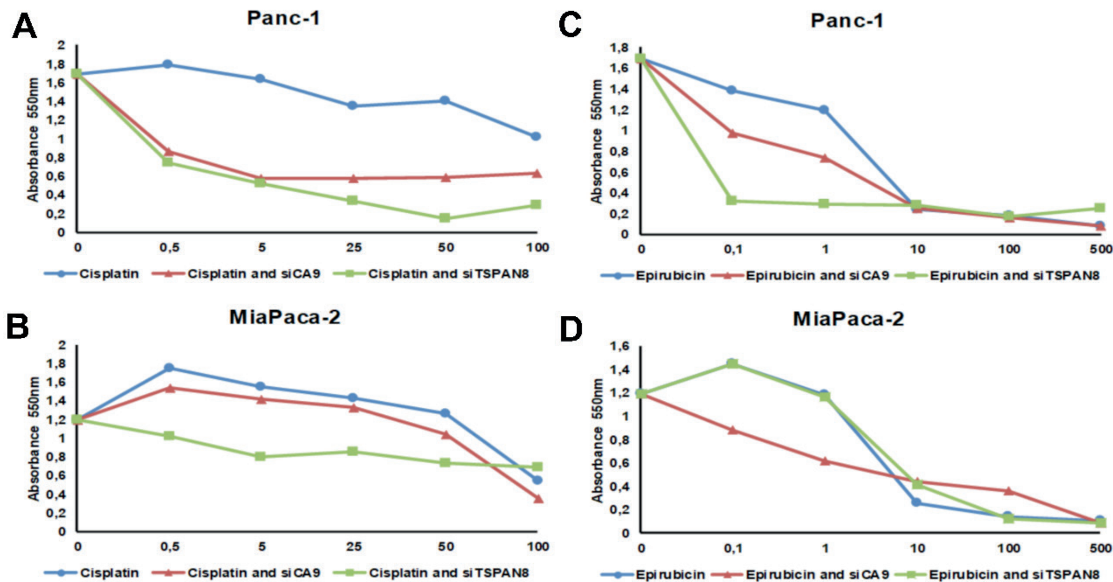

Figure 3. Silencing of CA9 and TSPAN8 sensitized Panc-1 cells and MiaPaca-2 cells to cisplatin and epirubicin. 24 hours after the siRNA transfection, (A), (C) Panc-1 cells and (B), (D) MiaPaca-2 cells were treated with (A), (B) cisplatin (0.5-5-25-50-100 µg/ml) or (C), (D) epirubicin (0.1-1-10-100-500 µg/ml) for 24 hours, and the cell viability was detected withMTT assay.

DISCUSSION

Pancreatic cancer aggressiveness is generally accompanied by overexpression of some proteins involved in tumor growth, metastasis and survival.Therefore,it is not only difficult to diagnose pancreatic cancer; it is also hard to overcome resistance to chemotherapy. Consequently, in combination with the chemotherapy agents, target siRNAs may play a role in sensitizing cancer cells[15, 16]. CA9 plays an important role in the regulation of acid base balance of cancer cells[14].Moreover, it has an important role in the development of the tumor and at the same time it participates in the formation of cell differentiation, migration and invasion[17].CA9 is overexpressed in many types of cancer, and its overexpression is declared to solid tumor aggressiveness and poor outcome[18].TSPAN8 is responsible for cell mobility and it has a role in cell adhesion and motility[6].

MiaPaca-2 cells were transfected with CA9 and TSPAN8 siRNA to achieve a dose-dependent silencing of total CA9 and TSPAN8 respectively, which did correlate with a decrease in their specific proteins (Figure 1). Accordingly, transfection for 48 hours was found more effective in decreasing CA9 and TSPAN8 siRNA treated cell proliferation in comparison to 24 hours (Figure 2).Researchers studied the effect of STEAP1 silencing on LNCaP cells.After 24 and 48 hours' transfection for STEAP1 silenced cells were compared with the control cell group, cell viability decreased by 33% and 44%, respectively [19].

Whitehurstand coworkers found that silencing of several genes sensitized lung cancer cells to a paclitaxel concentration much less than the dose needed that required for a significant response. These researchers also demonstrated that decrease in cell number was attributable to cell death[20].Cisplatin resistance in resistant pancreatic cancer cell lines were known to be associated with drug inactivation, drug transport, DNA damage response, DNA repair and the modulation of apoptosis[21]. Epirubicin (4'-epidoxorubicin), an analogue of doxorubicin (Adriamycin), has been used alone or in combination with other cytotoxic agents in the treatment of a variety of malignancies[22].

Co-delivery of TSPAN8or CA9 targeting siRNA and cisplatin to Panc-1 cells reduced the proliferation of cells more than 50% compared to only cisplatin treated cells. This effect seems to be cell specific as the response of MiaPaca-2 cells to CA9 targeting siRNA with cisplatin was not found significant. Similarly, treatment of TSPAN8 targeting siRNA with epirubicin also did not reduce the cell viability compared to only epirubicin treated cells (Figure 3).Many recent reports also show the similar results for the combination of chemotherapy agents and siRNAs. Their results underline that the combination of chemotherapy agent and siRNA offers superior anticancer effects when compared to chemotherapy agent alone or siRNA alone [23-25]. Beh and coworkers found that the combination of Bcl-2 siRNA

and paclitaxel treatment clearly chemo sensitized HeLa cells, resulting in significantly reduced of cell proliferation in a HeLa cell compared with untreated cells[23].

CONCLUSION

The present study demonstrates that silencing of TSPAN8 and CA9 genes sensitized pancreatic cancer cell to cisplatin and epirubicin.Our findings suggest that combinational treatment of cisplatin and TSPAN8 siRNA may be considered as a novel approach for pancreatic cancer and needs to be further analyzed in vitro and in vivo.

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Distribution and Factors Associated with Occurrence of Listeria monocytogenes in Table Size African Catfish, Pond Water and Sediment in Two Ecological Zones in Nigeria

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Abstract

Occurrence of *Listeria monocytogenes* (LM) and associated factors affecting its distribution in Oyo state, Nigeria was investigated in 2016. A total number of 972 (756 fish organs, 108 water and 108 sediments) samples were collected and examined for LM using molecular method. Descriptive statistics, t-test, Manny-Whitney U test and ANOVA were employed to analysed the data generated. It was found out, among other that 61 out of 972 samples had listeria infection. Forest zone had higher percentage of infection (66.67%) than the derived savannah zone (25.93%). However, there was no significant difference in LM occurrence between the sexes (p=0.333) of the fish. Although, there was significant difference in body weight (p=0.005). Out of 61 biochemically characterized isolates, only 18(29.51%) using *iap* primer and 12(19.67%) using *hly-A* primer were confirmed to be *L. monocytogenes* by PCR method. There was significant difference in the occurrence of *L. monocytogenes* in organs (p=0.001) with the spleen having the highest occurrence. Thus, the study concluded that LM was well distributed in fish farms in the ecological zones. Also, the highest occurrence was observed in forest zone revealing body weight and fish length as possible predisposing factors.

Keywords: Listeriosis; fish farms; infection; predisposing factors

INTRODUCTION

Fish is one of the cheapest and major sources of animal protein with high polyunsaturated fatty acid and essential amino acids for riverine dwellers and consumers in general [1]. The highly perishable nature of fish encourages the growth of microorganism including food poisoning pathogens such as *Listeria monocytogenes* leading to mortality, economic losses and infections. African catfish, *Clarias gariepinus* is the most cultured fish in Nigeria and is widely accepted as “common man” fish in the market. Its wide acceptance is connected to some important features which include high quality flesh, hardy nature, high tolerance level of water characteristics, production performance and attraction of appreciable market values. Table size fish (adult) is often consumed in many homes, hotels and relaxation centres hence it has become the most important stage of the fish with greater contact with human populace [2].

Listeriosis is a serious foodborne disease caused by bacteria in the genus *Listeria,* which has ten species: *L. grayii, L. innocua, L. ivanovii, L. monocytogenes, L. seeligeri,* and *L. welshimeri, L. marthii, L. rocourtiae, L. weihenstephanensis,* and *L. fleischmannii* [3]. Among these species, only *L. monocytogenes* is capable of causing illness in both human and animal [4]. *Listeria monocytogenes* is a gram positive, facultative, non-sporing, rod-like, motile and flagellated bacterium. It is an ubiquitous organism capable of surviving at refrigerating temperature [5] and it is accountable for serious illnesses like septicaemia, central nervous system infection, intra-uterine infection, and meningitis in matured human, severe systemic infection in new born and/or unborn child while pregnant women suffer a mild influenza-like illness. *Listeria monocytogenes* has been reported to account for mortality rate of about 20% to 50% of infection in human or even 100% in laboratory animals [6-7]. However, investigation of incidence of *Listeria monocytognees* in fish is highly necessary.

Listeria monocytogenes has been isolated from several environmental samples such as soil, vegetation, and human and animal faeces [8-9], signifying the pervasive occurrence of the pathogen in nature. Its occurrences in fish and fish products varied with respect to the form of the products. Occurrence ranges slightly above 4% to as high as 88% in fishes [10], 0 to 51% in shelf fish [11], 7.5% in fresh fish in Iran [6, 12] and 25% in smoked fishes in Nigeria [13]. Although the occurrence of *Listeria monocytogenes* in several foods including fish and fish products have been investigated in some countries, there is dearth of information on the *L. monocytogenes* occurrence and associated factors in cultured fish in Oyo State, Nigeria hence, the need for this study.

The aim of the study was to investigate the distribution and factors associated with occurrence of Listeria species especially *L. monocytogenes* in table size African catfish raised in pond earthen Oyo State, Nigeria and highlight any differences in the occurrence and associated factors.

MATERIALS AND METHODS

Study area

The study was carried out in Oyo State, in the South-West geopolitical zone of Nigeria. It lies within Latitudes $7^{o}3'$ S and $9^{o}12'$ E and longitudes $2^{0}47'$ and $4^{0}23''$ [14]. The two ecological-vegetation division (forest and derived savannah zones) of Oyo State were adopted for this study [14-15]. The forest zone comprises 11 local government areas

in Ibadan metropolis in the southern part of the state while derived savannah zone comprises 22 local government areas in the northern part of the state [16].

Water and Fish organs collection

Farms in each zone were sub-grouped into Large, Medium and Small scales using stratified methods [17]. A total number of thirty-six (36) farms (12 Large, 12 Medium and 12 Small scales farms) were used [2]. Water, soil, and fish samples were collected from these farms and analyzed for *Listeria* species. The protocol of the study was subjected to ethical consideration and was approved by Animal Care Use and Research Ethics Committee, University of Ibadan, Ibadan, Oyo state, Nigeria with reference number UI-ACU-REC/App/03/2017/008.

Water samples were collected using plastic bottles, fixed with Winkler A and B and transported to the Department of Aquaculture and Fisheries Management, University of Ibadan. A total number of one hundred and eight (108) (three fish per farm) fish were collected in early hours of the day (6:00 to 7:00 am) and transported to the laboratory in different plastic containers containing water from the respective ponds to avoid contamination. The fish were caught, condition (males and females were kept separate) to avoid stress and transported to the laboratory immediately. Seven (7) organs (liver, kidney, gill, intestine, flesh/muscle and spleen) were aseptically collected per fish and weighed into sterile universal bottles while skin samples were collected using skin swab [5]. Fish with body weight ranges between 300g to 1300g were used for this study as the group comprised stage of fish that are consumed by most individuals or processed for economic value. Organo-somatic index were determined using .

Water quality parameters

The water samples were taken and parameters were measured at an early hour (6:00hrs) of the day. Dissolved oxygen (DO) was determined by analytical method [18]. Water samples were fixed with 2 ml of Winkler A and B each. Exactly 100 ml of the fixed water was neutralized by addition of 2 ml of 10 sulphuric acid. Four drops of starch were added and titrated with 60% sulphuric acid. The DO level were calculated using , conductivity (ms.cm^{-1}) was measured with the aid of conductivity meter (LABTECH (R)] Model AVI-660 (Power: 220V, AC: 50 Hz: Sr./No. 376), nitrite (ppm) was measured using Auasol Nitrite kit (Nitrite High Level Test Kit; AE-207; MRP(Rs):1120; Rakiro Biotech Systems PVT Ltd, USA), pH was measured with the aid of a digital pH meter [LABTECH (R)] Model Photoic 20 (Power: 230V AC: 50 Hz: Sr./No. 1223) and temperature was measured using mercury thermometer.

Isolation of Listeria species

Sampled organs were pre-enriched in half-strength Fraser broth. One gram of each sample was added to universal bottles containing 9 mL of sterile half strength Fraser broth and supplements and incubated at 37ºC for 24 hours. From the pre-enrichment culture media 0.1mL was transferred into 10 mL of full strength Fraser broth with supplements added and was incubated at 37ºC for 24-48 hours (secondary enrichment) [4, 19]. The culture media were then streaked on PALCAM agar plates using wire loop and incubated for 48 hours. The wire loops were sterilized on spirit lamp before usage. The plates with a black sunken center and a black halo on a cherry-red background, following aesculin hydrolysis and mannitol fermentation were suspected to be listeria species colonies and selected for further confirmation and identification [4, 11]. The suspected colonies were sub-cultured on PALCAM agar and incubated for 24-48 hours to obtain pure colonies.

Macroscopic examination of culture plates

The presumptive identification of *L.* species was based on the cultural and evidence of aesculin hydrolysis or black – halo formation on PALCAM plates. The suspected organisms were kept on glycerol and PALCAM slants and stored in a freezer and refrigerator respectively, until use.

Biochemical test for Listeria species isolates

Biochemical tests adopted include gram stain reaction, beta-haemolysis, catalase reaction, sugar or carbohydrate fermentation (Rhamnose, Xylose, Lactose, Fructose and Mannitol) and motility at room temperature [20].

Confirmation of *L. monocytogenes*

Sixty-one (61) biochemically characterized isolates were further subjected to PCR method for identification of *L. monocytogenes* using primers specific to invasive associated protein (*iap*) and haemolysis (hlyA) [21] listed in table 1.

Table 1. Primers used for *Listeria monocytogenes* identification

Primers	Target gene	Length	Primer sequence	Amplification product bp
iap-F	Iap	20	5’ ACAAGCTGCACCTGTTGCAG 3’	131
iap-R	Iap	20	5’ TGACAGCGTGTGTAGTAGCA 3’	131
hlyA-F	hly	24	5’GCAGTTGCAAGCGCTTGGAGTGAA3	456
hlyA-R	hly	24	5’GCAACGTATCCTCCAGAGTGATCG 3	456

Source: adapted from Swetha *et al.* (2012).

DNA extraction

The *Listeria* isolates were suspended in 1.5 mL of enriched Fraser broths, grown on a shaker for 48 hours at 48ºC, 4600 x g for 5 minutes. The pellets were re-suspended in 520 µL of TE buffer (10 mMTris-HCl, 1 mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µL of Proteinase K (20 mg/ml) were added. The mixture was incubated for 1 hour at 37ºC, then 100 µL of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and mixed [4]. The suspension was incubated for 10 minutes at 65 ºC and kept on ice for 15 minutes. An equal volume of chloroform: isoamyl alcohol (24:1) was added, follow by incubation on ice for 5 minutes and centrifugation at 7200 x g for 20 minutes. The aqueous phase was transferred to a new tube, isopropanol (1: 0.6) was added and DNA was precipitated at –20 ºC for 16 hours. The DNA was collected by centrifugation at 7200 x g for 10 minutes, washed with 500 µL of 70% ethanol, air-dried at room temperature for approximately 3 hours and finally dissolved in 50 µL of TE buffer [4].

Polymerase Chain Reaction (PCR)

PCR reaction cocktail consisted of 2.5 µl of 5x Go-Taqgreen reaction buffer, 0.75 µl of $MgCl_2$, 0.25 µl of 10 mM of dNTPs mix, 0.25 µl of 10 pmol each of forward and backward primers and 0.06 µl of Taq DNA polymerase (8,000U) (Promega USA) made up to 10.5 µl with sterile distilled water then 2 µl DNA template. The PCR was carried out in a GeneAmp 9700 PCR System (Applied Biosystem Inc., USA) Thermalcycler while the cycling conditions are shown in Table 2 [21].

Table 2. Cycling conditions used for *iap* and *hly-A* primer

S/No	Step	iap (L. monocytogenes)	HlyA (L. monocytogenes)
1.	Initial denaturation	95^0C / 5 min	95^0C / 5 min
2.	Final denaturation	95^0C/30 sec	94^0C/60 sec
3.	Annealing	56^0C/30 sec	61^0C/45 sec
4.	Initial extension	72^0C/30 sec	72^0C/60 sec
5.	Final extension	72^0C/7 min	72^0C/7mins
6.	Hold	4^0C	4^0C

Integrity

The integrity of the amplified band for *iap* and *hlyA* genes fragment was checked on a 1.5% Agarose gel ran at 110V for 1 hour to confirm amplification and pictures were taken under gel electrophoresis to indicate amplification using *iap* and *hlyA* specific primers.

Statistical analysis

The data were analyzed using both descriptive statistics (percentage) to express the prevalence of bacterium occurrence. The values of water quality paremeter, organo-somatic index were analysed using one-way analysis of variance (ANOVA). The difference in the means were separated using student t-test for values between the zones, Mann-Whitney U test for values between the sexes of the fish and Duncan Multiple Range Test for values among the organs at p = 0.05 with the aid of Statistical Package for Social Science (SPSS) version 20.

RESULTS

Water quality

The result of the water quality of the selected farms were shown in Table 3. Farms in forest zone of the state had higher DO (6.04 ± 1.32 mg/l) and pH (7.28 ± 0.51) compared with savannah zone (5.78 ± 1.61 mg/l) and (7.27 ± 0.53) respectively. More so, savannah zone had higher conductivity (2.17 ± 1.33 $ms.cm^{-1}$), nitrite (46.85 ppm) and temperature (25.64 ± 1.41 ^oC) while farms from the forest zone had lower values 1.42 ± 1.07 $ms.cm^{-1}$, 42.78 ± 19.48 ppm and 25.31 ± 1.16 ^oC respectively. There were significant differences in conductivity, nitrite, pH and temperature ($p<0.05$) except DO ($p=0.84$)

Table 3. Water quality of the fish farms in Oyo State, Nigeria

Parameters	Forest zone (n=27)	Derived Savannah zone (n=81)	t-test (Sig)
DO (mgL^{-1})	6.04±1.32	5.78±1.61	0.84
Conductivity ($ms.cm^{-1}$)	1.42±1.07	2.17±1.33	-2.97
Nitrite (NO2-) (ppm)	42.78±19.48	46.85±20.30	-0.93
pH	7.28±0.51	7.27±0.53	0.05
Temperature (^{0}C)	25.31±1.16	25.64±1.41	-0.12

Values are represented as mean ± standard deviation (P < 0.05).

The organo-somatic index of the sampled fish were shown in Table 4. Organ-somatic index of gill and kidney were highest in fish from forest zone (4.30±1.15 and 0.11±0.04) while liver and intestine were highest in fishes from derived savannah (2.66±1.07 and 2.23±0.85). Organ-somatic index of gill and kidney were not significantly different ($p>0.05$) while there were statistically significant difference in organ-index of liver and intestine ($p<0.05$).

Table 4. Organ-somatic of table size Africa catfish in Oyo State, Nigeria

Parameters	Forest zone (n=27)	Derived Savannah zone (n=81)	t-test (Sig)
Gill	4.30±1.15	3.54±1.18	2.97
Liver	1.57±0.56	2.10±0.85	-3.70
Kidney	0.11±0.04	0.10±0.04	1.02
Intestine	1.30±0.50	1.85±0.70	-4.44

Vales are represented as mean ± standard deviation (P < 0.05).

Table 5 shows that the female fish from forest zone had higher percentage of infection (72.73%) and intensity of infection (0.73) than male (62.50% and 0.63 respectively). In derived savannah, male fish had higher percentage and intensity of infection (29.27 % and 0.29 respectively) than females (22.50% and 0.23 respectively). There was no statistically significant difference of *L. monocytogenes* occurrence between the males and females (p=0.333). In addition, fish with total length of 51-60cm had higher percentage and intensity of infection (100% and 1.00 respectively) in forest zone and least was observed in fish with total length of 30 – 40cm (46.15% and 0.46 respectively). In derived savannah zone, the highest percentage and intensity of infection (31.71% and 0.32 respectively) was observed in fish with 41 – 50 cm total length while the least (11.76% and 0.12) were recorded in fish with 30 – 40cm total length.

Table 5. Occurrence of *Listeria* by length, weight and sex of farm raised *C. gariepinus* in Oyo State, Nigeria

Parameter		No. of samples		No. of infection		Percentage		Intensity of infection		p-value
		F	S	F	S	F	S	F	S	
Sex	Male	16	41	10	12	62.50	29.27	0.63	0.29	p=0.333
	Female	11	40	8	9	72.73	22.50	0.73	0.23	
	Total	27	81	18	21	66.67	25.93	0.67	0.26	
Total length (cm)	30-40	13	17	6	2	46.15	11.76	0.46	0.12	p=0.099
	41-50	12	41	10	13	83.33	31.71	0.83	0.32	
	51-60	2	23	2	6	100.00	26.09	1.00	0.26	
	Total	27	81	18	21	66.67	25.93	0.67	0.26	
Body weight (g)	300-600	17	43	10	12	58.82	27.91	0.59	0.28	p=0.005
	601-900	8	35	6	7	75.00	20.00	0.75	0.20	
	≥ 900	2	3	2	2	100.00	66.67	1.00	0.67	
	Total	27	81	18	21	66.67	25.93	0.67	0.26	

Note: F = Forest zone, S = Derived savannah zone

(i) Intensity of infection $= \frac{\text{Number of infected samples}}{\text{Total samples examined}}$

More so, from the forest zone, fish with body weight of 900 g and above had highest percentage (100%) and intensity (1.00%) of infection and least (58.82% and 0.59), while in derived savannah zone, the highest percentage (66.67%) and intensity (0.67) of infection were observed in fish with body weight of 900g and above and lowest percentage (20.00%) and intensity (0.20) of infection in fish with body weight 601 – 900g. Hence, body weight significantly affected the percentage and intensity of infection (p=0.005) while sex and total length did not (p>0.05).

Table 6 shows that fish gill, kidney, skin, flesh and spleen of fish from forest zone had higher percentages of infection (22.22%, 22.22%, 33.33%, 22.22% and 11.11% respectively) while derived savannah zone had lower infection (3.70%, 11.11%, 7.40, 11.00 and 0.00%) respectively. Overall, organs of fish in forest zone had higher infection 15.86% compared with derived savannah (4.76). There are differences in the values of infection but there is no significant difference (p>0.05) as shown in Table 6.

Table 6. Distribution of Listeria species in fish by organs in Oyo state, Nigeria

Organ	Forest zone				Derived savannah zone			
	No. of sample	No. of Positive	%	Intensity of infection	No. of sample	No. of Positive	%	Intensity of infection
Gill	27.00	6.00	22.22	0.22	81.00	3.00	3.70	0.04
Liver	27.00	0.00	0.00	0.00	81.00	0.00	0.00	0.00
Kidney	27.00	6.00	22.22	0.22	81.00	9.00	11.11	0.11
Intestine	27.00	0.00	0.00	0.00	81.00	0.00	0.00	0.00
Skin	27.00	9.00	33.33	0.33	81.00	6.00	7.41	0.07
Flesh	27.00	6.00	22.22	0.22	81.00	9.00	11.11	0.11
Spleen	27.00	3.00	11.11	0.11	81.00	0.00	0.00	0.00
Total	189.00	30.00	15.87	0.16	567.00	27.00	4.76	0.05
p-value		0.842				0.503		

i) Intensity of infection $= \frac{\text{Number of infected samples}}{\text{Total samples examined}}$

Table 7 shows that pond water from forest zone had higher percentage (3.70%) and intensity (0.04) of infection while no infection was recorded in derived savannah zone. In the pond sediments, forest zone had higher percentage (7.41%) and intensity (0.07) of infection than 1.00% and 0.01 percentage and intensity of infection recorded in derived savannah respectively. Overall, percentage (5.56%) and intensity (0.06) of infection were higher in forest zone than 1.0% and 0.00 recorded in derived savannah. There were no significant difference in percentage of infection in pond water (p=0.221) and sediment (p=0.254).

Table 7. Distribution of *Listeria* in pond water and sediment in two ecological zones

Material	Forest zone				Derived savannah zone				
	No. of sample	No. of Positive %		Intensity of infection		No. of Positive %		Intensity of infection	p=value
Pond water	7.00	1.00	3.70	0.04	81.00	0.00	0.00	0.00	p=0.221
Sediment	27.00	2.00	7.41	0.07	81.00	1.00	1.23	0.01	
	54.00	3.00	5.56	0.06	162	1.00	0.62	0.00	

Note: Intensity of infection = $\frac{\text{Number of infected samples}}{\text{Total samples examined}}$

Table 8 shows that out of 61 isolates examined, only 18(29.51%) were confirmed *L. monocytogenes* using *iap* primer. Among the organs, spleen had highest occurrence (100%), and least was observed in kidney (20.00%). When using *hly-A* primer, only 12(19.67%) were confirmed *L. monocytogenes*. However, among the organs, *L. monocytogenes* were only identified from the skin and muscles (40.00%). There is significant difference in the occurrence of *L. monocytogenes* among the organs and between the *iap* and *hly-A* primers (p<0.05).

Table 8. Occurrence of *L. monocytogenes* using *iap* primer in two ecological zones.

Organs	Number of samples	*L. monocytogenes* using *iap* (%)	*L. monocytogenes* using *hly-A* (%)	Other Listeria species (%)
Skin swab	15	6 (40.00)	6(40.00)	9(60.00)
Kidney	15	3(20.00)	0(0.00)	12(80.00)
Muscles/flesh	15	6(40.00)	6(40.00)	9(60.00)
Spleen	3	3(100)	0(0.00)	0(0.00)
Gill	9	0(0.00)	(0.00)	9(100.00)
Water	1	0(0.00)	(0.00)	1(100.00)
Sediment	3	0(0.00)	0(0.00)	3(100.00)
Total	61	18(29.51)	12(19.67)	43(70.49)
p-value		0.002	0.001	

Figure 1 shows the PCR product with positive band (131bp) from skin, kidney, muscles and spleen. Isolates on Lanes 1 and 5, 4, 9, 12 and 15 were the only isolates that had equivalent and desired band which were isolates from skin, muscle, kidney and spleen using specific primer invasive associated protein (*iap*) that is targeting *iap* gene confirming it to be *L. monocytogenes*. Figure 2 shows the PCR product with positive band (456bp). Isolates on Lanes 1, 4, 9, and 15 were the only isolates that had equivalent and desired band which are isolates from skin and muscle using specific primer haemolysis (*hlyA*) that is targeting haemolysis gene confirming it to be *L. monocytogenes*.

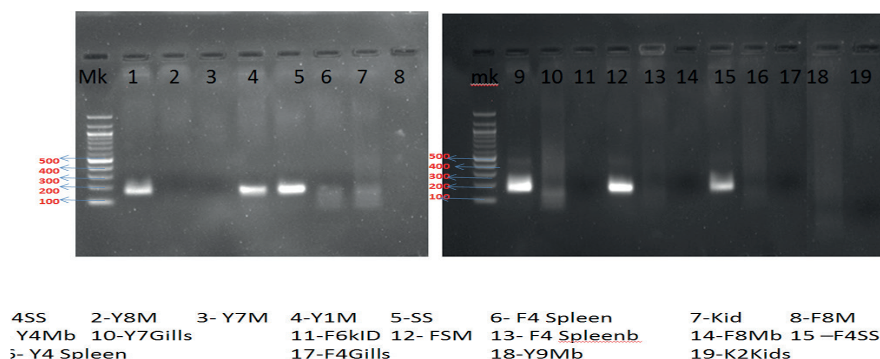

Figure 1. Standardization of PCR using assay for detection of Listeria monocytogenes (iap). Where: Lane 1= F2 Skin, Lane 2= F1 Muscle, Lane 3= F2 Gill, Lane 4 = Y1 Muscle, Lane 5 = O4 Skin, Lane 6 = F4 Skin, Lane 7 = F5 Kidney, Lane 8 = F6 Skin, Lane 9 = F6 Kidney, Lane 10 = F8 Muscle, Lane 11 = O9 Skin, Lane 12 = Y5 Muscle, Lane 13 = Y4 Kidney, Lane 14 = F4 Gill, Lane 15 = F4 Spleen, Lane 16 = Y7 Gill, Lane 17 = Y7 Muscle, Lane 18 = Y7 Kidney, Lane 19 = K7 Kidney.

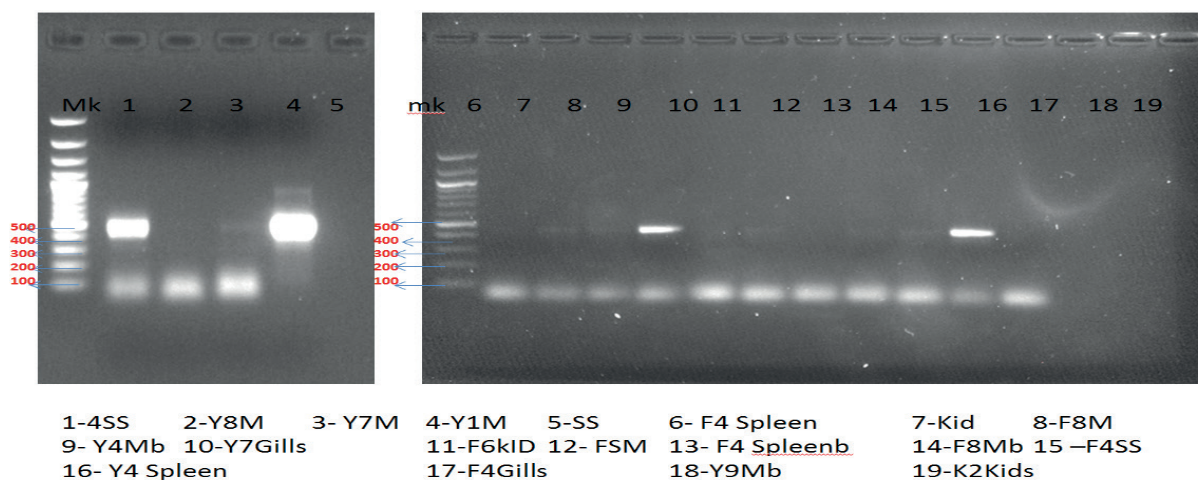

Figure 2. Standardization of PCR using assay for detection of *Listeria monocytogenes* (hly-A). Where: Lane 1= F2 Skin, Lane 2= F1 Muscle, Lane 3= F2 Gill, Lane 4 = Y1 Muscle, Lane 5 = O4 Skin, Lane 6 = F4 Spleen, Lane 7 = F5 Kidney, Lane 8 = F6 Skin, Lane 9 = Y5 Muscle, Lane 10 = F8 Muscle, Lane 11 = O9 Skin, Lane 12 = F6 Kidney, Lane 13 = Y4 Kidney, Lane 14 = F4 Gill, Lane 15 = F4 Skin, Lane 16 = Y7 Gill, Lane 17 = Y7 Muscle, Lane 18 = Y7 Kidney, Lane 19 = K7 Kidney.

DISCUSSION

This study describes the distribution and factors associated with occurrence of *Listeria spp* especially *L. monocytogenes* in table size African catfish, pond water and sediments in African catfish in Oyo state in Nigeria. Water is an important factor in fish farming as it predetermines the survival, growth and health of the fish since the entire life process of the fish wholly depends on the quality of its environment. DO, temperature and pH in this study fall within the recommend desirable values which is in agreement with works of [22-25]. The overall prevalence of *L. monocytogene*s in fish samples from Oyo State is similar to that reported for fresh fish [6, 26] but differ from 3% reported in fresh fish in Europe, 1%, and 56% of fresh fish on sale in Iceland [27]. The results further suggest that fish with longer total length have chance of being infected; this may be as a result of higher surface area. The infection is not sex related as there was no significant difference in the percentage of infection of the male to female. This suggests that the fish farms from the study area share similar management practices.

The result of the PCR showed that rate of occurrence of *L. monocytogenes* in fish sampled is high. The prevalence is in agreement with works of [26] who reported *L. monocytogenes* up to 21% from Salmon in USA, 22% from rainbow trout Switzerland, 30% in Salmon in USA and 23% from different species in USA fresh fish respectively. However, the present study observed lower percentage of *L. monocytogenes* compared to reports of [21, 28, 29] who reported up to 65% in India.

The significant difference in the occurrence with body weight and length further revealed that body weight and length of the fish are possible predisposing factors of *L. monocytogenes* occurrence in fish. However, sex may not have significant influence on the bacterium occurrence and therefore could not be used as an index to predict the presence of *L. monocytogenes* in fish. The insignificant difference in the farms also confirmed the ubiquitous nature of *L. monocytogenes* which is consistent with findings of [3, 6, 20, 26].

Also, the significant differences observed among the organs suggested that different organs are susceptible to *L. monocytogenes* than the other. In this study, spleen, skin and muscle have higher chance of being infected than others. This is in agreement with the work of [5] who reported significant variation in the occurrence of *L. monocytogenes* in gill, skin and muscle in rainbow trout. The higher intensity of infection observed in rainbow trout may be related to the small thinning scales in this fish which could serve as accumulation site with subsequent domiciling in gills, while the absence of scale in *C. gariepinus* may be responsible to the migration of the bacterium to the inner part such as spleen.

The low concentration of *Listeria* species in farm ponds water as compared to sediment may be as a result of precipitation, rainfall or direct addition of water by farmers when the water level is low. Although raw fish are not consumed in Nigeria, but the presence of *L. monocytogenes* in tissues of the fish across the chain pose a public health risk and the fact that the organism may be very difficult to eliminate.

From the result of this study it could be concluded that *L. monocytogenes* is well distributed in fish farms in fish farms in Oyo State, Nigeria. The prevalence by organs shows that fish flesh, skin, kidney and spleen are the affected organs which further buttresses the fact that infected fish may pose a public health risk to fish handlers and consumers. However, adequate awareness on significance of *L. monocytogenes* should be encourage within the fish handlers and consumers

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Paradoxical Advantage of Middle Aged Sedentary over Young Sedentary on Starting Exercise in Terms of GH/IGF-1 System

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Abstract

The aim of this study was to investigate the effects of the submaximal running exercise on the GH/IGF-1 axis in the middle aged sedentary non-obese and pre-obese males. Twenty-four healthy volunteer males were divided into two groups and then two subgroups according to their age ranges (20-25 and 35-43) and Body Mass Indexes (BMI) (non-obese - NO and pre-obese - PO) respectively. All subjects underwent a submaximal running exercise for 30 minutes a day / 4 days a week and for a period of 6 weeks. Serum GH, IGF-1, IGFBP-3, and insulin levels were measured in all subjects before and after the exercise program. IGFBP-3 levels were significantly decreased in the PO middle aged and NO young groups whereas IGF-1 levels were statistically increased in NO young group after the exercise treatment. Contrary to the results observed in young people, middle aged PO and NO groups had a tendency to have better insulin and GH responses to exercise. In the light of the findings, it could be suggested that the middle aged group's response to exercise on the base of GH/IGF-1 system was more advantageous than the young group, although it is not significantly important.

Keywords: Exercise, pre-obese, middle aged, sedentary, GH/IGF-1 system

INTRODUCTION

Physical exercise has a major role in sustaining a healthy life. As a result of the facilities provided by technological advancements, people have abandoned most of their physical exercise. Therefore, the idea of reintroducing physical activity to people's lives has been implemented by the governments. This implementation is mainly associated with the scientific fact that physical exercise not only enables a sustainable healthy life but also prevents the formation of numerous diseases.

Physical inactivity resulting from industrialization and the modern lifestyle can affect individuals at any age. Moreover, a sedentary lifestyle may lead to serious health problems including hypertension, obesity, muscle weakness, postural disorders, increased risk of diabetes and coronary artery disease. It has been suggested that regular exercise prevents and even treats so many diseases associated with sedentary lifestyle (1, 2).

It has been reported that acute physical exercise increases GH secretion, thereby regulating the expression of IGF-1 and insulin-like growth factor binding protein 3 (IGFBP-3). Some other studies have suggested that regular physical exercise can improve health-related quality of life in elderly people since it increases the IGF-1 concentration in circulation (3). However, it is reported that the extent of health benefits achieved through physical activity is associated with each individual's age at the time of exercise (4).

Although the importance of the GH/IGF-1 system and insulin sensitivity in sustaining a long and healthy life has been extensively documented in the literature (5), there have been rather limited number of studies reporting the effects of submaximal exercise on the GH/IGF-1 system and insulin sensitivity, particularly in sedentary non-obese and pre-obese middle-aged individuals. Present study aims to investigate the alterations in the GH/IGF-1 system and insulin sensitivity in sedentary young and middle-aged individuals who participated in regular submaximal running exercise program. It also aims to evaluate the effect of age and body weight on this system and to analyze probable alterations in the GH/IGF-1system in pre-obese participants.

MATERIALS AND METHODS

The twenty-four male participants were initially divided into two groups as young (20-25 years) and middle-aged (35-43 years) individuals and each group was subsequently divided into two subgroups as non-obese and pre-obese participants depending on their body weight and body mass index (BMI). The young group had twelve voluntary undergraduates studying at Van Yuzuncu Yil University who had no clinical complaint or finding. The middle-aged group included twelve voluntary healthy participants who did not have any regular exercise. The participants in both groups underwent general health screening at Van Yuzuncu Yil University Medical School Hospital for the assessment of fitness for exercise. The participants were requested to avoid any physical exercise the week before the exercise program. A 6-week exercise program was applied which included 30 minute running session/day, four days/week. Intravenous blood samples were collected both before and after the exercise program at fasted state. Blood samples were analyzed for the assessment of GH, IGF-1, IGFBP-3, and insulin at Van Yuzuncu Yil University Medical School Biochemical Laboratory.

Calculation of BMI for the Identification of Pre-obese Participants

Body mass index (BMI) is the most common measurement used for the assessment of obesity. BMI is calculated by dividing body weight in kilograms (kg) by the square of the body height in meters (m^2).

Table 1. Pre-exercise age, height, weight, and BMI values of the groups.

	Group	Age	Height (m)	Kg	BMI
20-25 Years	NON-OBESE	23	1.73	61	20.4
		22	1.78	65	20.5
		22	1.72	60	20.3
		23	1.75	60	19.6
		21	1.75	56	18.3
		22	1.70	60	20.7
35-43 Years	NON-OBESE	40	1.89	88	24.6
		37	1.70	71	24.5
		38	1.69	70	24.5
		38	1.83	81	24.2
		35	1.73	73	24.4
		35	1.65	66	24.2
20-25 Years	PRE-OBESE	24	1.85	87	25.4
		25	1.80	83	25.6
		21	1.75	77	25.1
		22	1.68	73	25.8
		21	1.76	78	25.2
		22	1.75	80	26.01
35 -43 Years	PRE-OBESE	36	1.65	74	27.2
		37	1.78	87	27.5
		36	1.78	87	27.5
		42	1.65	74	27.2
		35	1.64	70	26.1
		39	1.85	100	29.2

Blood Sampling and Analysis

Prior to exercise, 5 cc of blood was intravenously sampled from each participant at fasted state. The samples were studied for the assessment of the parameters defined in the study and the results were used as pre-exercise values. At the end of the 6-week program, second blood samples were collected using the same protocol and the results were accepted as post-exercise values. The alterations were calculated based on the pre- and post-exercise values. For the assessment of GH, IGF-1, IGFBP-3, and insulin levels, immunoassay test was performed using Immulite 2000 with commercially available Biodepc kits.

Statistical Analysis

Independent-samples t-test was performed for binary comparisons and paired-samples t-test was performed for comparing pre- and post-exercise values. For the variables with normal distribution, appropriate tests were performed. Kolmogorov-Smirnov test and the homogeneity of the variables were taken into consideration. The assumptions of parametric tests were fulfilled. $p \leq 0.05$ accepted as statistically significant.

RESULTS

In the non-obese young subgroup, IGFBP-3 significantly decreased and IGF-1 significantly increased ($p>0.05$ and $p<0.01$, respectively) (Figure 1 and Figure 2). On the other hand, although GH decreased (Figure 3) and insulin level increased (Figure 4), no significant change was observed. Table 2 presents the pre- and post-exercise values of the parameters in all participants.

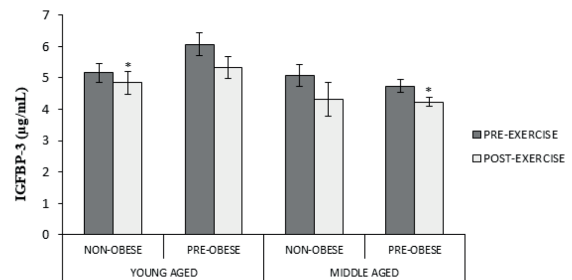

Figure 1. Comparison of pre- and post-exercise IGFBP-3 values in all participants.

* Statistically significant

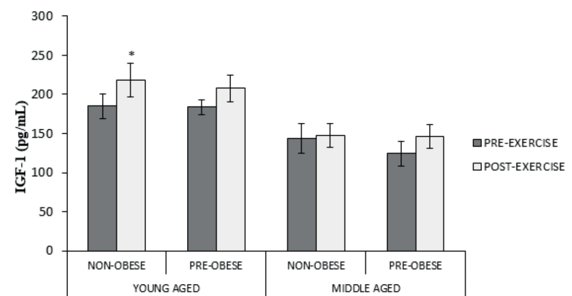

Figure 2. Comparison of pre- and post-exercise IGF-1 values in all participants.

* Statistically significant

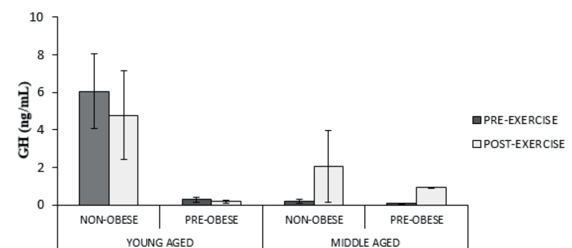

Figure 3. Comparison of pre- and post-exercise GH values in all participants.

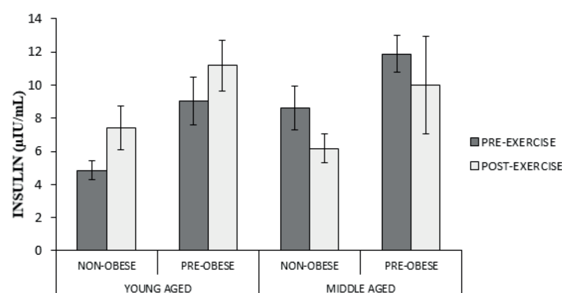

Figure 4. Comparison of pre- and post-exercise insulin values in all participants.

		N	PRE-EXERCISE GH (ng/mL)	PRE-EXERCISE INSULIN (µIU/mL)	PRE-EXERCISE IGF-1 (pg/mL)	PRE-EXERCISE IGFBP-3 (µg/mL)	POST-EXERCISE GH (ng/mL)	POST-EXERCISE INSULIN (µIU/mL)	POST-EXERCISE IGF-1 (pg/mL)	POST-EXERCISE IGFBP-3 (µg/mL)
YOUNG AGED	NON-OBESE	6	6.04±1.99	4.86±0.56	184.83±15.61	5.16±0.3	4.76±2.36	7.43±1.31	218.17±22.02 [a]	4.84±0.36 [b]
	PRE-OBESE	6	0.28±0.13	9.03±1.42	183.50±8.98	6.07±0.36	0.17±0.07	11.17±1.52	207.83±17.20	5.34±0.35
MIDDLE AGED	NON-OBESE	6	0.17±0.10	8.60±1.32	143.68±18.73	5.08±0.34	2.05±1.93	6.18±0.89	147.41±14.82	4.31±0.54
	PRE-OBESE	6	0.06±0.01	11.87±1.11	124.47±15.79	4.74±0.20	0.92±0.02	9.99±2.92	146.00±14.93	4.23±0.15 [a]

Similarly, in the pre-obese young subgroup, although IGFBP-3 and GH were decreased, IGF-1 and insulin were increased, but these changes were not significant.

In the non-obese middle-aged subgroup, insulin and IGFBP-3 were decreased and, GH and IGF-1 were increased in the post-exercise period insignificantly.

In the pre-obese middle-aged subgroup, a significant decrease was found in IGFBP-3 ($p<0.05$), whereas no significant decrease was observed in insulin level. However, GH and IGF-1 increased insignificantly.

DISCUSSION

It is well-known that sedentary lifestyle could be accounted for many chronic diseases. Even though many studies are available on the effects of exercise young and elderly people, the age groups in between are mostly neglected. However, any kind of exercise interventions in these age groups might have a greater chance to prevent and reverse age-related deterioration of overall health as metabolic parameters do not change significantly at those age groups yet. GH/IGF-1 axis and insulin levels are regarded as important parameters for healthy aging (6, 7). The present study investigated the effect of regular submaximal running exercise on the alterations in the GH/IGF-1 system and on insulin and IGFBP-3 in sedentary non-obese and pre-obese young and middle-aged individuals.

Previous studies suggest that short-term exercise programs that last only for several weeks could not be sufficient for evaluating general health parameters since they can only change by long-term exercise programs that last for several months. It is also proposed that adaptation to any exercise program takes several weeks, particularly in middle-aged or older individual. Additionally, a minimum of 15-20 weeks are necessary to achieve health benefits of the exercise program (8). However, some other studies indicated that 4-6 weeks could be sufficient for evaluating the time-dependent effects of an exercise program, particularly after the adaptation period, and also for assessing the relative alterations in several parameters (9).

The GH/IGF-1 system has a direct effect on the glucose, lipid, and protein metabolism in human subjects. During physical exercise, the need for energy increases and a number of hormonal changes occur. It is also known that regular exercise alters the body composition depending on the type of the exercise performed. Moreover, acute changes are likely to occur in the GH/IGF-1 system in adults with GH deficiency (10), runners (11, 12), strength athletes (13), and in healthy volunteers (14). But these hormonal changes have not been reported in all studies. For example Grandys et al observed no changes in resting serum concentrations of GH, IGF-I, IGFBP-3, and IGF-I/IGFBP-3 ratio after the 20 weeks of endurance running training (12).

Thomas et al. evaluated 19 healthy volunteers including class 1 obese (BMI, 30.00-34.99), class 2 (BMI, 35-39.99)/ class 3 (BMI, ≥40) obese, and 9 lean men and reported that no increase was observed in the post-exercise insulin levels in all these groups (15). Gregory et al. evaluated non-obese women (mean age, 20.3 ± 0.3 years) and reported that GH, total IGF-1, and IGFBP-3 significantly increased ($p<0.05$) and free IGF-1 concentration decreased following endurance

and resistance exercises (16). Mohajeri Tehrani et al. evaluated 20 middle-aged participants and reported that IGF-1 and IGFBP-3 increased significantly and insulin resistance decreased insignificantly following submaximal endurance training using a cycle ergometer (17). In the present study, insulin level increased and GH decreased, whereas IGFBP-3 decreased significantly ($p<0.05$) and IGF-1 value increased significantly ($p<0.01$) in non-obese young participants. Similarly, in pre-obese young participants, insulin level increased and GH decreased, whereas levels of IGFBP-3 and IGF-1 did not change.

Studies on physical exercise indicate that acute hormonal response to exercise is weakened with age and this occurrence can be explained by the lower exercise intensity in elderly women. It is also postulated that physical exercise has the capacity to affect the hormones as a result of changes in protein carriers and receptors. Therefore, the value and safety of hormone supplements should be carefully examined, particularly when used in combination with an exercise program (18). In the present study, the middle-aged individuals had higher positive hormone sensitivity to exercise compared to young individuals. However, another study reported that elderly participants (mean age, 65.5 years) exhibited similar hormonal response with young (mean age, 22.9 years) and middle-aged participants (mean age, 44.9 years) following an acute submaximal cycling training (19). Contrary to the findings of this study, the middle aged participants of our study showed a more effective response to exercise when compared to the young. This could be due to the longer duration and different types of exercise in the other study.

Poehlman and Copeland assessed serum concentrations in healthy non-obese young and elderly men, characterized for maximal aerobic capacity and energy expended in leisure-time physical activity, in order to investigate the hypothesis that a lower level of physical activity affects the age-related reduction in IGF-1. The authors concluded that the age-related decrease in IGF-1 can be affected by various factors but the reduction in IGF-1 in elderly men is partially associated with reduced physical activity (3). In the present study, IGF-1 significantly increased in non-obese young participants ($p<0.01$) but increased insignificantly in pre-obese young participants. Yamaguchi et al. evaluated 19 individuals aged 46-54 years with a BMI >25 kg/m^2 and reported that IGF-1 increased by 5.8% following the exercise and serum GH increased from 0.69±0.59 to 1.25±0.78 following the training (20). However, Kanaley et al. investigated the effect of submaximal exercise on IGF-1 activity in patients with GH-deficiency and healthy subjects and reported that no change was observed in IGFBP-3 and IGF-1 but the GH levels increased in healthy subjects (21). In our study, insulin levels tended to decrease, whereas GH levels tended to increase in pre-obese middle-aged participants. However, IGFBP-3 levels decreased significantly, and IGF-1 levels increased insignificantly in the same group. In another study, Chadan et al. evaluated healthy elderly women and reported that GH increased by 1.3-2.6 times ($p<0.05$) following physical activity but IGF-1 was not affected by physical activity (22). In our study, IGF-1 was found to be affected by physical exercise in middle-aged participants.

Studies show that plasma insulin levels may decrease by 35% following any type of physical activity including low- and high-intensity and short- and long-term exercise programs (21). Likewise, in this study, the plasma insulin levels after exercise decreased in the non-obese and pre-obese subgroups of the middle-age group.

In our study, the GH/IGF-1 system in the middle-aged group exhibited a more effective hormonal response to exercise both in non-obese and pre-obese participants compared to young participants and their insulin levels decreased and GH levels increased as well. Thomas et al. also reported that implementing certain exercise program variables can ameliorate attenuated GH response in obese people, although obesity has negative effect on circulating GH-IGF-1 axis (15). Under the lights of the present study and the others, it is likely that the GH/IGF1 system has a dynamic activity in which changes occur with age and sexual maturation, and is affected by body composition and other factors (10). However, the IGFBP-3 and IGF-1 levels in the young group exhibited a more effective response in non-obese participants. It was also revealed that the young and middle-aged groups exhibited different responses to the same exercise intensities. Moreover, the results also indicated that the pre-obese participants had the initial signs of obesity.

Results of the present study reveal hormonal responses of middle-aged non-obese and pre-obese subjects have increased GH and IGF-1 levels and also decreased insulin levels. These finding suggest that normal age related deterioration in sedentary middle age groups could be improved through exercise intervention programs, by which disturbed metabolic processes could be diverted toward normal course. In another study, similar to the present findings, whole-body insulin sensitivity index was found to be increased after acute exercise in sedentary obese group but not in obese group who have regular physical activity (23). Acute exercise ameliorates differences in insulin resistance between physically active and sedentary overweight adults. The results obtained from pre-obese and non-obese young subjects were contrary to the expected outcome which could be explained by different metabolic activity levels between young and middle aged groups.

In conclusion, it could be said that it would not be too late for middle-aged individuals who had no regular exercise during early adolescence to start having regular exercise in middle age, in which the adverse effects of sedentary lifestyle on the health can be seen. Moreover, individuals with higher body weight, compared to non-obese individuals who are not classified as obese despite having metabolic signs of obesity, are more likely to reverse their situation by performing regular exercise. In particular, it seems that performing regular submaximal exercise with an intensity of 30 min session/day is essential for sustaining a healthier life especially for middle-age people. More research is needed to study the effects of different types of exercise in middle-age sedentary subjects with a larger number of groups.

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Laboratory Rearing of Cotton Bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera:Noctuidae) Wild Colony on Different Artificial Diets

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Abstract

The cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) is a major pest in agricultural areas in the world. It is crucial to study the pest's biology, behaviour, insecticides resistance and control methods in the laboratory conditions in all year around without depending on the natural host plant. In order to do that, the pest should be reared on artificial diet continously. The aim of the study is to test known artificial diets for laboratory rearing and adaptation of the cotton bollworm and also modify them if necessary to find out the best larval diet. The bollworm larvae were brought to the laboratory with infested tomatoes from Çanakkale provinces. Six artificial diets were prepared with basic ingredients including pinto bean flour, soybean meal, corn meal, chickpea meal, wheat germ, brewer's yeast, torula yeast, sucrose, vitamins and mold inhibitors. Larvae also reared on sliced tomato fruits as control during the experiments. Some biological parameters such as number of larval stages, survival of larval and pupal stages and development times, pupal weights and adult emergence rate were determined on six different artificial diets in the laboratory. As a result, Diet I, originally developed for *Grapholita molesta* that supported larval development succesfully and completed 11.6 ± 1.5 days and had 331.8 mg pupal weights. Another diet called as Diet II, originally developed for Lepidopteran species, also succesfully used to rear the cotton bollworm larvae having 16.6 ± 1.4 days of larval durations. The cotton bollworm was reared consecutive four generations on both diets. The study results showed that out of six tested diets, Diet I and Diet II were sufficient and nutrionally suitable for laboratory uses and adaptation of wild colony.

Keywords: Artificial diet, *Helicoverpa armigera*, Lepidoptera, cotton bollworm, rearing

INTRODUCTION

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is a major pest and well-known polyphagous insect in agricultural areas in worldwide [1,2]. The common names are known as old world bollworm, African bollworm, cotton bollworm, tomato fruit worm, the gram pod borer and American bollworm [1,3]. Cotton bollworm was first recorded in 1913 in Bergama, Turkey [4]. This pest has a wide geographical distribution, reported in Asia, Africa, Ocenia, Europe, Australia and recently in South America [1,5,6]. Larvae of *H. armigera* have been reported more than 181 cultivated and over 67 hosts, including Fabaceae, Malvaceae, Poaceae, Asteraceae and Solanaceae [1,7,8,9]. It has caused damages on economically important agricultural crops such as tomato, maize, cotton, sorghum, soybean, legumes and ornamental plants [1,10,11].

H. armigera feeds on various parts of the host plants, leaves and also reproductive structures [10,12]. It is easily adapted to environmental changes, having high mobility and surviving in different habitats [2]. Females deposit numerous eggs with high fecundity causing population increases [6].

It is a well known pest and several studies have been published on the cotton bollworm such as genetic diversity [13,14,15,16,17,18], biology [2,8,19], pesticide resistances [20,21,22], life table [1,6,23], geographic distributions [24,25] and rearing on different artificial diets [3,26,27,28]. These studies help to understand pest's behaviour, population dynamics analysis, life cycle and other control approaches.

Artificial diets are important to know the basic biology of the pest and to understand the factors involved in insects nutrition and insect rearing studies. Therefore, such informations could be critical for the pest nutritional requirements. Several diets slightly modified and reported by Vanderzant et al. [29], have been developed for the cotton bollworm. Understanding of pest nutritional requirements could allow accelerate laboratory rearing, adaptation and developing artificial diets to study without depending on the host plants. It is important to have large numbers of larvae in health and uniformly developed insects with low cost.

Rearing of cotton bollworm in the laboratory depends mostly on natural host plants. Susceptibility of larval rearing, maintaining of host plant year around are important issues to be considered [30] as well as the labor and cost.

The objective of the present study is to test known artificial diets to rear the cotton bollworm in the laboratory and to study some biological parameters by comparing natural host in order to find out the best larval diet.

MATERIALS AND METHODS

The present study was carried out at Çanakkale Onsekiz Mart University, Faculty of Agriculture, Insect Molecular Biology Laboratory, to study the effect of host plants and artificial diets on the development of the different biological stages of cotton bollworm. All experiments were conducted in controlled laboratory conditions, at 24±1˚C, 65 % RH and 16:8h (light: dark) photoperiod.

Cotton Bollworm Colony

Bollworm larvae were collected from infested tomato orchards in Çanakkale province and brought to the laboratory. They were reared about 7 generations on tomato fruits at 24±1˚C, 65 % RH and 16:8h (light: dark) photoperiod in the growth chamber (Figure 1A). The larvae were reared on sliced tomatoes in Tupperware® plastic container (30cm x 18 cm x 7 cm) having sterilized moist soil and a paper towel at the bottom (Figure 1B). Pupae were collected with soft forceps and transferred to petri dish (9 cm diameter) and incubated until adult emergence. Emerged adults were sexed and kept in rearing cages (30 x 17 cm) with 10% honey solution. Eggs were laid individually on tomato leaves in adult rearing cages. The eggs were collected daily and incubated in moisturized black filter paper in a petri dish. Newly hatched (neonate) larvae were used to set up the experiments.

Tested Artificial Diets and Ingredients

Six artificial diet formulations were experimentally tested. The composition of the diets and their quantity in a diet mixture were shown in Table 1. The tested diets were offered to newly hatched cotton bollworm larvae. Diet formulations reported by Diet I, originally developed for *Grapholita molesta* [31], Diet II formulated for Lepidopteran species [32], Diet III was a commercial diet developed for Gypsy Moth Diet; (https://www.mpbio.com), Diet IV developed for noctuid species [27], Diet V [33] and Diet VI were originally also developed for noctuid species [28]. All ingredients were weighed and mixed until having homogeneous mixture. Mold inhibitors and vitamins were added lastly. Diets were freshly prepared before each experiment and poured into plastic cups then kept at 4˚C until used. The tomato fruit was used as control. Sliced tomatoes were used to rear larvae until third instar then whole fruits were given to test and complete their developments.

Table 1. Composition of artificial diets used to rear *Helicoverpa armigera* larvae in this study.

Diet Ingredients	Diet I (Ivaldi-Sender, 1974)	Diet II (Poitout and Bues, 1970)	Diet III (MP #960293)	Diet IV (Hamed and Nadeem, 2008)	Diet V (Daguang et al., 2002)	Diet VI (Jha et al., 2012)
Pinto Bean	-	-	-	-	25 g	37.5 g
Soybean Meal	-	-	-	-	25 g	-
Corn Meal	-	56 g	-	-	100 g	-
Corn Semolina	25 g					
Chickpea Meal	-	-	-	-	-	37.5 g
Brewer's Yeast	-	15 g	-	-	45 g	30 g
Agar	10 g	10 g	6 g	4.5 g	6 g	18.75 g
Wheat Germ	25 g	14 g	-	6 g	-	27.5 g
Torula Yeast	25 g	-	-	6 g	-	-
Sucrose	-	-	-	6 g	20 g	-
Methyl paraben	0.9 g	-	-	0.75 g	-	0.875 g
Sorbic Acid	-	-	-	0.45 g	1 g	0.75 g
Cholesterol	-	-	-	0.06 g	-	-
Ascorbic Acid	2.25 g	2 g	-	1.2 g	-	3 g
Benzoic Acid	0.9 g	0.4 g	-	-	-	-
Streptomycin	-	-	-	0.01 g	-	-
Formaldehyde (10%)	-	-	-	1.5 ml	1 ml	-
Vitamin Mix USDA	-	-	-	0.12 g	-	-
Choline Chloride (20%)	-	-	-	3 ml	-	-
Vanderzant Vitamin Mixture	-	-	-	-	2 g	-
Olive Oil	-	-	-	0.06 ml	2 ml	-
Nipagine	-	0.5 g	-	-	3 g	-
L-cysteine	-	-	-	-	-	0.3 g
Diet Mix Gypsy Moth (MP #960293)	-	-	66.4 g	-	-	-
Water	390 ml	340 ml	400 ml	220 ml	650 ml	650 ml

Rearing Procedure

Diets were cut into small pieces as 3 cm in diameter, weighing and placed into petri dish having white filter papers at the bottom. To test each diet, 10 neonate larvae were used with three replications. Neonate larvae were transferred with a camel hair brush to each diet (Figure 1C). Diets and tomato fruits were renewed every other day. Larval development and survivorship were monitored daily under Olympus SZX9 stereozoom microscope. Biological stages and development periods were observed, measured and photographed. Mature larvae left tomato fruits or diets and pupated in sterilized moist soil. The pupae were sexed by examining externally the last abdominal segments [34,35,36]. Newly emerged adults were kept in adult cages (30 x 17 cm) containing fresh tomato leaves for oviposition (Figure 1D).

For each diet, the following biological parameters were reported: (1) larval development time (from hatching to pupation), (2) pupal development time (from the onset of pupariation of the mature larvae until the emergence of the first adult), (3) pre-pupa duration (day), (4) pupal recovery (total number of pupae produced from the number of original larvae), (5) percentage of adult emergence, (6) pupal width, length and weight (at day 5 after pupariation) and (7) the sizes of the head capsules. Additionally, after testing each artificial diet based on biological parameters, if the diets support larval development and growth, larvae keep rearing on that diet for four continous generations in order to adapt and see any nutritional deficieny.

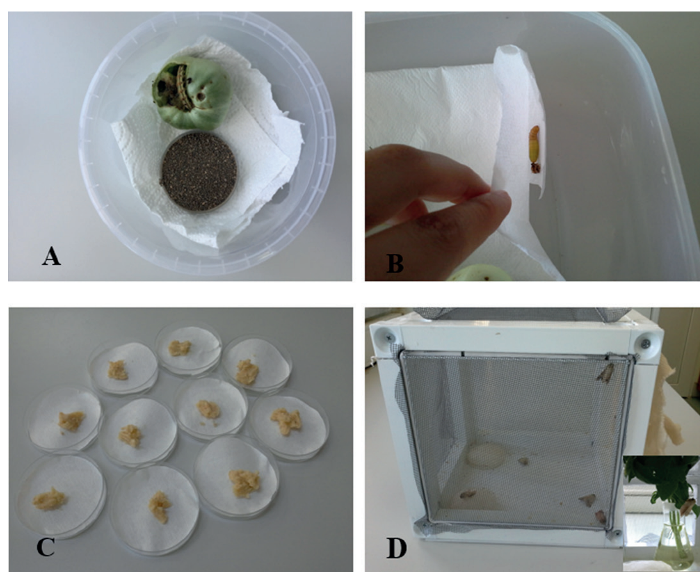

Figure 1. A view of *H. armigera* larvae reared on tomato as positive control (A), pupae on paper towel at the bottom (B), tested larval diets on petri dishes (C) and adult rearing cages (D).

Statistical Analysis

Descriptive statistics as mean values ± standard error (SE) were calculated. The differences among the diets quality control parameters were determined by analysis of variance (ANOVA) and Tukeys's test was used. Significance was accepted with $p \leq 0.05$. The biological parameters were analyzed with Minitab 17 statistical software.

RESULTS AND DISCUSSION

Six different artificial diets along with tomato fruit as positive control were tested in this study. Diet evaluations based on the duration of larval and pupal stages, pre-pupa duration, pupal recovery (%), adult emergence (%), weight, width and length of pupae were given in Table 2. The results of all tested diets were compared to natural host to order to find out the best artificial diets.

Table 2. Biological parameters of cotton bollworm reared on artificial diets and natural host (tomato fruit) (mean±SD) (n=30).

Parameters	Diet Type					
	Diet I	Diet II	Diet III	Diet V	Diet VI	Control (tomato)
Larval duration (day)	11.6 ± 1.5 e	16.6 ± 1.4 d	25.1 ± 0.9 a	25.5 ± 3.2 a	22.1 ± 1.7 b	18.2 ± 0.8 c
Pupal duration (day)	9.1 ± 0.5 c	10.1 ± 1.6 c	31.2 ± 4.2 a	29.5 ± 4.8 a	19.9 ± 4.1 b	9.3 ± 0.4 c
Pre-pupal duration (day)	2.1 ± 0.3 b	2.2 ± 0.7 b	3.3 ± 0.5 a	3.4 ± 0.7 a	3.5 ± 0.9 a	2.3 ± 1.1 b
Pupal recovery (%)	76.6 ± 2.1 c	80.1 ± 1.1 b	76.6 ± 1.5 c	83.3 ± 1.5 b	83.3 ± 2.8 b	90.1 ± 1.1 a
Adult emergence (%)	85.9 ± 2.7 a	86.2 ± 5.4 a	73.3 ± 2.8 b	66.6 ± 1.2 c	83.3 ± 2.8 a	88.4 ± 11.1 a
Pupal weight (g)	0.33 ± 0.04 b	0.31 ± 0.03 ab	0.26 ± 0.04 c	0.35 ± 0.08 ab	0.34 ± 0.04 ab	0.36 ± 0.05 a
Pupal width (mm)	5.72 ± 0.21 ab	5.62 ± 0.27 b	5.5 ± 0.34 b	5.85 ± 0.44 ab	5.77 ± 0.31 ab	6.05 ± 0.3 a
Pupal length (mm)	19.45 ± 1.02 ab	19.2 ± 1.12 ab	18.8 ± 1.29 b	18.62 ± 1.34 b	18.97 ± 1.35 b	19.97 ± 0.58 a

Means within a row followed by the same letter do not differ significantly by Tukey test ($P<0.05$).

Larvae survived on Diet I, completed their development successfully and the larval feeding behaviours were shown in Figure 2. We clearly observed second instar (Figure 2A), third instar (Figure 2B), fourth instar (Figure 2C) and mature larvae (Figure 2D) on Diet I. They prepared silken filament and attached themselves to diet to feed on. The sizes of the head capsules on Diet I were 0.26±0.03 mm for the first instar, 0.45±0.04 mm for the second instar, 0.85±0.05 mm for the third instar, 1.52±0.04 mm for the fourth instar and 2.96±0.32 mm for the fifth instar (Table 3). Variance analysis of head capsule width among different larval stages in column indicated that the differences were significant ($p<0.05$). So, the cotton bollworm has five instars on Diet I. The larval duration period was 11.6±1.5 days and pupal duration was about 9.1±0.5 days. No significant differences in pupal duration between artificial diet and tomato, but larval development time on artificial diet was shorter than control. Pre-pupal duration was about 2.1±0.3 days. Pupae measured about 5.72±0.21 mm in width, 19.45±1.02 mm in length and weighed about 0.33±0.04 g on Diet I. Adult emergence rate was 85.9±2.7%. The number of emergenced adults was 21 (11♀:10♂). Adults were mated in rearing cages and females laid eggs on tomato leaflets. The hatched egg viability was about 87% (Table 2).

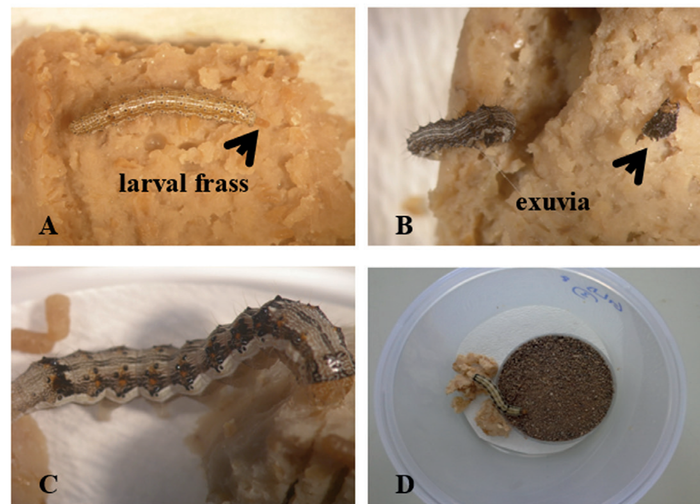

Figure 2. Larval feeding behaviour of *H. armigera* on Diet I, (A) 2nd instar, (B) 3rd instar, (C) 4th instar and (D) mature larva about to pupate.

Table 3. Head capsule width of bollworm larvae reared on artificial diets and tomato (n=15).

	Diet Type					
	Diet I	**Diet II**	**Diet III**	**Diet V**	**Diet VI**	**Control (tomato)**
1st instar	0.26±0.03 Aab	0.28±0.04 Aab	0.30±0.01 Aa	0.29±0.01 Aa	0.29±0.01 Aa	0.25±0.05 Ab
2nd instar	0.45±0.04 Bbc	0.41±0.03 Bd	0.47±0.03 Bab	0.47±0.03 Bab	0.50±0.01 Ba	0.42±0.02 Acd
3rd instar	0.85±0.05 Cbc	0.77±0.06 Cc	0.82±0.04 Cbc	0.80±0.12 Cbc	0.88±0.03 Cb	1.00±0.12 Ba
4th instar	1.52±0.04 Dab	1.63±0.08 Da	1.47±0.08 Db	1.37±0.16 Dc	1.56±0.06 Dab	1.48±0.13 Db
5th instar	2.96±0.32 Eb	1.85±0.08 Ec	3.13±0.23 Eab	1.81±0.07 Ec	3.27±0.19 Ea	1.85±0.10 Dc
6th instar	-	2.82±0.20 Fb	-	3.48±0.12 Fa	-	3.06±0.40 Eb

*Means within a row followed by the same small letter do not differ significantly by Tukey test ($P<0.05$).
**Means within a column followed by the same big letter do not differ significantly by Tukey test ($P<0.05$).

Bollworm larvae survived and completed their development with having six instars on Diet II. The larval frasses were observed during feeding inside the silky area of the diet. Larval duration was about 16.6±1.4 days which was longer than Diet I. The pupal recovery was 80.1±1.1%. Pre-pupal duration was 2.2± 0.7 days and there were no significant differences for pre-pupal duration between Diet II and control. Both diets were supported larval development. Pupal duration was about 10.1±1.6 days. The number of adult emergence was 21 (11♀:10♂) on Diet II. No significant differences in adult emergence rate were found between Diet II and tomato. Pupae were measured 5.62±0.27 mm in width, 19.2 ±1.12 mm in length and weighed about 0.31±0.03 g (Table 2). The larval frasses and head capsules were visible indicating that larvae were feeding (Figure 3). The sizes of the head capsules were 0.28±0.04 mm for the

first instar, 0.41±0.03 mm for the second instar, 0.77±0.06 mm for the third instar, 1.63±0.08 mm for the fourth instar, 1.85±0.08 mm for the fifth instar and 2.82±0.20 mm for the sixth instar (Table 3). Mean comparison revealed significant differences among the bollworm instars feeding on Diet II.

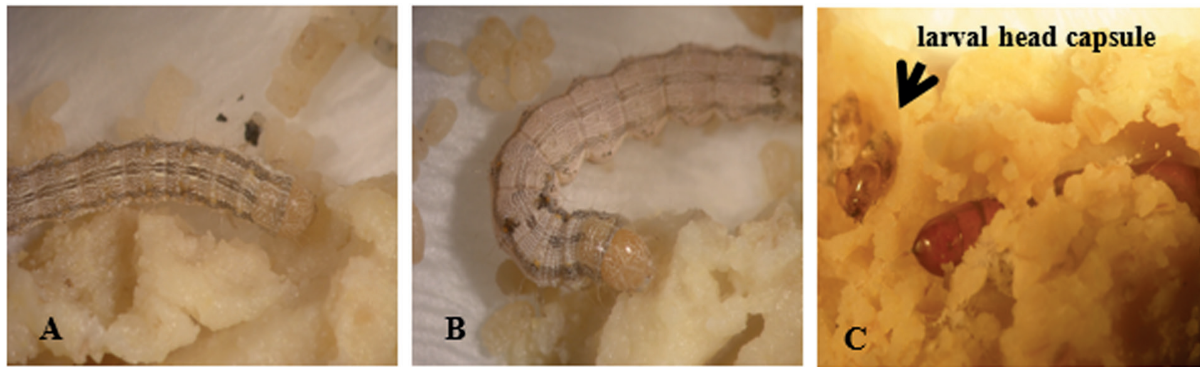

Figure 3. Larval feeding of *H. armigera* on Diet II, (A) 4th instar, (B) 5th instar and (C) pupa with the shredded larval head capsule.

Bollworm larvae had five instars based on head capsule measurements on Diet III (Table 3). The larval and pupal durations were longer than Diet I and Diet II. Larval duration was about 25.1±0.9 days. The pupal recovery was 76. ±1.5 %. Adult emergence rate was 73.3±2.8 %. Pupae were measured 5.5 ±0.34 mm in width, 18.8±1.29 mm in length and weighed about 0.26±0.04 g (Table 2). There were significant differences in pupal weights ($p<0.05$).

Larvae refused to feed on Diet IV and survived only first 10 days. Early instars had the highest mortality as 90%. This diet was not supported larval growth and development. Therefore, Diet IV was not suitable for rearing cotton bollworm larvae.

Diet V was also tested to rear the cotton bollworm. Larval development duration was 25.5±3.2 days. Larval development time on this diet was significantly longer than other diets. Pupal duration was about 29.5±4.8 days. Pre-pupal duration was 3.4±0.7 days and there were significant differences for pre-pupal duration between Diet I, Diet II and control. Pupae were measured 5.85±0.44 mm in width, 18.62±1.34 mm in length and weighed about 0.35±0.08 g. We observed that cotton bollworm has six instars on diet V (Table 2).

Bollworm larvae survived on Diet VI. Larval duration was about 22.1±1.7 days which was longer than Diet I and Diet II. Pupal development time was also longer compared to other diets. Adult emergence rate was 83.3±2.8 %. Pupae measured 5.77 ± 0.31 mm in width, 18.97±1.35 mm in length and weighed about 0.34±0.04 g. The cotton bollworm larvae had five instars on Diet V. Pre-pupal duration was about 2.3±1.1 days (Table 2).

The cotton bollworm larvae were also reared on tomato fruits as control and they had six instars. The adult emergence was 88.4 ± 1.1 %. Larval period lasted about 18.2±0.8 days, pupal period was about 9.3±0.4 days and pre-pupal duration was 2.3 ±1.1 days. Pupal recovery was 90.1± 1.1%. Pupae measured 6.05±0.3 mm in width, 19.97±0.58 mm in length and weighed about 0.36±0.05 g (Table 2).

The development larval period of the bollworm on tomato fruits was reported as 19.23 days by Kumar et al. [37], 16.17 days by Gupta et al. [38], 17 days by Guerra and Ouye [39] and 15.04 days by Casimero et al. [40]. According to Tamer [41], larval period was 12.7 to 13.5 days. Barbosa et al. [3] and Mironidis and Savopoulou-Soultani [2] tested the rearing of cotton bollworm on artificial diets and larval duration were ranged from 12.7 ± 0.3 days to 15.5 ± 0.2 days.

Kumar et al. [37] observed pupal period of bollworm on tomato fruits as 11.57 days. Additionally, it was reported as 11.34 days by Devi and Singh [42]. Liu et al. [8] and Tamer [41] reported pupal duration were 9.35 ± 0.14 days and 11.2 days respectively. Guerra and Ouye [39] were reported as 14 days and Brewer [43] results indicated as 9.56 days when bollworm were reared on artificial diets. Our results were similar to previous studies.

According to Kumar et al. [37], pre-pupal duration of bollworm was 2.27 days on tomato fruit and 1.13 days on artificial diet. Similar results have also been reported by Liu et al. [8,23], as 2.35 days.

Pupal weights are an important parameter in terms of artificial rearing studies. The weight of bollworm pupae exhibited significant differences among tested different diets. The highest weight of pupae was 0.361 g when the larvae fed on tomato. On the other hand, the lowest pupal weight was 0.267 g observed on Diet III. According to Barbosa et al. [3], pupal weight of cotton bollworm was 0.358 g on the artificial diet. Similar findings have also been reported by Amer and Sayed [26], as 0.325 g and Kumar et al. [37] recorded that pupal weight as 0.237 g on tomato fruit and 0.321 g on artificial diet.

Data were presented in Table 2 showed that pupal recovery was 90.1 % on tomato. The average pupal recovery was 76.6 % and 80.1 % on Diet I and Diet II, respectively. These results were similar to Kumar et al. [37] recorded that pupal recovery was 91.86%. In addition to that, Devi and Singh [42] found pupal recovery as 75.05%. Ahmed [44] reported that the highest pupal recovery was 83.7 % in the first generation and 71.2% in the field generation.

Ahmed [44] and Ahmed et al. [45] also reported bollworm adult emergence as 92.8% in the fourth generation and 78.4% in the first generation, respectively. Amer and Sayed [26] reported adult emergence rate was 92.73% on artificial diet. Kumar et al. [37] also reported highest adult emergence were 70.61% on tomato and 83.46% on artificial diet.

The cotton bollworm had different number of instars depending on larval food. We determined 6 instars on Diet II, Diet V and control, whereas, it had 5 instars on Diet I, Diet III and Diet VI. The other investigators also reported different number of instars such as Kumar et al. [22] observed 5 instars Jha et al. [46] reported 6 instars whereas Barbosa et

al. [3] observed 3 to 7 instars and Liu et al. [8] observed 7 instars on different diets.

There are many factors which effect on the biological parameters as larval feeding on artificial diet [47,48] which are mostly depended on the host plants, temperatures, humidities, rearing conditions, mating behaviours as well as the structure, composition and nutritional values of artificial diet. The nutritional content of a diet can considerably affect development time, fecundity, survival and growth of lepidopteran species [49]. In this study, Diet I was tested for the first time to rear the cotton bollworm larvae. Diet I and Diet II were suitable and sufficient for adaptation of wild colony in the laboratory conditions.

CONCLUSION

In this study, we evaluated six different artificial diets in the controlled laboratory conditions. Diet I and Diet II were sufficient and nutrionally suitable for laboratory uses and adaptation of wild colony. Diet I was the best diet out of six tested artificial diets which was also used the first time to rear cotton bollworm larvae. Diet I is simple to prepare, low costed and has a good texture supporting larval development of cotton bollworm.

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Optimization of Recombinant Novel Esterase Expression from Extremophiles

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Abstract

Esterases, which are a sub-group of lipolytic enzymes, are important biocatalysts for many industrial applications. In this study, optimization for the recombinant expression of a novel esterase, which was previously obtained by metagenomic approach, was studied. To optimize the expression, 0.1, 0.5 and 1 mM of isopropyl β-D-1 thiogalactopyranoside (IPTG) concentrations were applied. In addition, induction at 25 ºC for 16 hours, 30 ºC for 6 hours and 37 ºC for 3 hours were tested. According to the results, induction at 30 °C for 6 hours by 0.1 mM of IPTG yielded high amount of protein with maximum catalytic activity. After the gene was successfully expressed, purification studies were conducted. The protein was purified using His-tag method. Native and SDS-PAGE analysis showed that protein which is present as a monomer was successfully purified.

Keywords: Protein expression, esterase, optimization

INTRODUCTION

Lipolytic enzymes are widely used biocatalysts in many industries such as pharmaceutical, detergent, food and paper industry. They find application in a wide variety of reactions such as hydrolysis, esterification, interesterification, alcoholysis and acidolysis [1]. These enzymes are widely distributed in nature and present in animals, plants and microorganisms. Lipolytic enzymes are divided into two groups as lipases and esterases according to their substrate preference. Esterases (EC 3.1.1.1) are the enzymes that catalyze the hydrolysis of ester bonds of partly water soluble molecules, with relatively short carbon chain [2].

Metagenomics is the culture independent study of mixed microbial populations, without using the traditional culturing techniques [3]. Metagenomics is an effective approach for studies in which the culturing is troublesome, especially for studies with extremophiles. Halophiles are extremophilic microorganisms that can live in high salt concentrations [4]. From industrial aspect, they are especially important for their enzymes which are usually exploitable in organic solvents and receive the attention of researchers due to their ability to maintain the activity in low water content medium.

The gram negative bacterium *E. coli* has been used for many years as a host organism to produce recombinant proteins rapidly and with a high yield [5]. Its physiology is well known and due to its rapid growth kinetics, high cell densities can be achieved in a relatively short time. In addition, procedures for transformation with exogenous DNA are well established and efficiency is usually high [6]. There are different strains of *E. coli* available for recombinant protein expression. The strain C43 (DE3) used in this study was generated from *E. coli* BL21 (DE3) strain by Mirous and Walker [7]. The strain was especially designed for the expression of toxic or membrane proteins at high levels. To minimize the risk of toxicity of the recombinant protein to the host cell, *E. coli* C43 (DE3) was used [6]. pET vector systems, which are used in this study, are widely used for recombinant protein expression. They use T7 promoter system and in some cases, the recombinant protein can possess 50% of total proteins within the cells [8]. In this system, the transcriptional control in under the *lac*UV5 promoter, which makes the system inducible by IPTG [6]. Along with IPTG concentration, induction temperature has important effect on the production of recombinant protein. It is known that although it is a highly efficient host, recombinant proteins produced by *E. coli* tend to form inclusion bodies, which obligate more steps to be applied in downstream processing to obtain a soluble and active protein. An effective way to reduce the formation of inclusion bodies is to reduce the expression temperature and extend the time [9]. As can be concluded, recombinant protein production requires optimization of inducer concentration and time/temperature for induction to be able to obtain active proteins with a high yield.

The novel esterase enzyme related to this study was previously isolated from the metagenome of a hypersaline lake, Acıgol (Denizli), by metagenomic approach [10]. Within the scope of this study, it was aimed to optimize the conditions for the recombinant expression and purification of this protein in active form with a high yield.

MATERIALS AND METHODS

Bacterial strains and plasmids

The gene encoding the esterase was previously cloned in pET-28(+) vector (Novagen, USA) and transformed into OverExpress™ C43 (DE3) strain of *E. coli* (Lucigen, USA).

Optimization of recombinant protein expression

Single colony from transformed *E. coli* C43 (DE3) cells was inoculated into 10 mL of LB containing 40 µg/mL ka-

namycin. After 16 hours of incubation at 37°C with 200 rpm orbital shaking, 200 mL of medium was inoculated with the starter culture. The plasmid used in cloning (pET-28a(+)) contains a *lac* operon and the expression is inducible with IPTG. To determine the optimum IPTG concentration, cells were induced with 0.1, 0.5 and 1 mM of IPTG when OD_{600} was 0.8 and incubated at 37 °C for 1, 3 and 4 more hours.

After optimum IPTG concentration was found, the expression was conducted at different time and temperatures (16 hours at 25 ºC, 6 hours at 30 ºC and 3 hours at 37 ºC) to assess the effect of expression temperature on the activity of esterase enzyme. Recombinant protein was purified as explained below, analyzed on SDS-PAGE and kinetic analysis was performed in the presence of para nitro phenyl octanoate as substrate. After optimization of the IPTG concentration, expression time and expression temperature were also optimized. The volume of the starter culture was fixed to 50 mL and proteins were expressed in 1L of medium. At the end of incubation, cells were harvested by centrifugation at 5000 rpm for 20 min at 4°C. Cell pellets were stored at -20°C.

Protein purification

Cell pellet from 1 L of culture was resuspended in 15 mL of ice cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8). Lysozyme was added and incubated on ice for 30 min. Cell suspension was sonicated (Bandelin SonoPuls) 10 times with 10 s on / 20 s off cycle. Cell debris was pelleted at 12000g for 15 min at 4 °C. To bind the histidine-tagged proteins, 1.2 mL of Ni-NTA Agarose (Qiagen, #30210) was added to the cell free lysate and incubated for 1 hour at 4 °C on rotary shaker. Proteins bound to resin was pelleted by centrifugation at 3000 g at 4 °C. Resin was washed with 4 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) for three times and proteins were eluted with 1 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8) for six times.

SDS and native polyacrylamide gel electrophoresis

Crude lysates or purified protein samples were electrophoresed on polyacrylamide gel. Stacking gel concentration was 5% and resolving gel concentration was 12%. For SDS-PAGE, 20 µL of samples were mixed with 5 µL of sample application buffer and heated at 95ºC for 15 min. Molecular weight marker (Pierce™, #26610) was denatured at 95 ºC for 5 min. For native PAGE, same buffers were used except that denaturing agents were not included in any buffer and heating step was omitted. Electrophoresis was initiated with a voltage of 80 V, and after proteins enter the resolving gel, a constant voltage of 120 V was applied until the front dye reached at the end of the gel. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250.

After purification of the protein was verified by SDS-PAGE, pure protein fractions were collected and applied to ultrafiltration tubes (Amicon®, # UFC901008) with 10 kDa molecular weight cut-off value. Elution buffer was changed to 50 mM Tris-HCl, containing 10% NaCl, pH 8 and protein was concentrated to a total volume of 1.5 mL. For protein quantification, Bradford method was used [11]. 5 µL of sample was mixed with 195 µL of Bradford reagent (Sigma, #B6916). BSA was used as standard and absorbance measurement at 595 nm was done triplicate.

Activity Assay

The activity assay was performed according to Winkler and Stuckman [12]. 1 µM of enzyme and 1 mM of para nitrophenyl octanoate was used in the reaction. Reaction was incubated at 30 °C and the absorbance at 410 nm was measured at 20th minute.

RESULTS AND DISCUSSION

A novel metagenome derived esterase gene was previously cloned into pET-28(a) and transformed into *E. coli* C43(DE3) cells [10]. To determine the optimum conditions for protein expression, different IPTG concentrations (0.1, 0.5 and 1 mM IPTG) were tried. Expression was conducted for 1, 3 and 4 hours to determine the optimum duration of expression. At the end of incubation, cells were harvested, sonicated and cleared lysates were analyzed on SDS-PAGE (Figure 1).

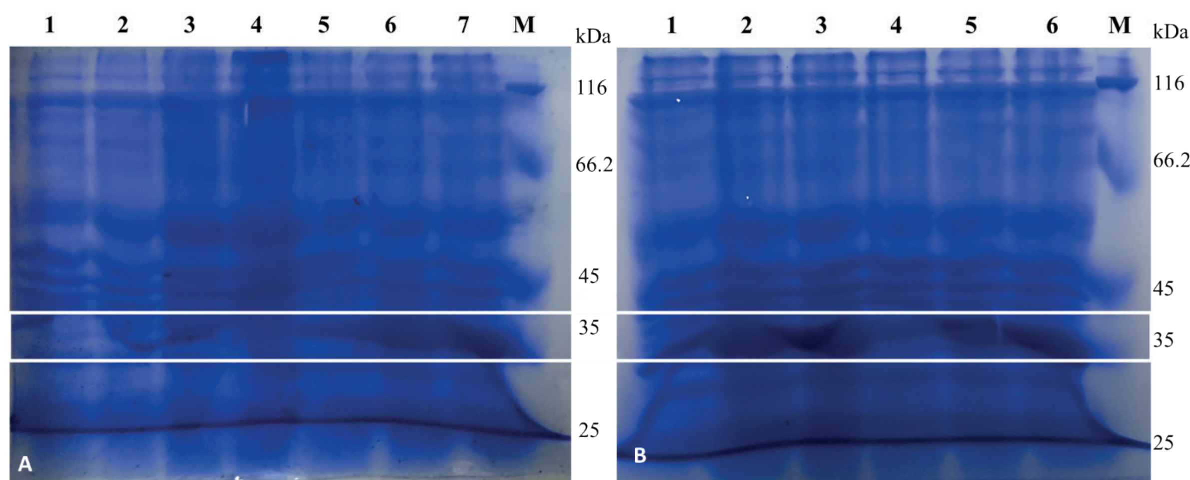

Figure 1. SDS-PAGE analysis of crude lysates samples for IPTG optimization. A. Lane 1, 2, 3 and 4: no IPTG was added, incubation time was 0, 1, 3 and 4 hours, respectively. Lane 5, 6 and 7: 0.1 mM of IPTG was added, incubation time was 1, 3 and 4 hours, respectively. B. Lane 1, 2 and 3: 0.5 mM of IPTG was added, incubation time was 1, 3 and 4 hours, respectively. Lane 4, 5 and 6: 1 mM of IPTG was added, incubation time was 1, 3 and 4 hours, respectively. Lane M: Molecular weight marker. Position of respective bands are shown inside the white rectangle.

As shown in Figure 1, protein was not expressed in the absence of IPTG (Figure 1A, Lane 1-4) as expected. The protein was expressed in high amount with 0.1 mM IPTG. The yield did not improved significantly with 0.5 mM and 1 mM of IPTG. Protein amount expressed in 1 hour was lower than 3 and 4 hours, as expected. Optimum conditions at 37 ºC was found to be 0.1 mM IPTG and 3 hours of incubation. However, it is known that the recombinant proteins expressed in *E. coli* tend to form inclusion bodies [6]. To overcome this problem, induction at lower temperatures are recommended. In this study, expression level and activity of the enzyme expressed at 25 ºC for 16 hours, at 30 ºC for 6 hours and 37 ºC for 3 hours were also compared. Following the appropriate amount of expression, the recombinant protein from each sample was purified by His-tag method. The results are given in Figure 2.

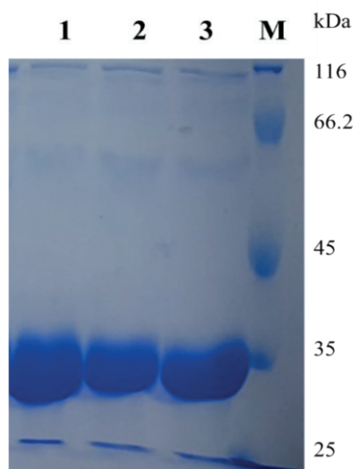

Figure 2. SDS-PAGE analysis of purified proteins for optimum induction temperature. Lane 1: 25 °C for 16 hours; Lane 2: 30 °C for 6 hours, Lane 3: 37 °C for 3 hours, Lane M: molecular weight marker.

The concentration of the purified proteins were measured by Bradford method, using BSA as standard. The protein amounts were calculated as 8.8 g/mL, 7.7 g/mL and 11.8 g/mL for the proteins expressed at 25°C for 16 hours, 30°C for 6 hours and 37°C for 3 hours, respectively. As can be interpreted from the concentration values, the recombinant protein was successfully expressed in all conditions. However, expression of the protein does not always guarantee the high activity of the protein. To assess the effect of incubation temperature on enzyme activity, purified enzymes were used in kinetic assay using pNP-octanoate as substrate. As can be seen in Figure 3, the enzyme expressed at 30 °C showed maximum activity. Lower activity in the protein expressed at 37 °C can be explained with improper folding of the protein. However, reducing the temperature to 25 °C did not improve the activity of the protein. This enzyme was isolated from enrichment culture of organisms which are grown at 30 °C. Additionally, optimum temperature of the enzyme was found as 30 °C [10]. So, it is predicted that the enzyme can gain its best conformation when the temperature is fixed to 30 °C.

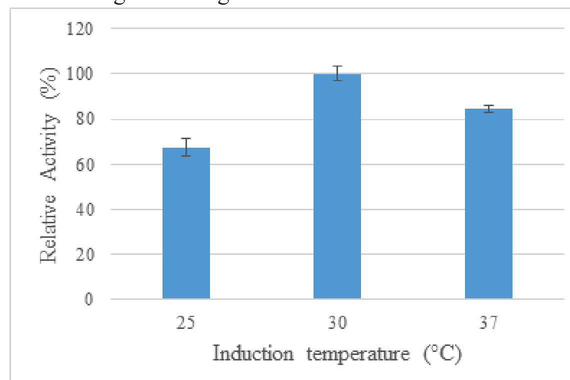

Figure 3. Effect of induction temperature on the activity of the enzyme.

The optimized conditions for recombinant protein expression in *E. coli* varies according to the host strain, vector type and the nature of the protein. In a similar study, in which a metagenomic esterase was isolated, expression was induced by 0.4 mM of IPTG for 18 hours at 20 °C [13]. In another study, *E. coli* BL21 cells bearing a metagenomic esterase coding gene, which was cloned into pET-22b, was induced by 1 mM of IPTG at 20 °C for 20 hours [14]. Another metagenome derived esterase was expressed in *E. coli* BL21 (DE3) using pET28a(+) and induced by 0.1 mM IPTG at 30 °C for 6 hours, which are the same conditions used in present study [15].

Affinity tags have been widely used for recombinant protein purification. In this work, we used an N-terminal 6 histidine tag and purified the protein using the principle of affinity of nickel to histidine. Proteins were bound to Ni-NTA matrix, then eluted with high amount of imidazole. During purification, samples from each fraction were collected and analyzed on SDS-PAGE. Elution samples were combined and concentrated by ultrafiltration membranes. 20 µg of protein samples were analyzed on both native and SDS-PAGE, as results are shown in Figure 4.

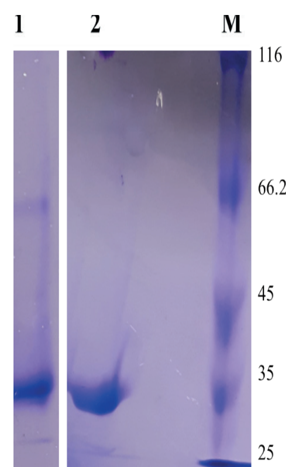

Figure 4. Native (Lane 1) and SDS PAGE (Lane 2) result of purified protein. Lane M: Molecular weight marker.

According to the gel images obtained after native and SDS-PAGE analysis, it is concluded that the enzyme is present as a monomer. The molecular weight of microbial esterases range from 27 to 54 kDa [16]. It is observed that the band corresponding to the purified protein on SDS-PAGE is between 35 and 25 kDa, compatible with the estimated molecular weight which is 28.4 kDa.

As a conclusion, optimum parameters for the expression of an esterase were evaluated with this study. Different IPTG concentrations, expression durations and temperatures were tried to find the optimum condition that yields not only highest amount of purified protein, but also highest activity. According to the results, the best condition was determined as 0.1 mM of IPTG, and induction at 30 °C for 6 hours. Although the amount of purified protein is much higher when induced at 37 °C for 3 hours, highest activity was observed when induction was done at 30 °C for 6 hours. In addition, native and SDS-PAGE analysis of the purified protein revealed that the protein is a monomer, giving a single band between 25 kDa – 35 kDa. After optimization of the expression conditions, studies focusing on detailed biochemical characterization of the enzyme are planned as future work.

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Screening of Y Chromosome Microdeletions and Cytogenetic Analysis in Infertile Men

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Abstract
In our study we aimed to demonstrate Y microdeletions with using sY254 sequence tagged sites (STS) marker in primary infertile male patients by using Polymerase Chain Reaction (PCR) method and determine the correlation between cytogenetic evaluation of patients. Twenty five male patients referred from Manisa and it's regions, who were diagnosed with primary infertility in the urology department of Manisa Celal Bayar University were included in our study. Thirteen individuals had azoospermia, twelve individuals had oligozoospermia. Ethics committee approval of our patients were recieved from Manisa Celal Bayar University. Blood material was taken under sterile conditions from individuals. Total DNA was isolated from peripheral blood. DNA purity was checked. Target gene regions of total DNA were amplified with PCR method by using oligonucleotide primers. sY14 STS marker used as internal control and pure water used as negative control. Cytogenetic study was made with lymphocyte cultures, human metaphase chromosomes were analysed with light microscopy. According to our analysis results no microdeletion was determined and all of the patients had normal karyotype (46, XY).

Keywords: Y microdeletion, sY254, STS, Infertility, Azoospermia, Oligozoospermia

INTRODUCTION

Infertility, which is defined as the inability to conceive after a year of unprotected sexual intercourse, affects almost 15% of couples. Male factors are responsible in about 50% of the cases [1,2,3,4]. Male infertility is a multifactorial syndrome. The major factors who contribute to infertility are; erectile dysfunction, infections of the reproductive system, gonadal endocrine disorders [Follicle Stimulating Hormone (FSH), Leutinizing Hormone (LH), testosterone], antisperm antibodies, exposure to chemical agents and radiations, testicular cancer, varicocele, anatomic abnormalities (hypogonadism, anorchia), genetic aberrations and others [4, 5, 6, 7, 8]. However, in many cases (in around 30%-50%) the causes of male infertility is still unknown, this condition is defined as idiopathic infertility [1, 4, 7]. Both genetic and environmental factors are responsible for reducing male infertility [1,9].

Genetic factors decrease reproductivity in males around 10-15% by affecting physiological processes of hormonal homeostasis, spermatogenesis, and sperm quality [3,10]. Some genetic causes of male infertility are chromosomal abnormalities, Y chromosome microdeletions, X-linked and autosomal gene mutations. After Klinefelter syndrome, Y chromosome microdeletions are the most frequent genetic cause of male infertility [1, 2, 10].

Between all human chromosome, Y chromosome is the shortest chromosome and it has the least number of genes. Y chromosome lenght is 60 Mb, 3 Mb associated with pseudoautosomal regions (PAR1 on short arm of the Y chromosome (Yp) and PAR2 on long arm of the Y chromosome (Yq)) and 57 Mb associated with nonrecombining regions (NRY) also known male specific Y (MSY). On the NRY region, heterochromatic and euchromatic regions are found. Known Y chromosome genes are in the euchromatic region. Euchromatic regions has nearly 23 Mb length; 8 Mb is located on the Yp and 14,5 Mb is located on the Yq [1, 6, 10, 11]. Y chromosome is essential for human sex determination, male germ cell improvement and continuation for normal spermatogenesis [1, 6, 9, 11, 12]. In 1976 Tiepolo and Zuffardi described the correlation among Y chromosome deletions and male infertility for the first time [10, 11, 12]. Y chromosome microdeletions are one of the most important genetic etiology of male infertility in the world. Generally male infertility is a de novo event of genetic origin and come to exist during spermatogenesis which involved more than 4000 genes. The long arm of the Y chromosome includes genes and gene families comprised in spermatogenesis [3, 9, 10]. Y chromosome microdeletions are responsible for not only to spermatogenesis defect, but also in recurrent pregnancy loss [14]. On the euchromatic portion of the Y chromosome long arm, microdeletions happen in 10%-15% of idiopathic primary testiculopathies (azoospermia and severe oligozoospermia) [10, 11, 12, 13]. These microdeletions present in the pathogenic mechanisms for infertile males [15].

Genes located on the euchromatic region of the long arm of the Y chromosome, interval 5 and 6, locus 11 (Yq11), called azoospermia factor (AZF) region and was genetically mapped in 1996 by Vogt et al., plays an essential role in spermatogenesis [1, 2, 6, 9, 11, 12]. Genes of AZF region are organized in three different nonoverlapping subregions; AZFa, AZFb and AZFc, these are necessary for normal spermatogenesis [4, 5, 9]. In recent years, the result of the sequence of the MSY and the molecular mechanism, a new AZF region which called AZFd was discovered. AZFd is located inthe place which AZFb and AZFc regions are overlapping [1, 5, 10]. Microdeletions of AZF regions result in several spermatogenetic changes and these microdeletions are associated with

azoospermia, oligozoospermia, diverse testis histology ranging from sertoli cell-only (SCO) syndrome, hypospermatogenesis (HSG) and maturation arrest [4, 9, 15].

AZFa region include ubiquitin-specific protease 9 (USP9Y) and dead box on the Y chromosome (DBY) [6]. This region is located on proximal Yq11 (Yq11.21) [6, 9]. Complete AZFa deletions are relevant to severe testicular phenotype, SCO syndrome, azoospermia. Partial AZFa deletions are extremely rare [1, 4, 14].

AZFb region include RNA binding motif on the Y chromosome (RBMY) [6]. This region is located on distal Yq11 (Yq11.23) [6, 9]. Complete AZFb deletions are associated with maturation arrest at meiosis I, and azoospermia and oligozoospermia. Partial AZFb deletions are changeable phenotypes from HSG to SCO syndrome extremely rare [1, 4, 6, 14].

AZFc region has the deleted in azoospermia (DAZ) gene cluster [6]. This region is located on distal Yq11 (Yq11.23) [6, 9]. Complete AZFc deletions are variable clinical and histological phenotypes which may range from severe azoospermia to mild oligozoospermia and SCO syndrome. Partial AZFc deletions are changeable phenotypes from HSG to SCO syndrome [1, 4, 6, 11]. There are many STS markers which are related with AZF regions for screening Y microdeletions. sY254 is one of the STS marker is mainly associated with DAZ gene of the AZFc region (Figure 1.) The absence of sY254 demonstrate complete deletion of AZFc region [15, 16].

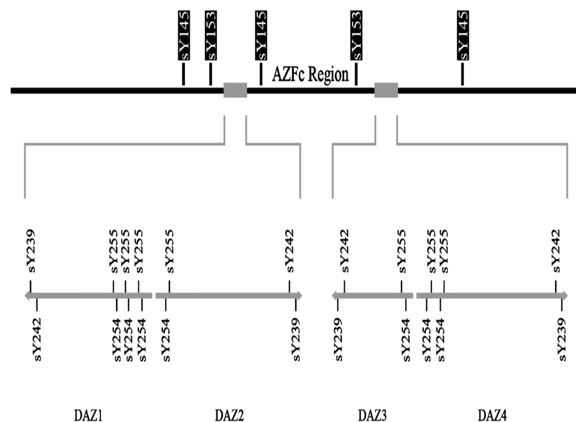

Figure 1. AZFc and putative AZFd region's relative STSs positions (STSs marked in bold belong to the putative AZFd region) [16]

In present, assisted reproductive technologies (ART) like testicular sperm extraction (TESE), intracytoplasmic sperm injection (ICSI), in vitro fertilization (IVF) help infertile men with azoospermia and oligozoospermia for succesful fertilizations and pregnancies. So, before performing ART procedure, determine Y microdeletions, especially AZFc region deletions, is very important diagnostic tool for obtain favourable results [2, 4, 5].

In this study, aimed to determine Y chromosome microdeletions with using sY254 STS marker and f method in 25 infertile men who were diagnosed with primary infertility in the urology department of Manisa Celal Bayar University and also ethics committee approval were recieved from Manisa Celal Bayar University.

MATERIALS AND METHODS

Material Collection

Semen analysis, reproductive hormone estimation (FSH and LH), karyotype and Y chromosome microdeletion analysis were examined for all patients.

Molecular Analysis

Peripheral blood materials was taken under sterile conditions from 25 patients in EDTA vacutainer tubes (BD Vacutainer) and were stored at -20 ^{O}C until DNA extraction. Total DNA was isolated from peripheral blood using DNA isolation kit (Macherey Nagel) according to manufacturer instructions. DNA samples were stored at -20 ^{O}C until PCR application. DNA samples purity were measured with spectrofotometre (Perkin Elmer). DNAs with an OD260 / OD280 ratio between 1.8-2.0 were taken into the study. Target gene regions were amplified with PCR method by using sY254 STS marker oligonucleotide primers (MWG Eurofins) (Table 1), reaction buffers and appropriate PCR programme. sY14 STS marker oligonucleotide primers (MWG Eurofins) used as internal control and pure water used as negative control. PCR products were run on 2 % agarose gel (Serva) and then imaged with a gel imaging system (Syngene).

Table 1. STS marker, primer sequences and PCR product size [5]

STS Marker	Primer sequence	AZF region	PCR product size (bp)
sY 254	F-GGGTGTTACCAGAAGGCAAA R-GAACCGTATCTACCAAAGCAGC	AZFc	370
sY 14	F-GAATATTCCCGCTCTCCGGA R-GCTGCTGCTCCATTCTTGAG	Internal control SRY	472

Cytogenetic Analysis

Peripheral blood materials was taken under sterile conditions from 25 patients in sodium heparin vacutainer tubes (BD Vacutainer) and were stored at -20 ^{O}C. Lymphocyte cultures were made from peripheral blood. GTG banding process was applied to preparates. Human metaphase chromosomes obtained by cytogenetic analysis were observed with light microscopy. For each patient 20 metaphases area were analyzed.

Semen and Hormone Analysis

Semen samples were collected after 3-5 days period of sexual abstinence into a sterile container (Falcon). Samples were analyzed for semen volume and sperm concentration, measured according to the reference values.

Blood materials was taken under sterile conditions from 25 patients in vacutainer serum separator tubes (BD Vacutainer) and were stored at -20 ^{O}C. Levels of serum FSH and LH were detected with biochemicals methods and measured according to the reference values. All semen and hormone analysis were done in the same laboratory.

RESULTS

Our patient group consisted of twenty five infertile men. Thirteen individuals were diagnosed with azoospermia and twelve individuals were diagnosed with oligozoospermia.

Clinical diagnosis, age, semen volume, sperm concentration, FSH and LH values of the patient group were given in Table 2.

Table 2. Clinical diagnosis, age, semen volume, sperm concentration, FSH, LH values of the patient group

Patient no	Clinical diagnosis	Age	Semen volume (ml)	Sperm concentration (x 10^6/ml)	FSH (mIU/ml)	LH (mIU/ml)
1	Azoospermia	28	3	0	24.47	13.47
2	Azoospermia	32	5	0	26.00	14.54
3	Azoospermia	35	4	0	26.66	15.44
4	Azoospermia	40	2	0	23.45	13.78
5	Azoospermia	21	4	0	25.45	14.66
6	Azoospermia	26	6	0	27.66	15.44
7	Azoospermia	23	5	0	25.00	17.54
8	Azoospermia	24	5	0	26.55	14.44
9	Azoospermia	33	6	0	24.44	13.77
10	Azoospermia	35	3	0	20.46	14.76
11	Azoospermia	36	4	0	23.55	13.54
12	Azoospermia	40	5	0	20.13	13.76
13	Azoospermia	23	6	0	26.99	16.55
14	Oligozoospermia	26	3	13	26.33	17.43
15	Oligozoospermia	29	4	10	-	-
16	Oligozoospermia	32	5	6	-	-
17	Oligozoospermia	37	5	4	-	-
18	Oligozoospermia	40	5	7	-	-
19	Oligozoospermia	32	5	15	-	-
20	Oligozoospermia	23	6	12	-	-
21	Oligozoospermia	20	4	15	-	-
22	Oligozoospermia	40	6	6	27.13	15.64
23	Oligozoospermia	23	5	4	24.13	14.34
24	Oligozoospermia	28	5	10	25.78	15.23
25	Oligozoospermia	33	4	16	19.54	15.00

The average age of patient group was calculated as 30,36. The average age of patient group who had azoospermia was calculated as 30,46. The average age of patient group who had oligozoospermia was calculated as 30,25.

Semen volume average of patients group was 4,6 ml. Semen volume average of patients who had azoospermia was calculated as 4,46 ml but sperm was not found. Semen volume average of patients who had oligozoospermia was calculated as 4,75 ml and sperm concentration was between 4 to 16 (x 10^6/ml).

In the cytogenetic analysis, 20 metaphase areas of each patient preparate were examined and they were observed to have normal karyotype (46, XY) (Table 3).

Table 3. Cytogenetic analysis results

Clinical diagnosis	Analyzed field count	Karyotype
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY

As the results of our PCR analysis using sY254 STS marker oligonucleotide primers, on Y chromosome no microdeletion was determined (Figure 2).

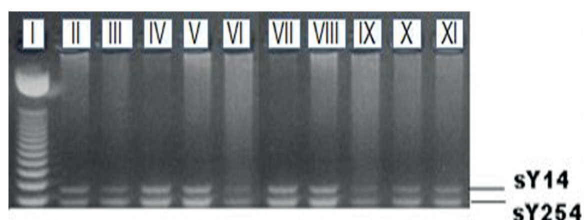

Figure 2. I; 100 bp marker, II-XI, infertile patient AZFc sY2454 region and sY14 region

DISCUSSION

Y chromosome microdeletions and chromosomal abberrations are the most important genetic cause in infertile men affected with azoospermia and severe oligozoospermia [3]. The most common genetic reason of male infertility due to spermatogenesis deficiency is Y chromosome microdeletions, the incidence is 2.7-55.5% in infertile men [15].

The AZF region microdeletions in some cases of male infertility who have azoospermia or oligozoospermia are thought to be pathogenetically involved [1,3]. Deletions of Yq11.23 in AZF region lead to idiopathic azoospermia and oligozoospermia, the rate of incidence is about 8-12% in worldwide [6].According to the studies, AZF region deletions affect approximately 13% of men with non-obstructive azoospermia and in approximately 7 to 10% of men with oligozoospermia. Testicular tissue sections

taken from infertile men who has azoospermia with Yq11 aberrations were analyzed and intense spermatogenesis disruption were determined. Thus AZF region functions are absolutely necessary for differentiation and proliferation of human male germ cells [1]. In our study, we analyzed infertile men patients group who had 13 (52%) azoospermic patients and 12 (48%) oligozoospermic patients.

Even though chromosomal abnormalities in infertile men may lead to recurrent pregnancy loss, microdeletions in the AZFc region of the Y chromosome may have an important function in embryo efficiency or in protection of pregnancy [1]. The other important genetic factors which can result with male infertility are numerical and structural chromosomal abnormalities. This abnormalites can be detected with cytogenetic analysis. If a men have non-obstructive azoospermia and their total motile sperm count <5 million, American Urological Association and European Academy of Andrology Guidelines recommend cytogenetic analysis [13]. Male infertility which come to exist with Y chromosome abnormalities is characterized by azoospermia (absence of sperm), severe oligozoospermia ($<5\times10^6$ sperm/ml semen) and moderate oligozoospermia ($5–20\times10^6$ sperm/ml semen) [5]. In our patients group, thirteen patients had azoospermia and when we analyzed their semen samples, semen volume average was calculated as 4,46 ml but sperm was not found. Twelve patients had oligozoospermia and when we analyzed their semen samples, semen volume average was calculated as 4,75 ml and sperm concentration was between 4 to 16 (x 10^6/ml).

Since 1970, researches have shown that deletion of the long arm of the Y chromosome is associated with spermatogenic failure and leads to partial or complete spermatogenic arrest. The loci involved in sperm production and differentiation using molecular methods have been defined [9]. Small deletions, which are located on the Yq, are considerably smaller than other structural chromosomal abnormalities, can not be seen with microscope, can not be detected by karyotyping so these are called microdeletions. They can only be detected by using PCR method [2, 10]. Y chromosome microdeletions can not be detected with cytogenetic analysis, but rarely Y chromosome abnormalities may refer Y chromosome microdeletions presence [13]. The incidence of Y chromosome microdeletions in patients who affected with azoospermia (they have no sperm count) or affected with oligozoospermia (they have sperm count less than 5 million per milliliter) is 2-10% or more. Frequency of Y chromosome microdeletions may changeable because of the patient selection criteria and population composition. Sometimes it can be encountered with many false diagnosis protocols. The frequency of Y chromosome microdeletions observed in various studies may vary with heterogeneity in the type and number of PCR markers which were applied [14]. In this study, our patients group consisted of infertile men who had oligozoospermia and azoospermia. We used the PCR method to investigate microdeletions of Y chromosome. In PCR analysis using sY254 STS marker oligonucleotide primers, Y chromosome microdeletion was not detected in their related gene region. Also we used cytogenetic analysis method to investigate chromosomal abnormality on the Y chromosome. Altough we analyzed 20 metaphase area for each patient, chromosomal abnormality was not found. All of the patients had normal karyotype (46, XY).

According to our study results, possible microdeletions that may cause infertility may be found in other gene regions. It is thought that the probability of microdeletion detection may increase with increasing the number of patients and the number of genes regions which were screened.

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Removal of Cu (II), Co (II) and Ni (II) Ions from Aqueous Solutions Using Modified Sporopollenin

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Abstract
In this study, (E)-2-((2-hydroxynaphthalen-1-yl)methyleneamino)pyridin-3-ol(HNMAP) compound was immobilized on sporopollenin of Lycopodium clavatum spores. The obtained structure was characterized by infrared spectroscopy (IR) and scanning electron microscopy (SEM). This newly synthesized compound has been used as an adsorbent in the separation of heavy metals. Batch method was used to separate Cu (II), Co (II) and Ni (II) ions from waste waters. In addition, different experimental parameters such as pH, concentration and duration of interaction were selected to effectively separate heavy metals from aqueous solution and wastewater. The experimental results were applied to Langmuir, Freundlich and Dubinin-Radushkevich (D-R) isotherm equations. Thermodynamic parameters such as free energy (ΔG^0), entropy (ΔS^0) and enthalpy (ΔH^0) were calculated from the experimental results to explain the mechanism of the sorption. As a result, this adsorbent has been successfully used to separate Cu (II), Co (II) and Ni (II) from aqueous solutions and waste water.

Keyword: *Immobilization, Wastewater, Heavy metals, Sporopollenin*

INTRODUCTION

Heavy metal levels are increasing day by day in drinking and irrigation waters. This increase leads to a serious threat to human health and ecological systems [1]. Many heavy metals such as Pb, Cd, Co, Ni, Cr and Hg have toxic effects on living organisms.

These metals can harm flora, fauna and other living things even in very small quantities. Their accumulation in humans causes kidney failure, nervous system damage and bone softening, as well as other serious illnesses [2].

Many methods such as precipitation, adsorption, ion exchange, photocatalyst and membrane are used to separate heavy metal ions. Adorption method is widely used for elimination of the heavy metal ions. For this method, adsorbent materials such as resins, clays, silica gel, activated carbon and biological materials [1] are used. For this purpose, one of the most widely used materials in recent times is the sporopollen [3].

Sporopollenin of Lycopodium clavatum spores has a stable aromatic structure and contains carbon, hydrogen, and oxygen. Sporopollenin is produced by monomer structures of the macromolecular sporopollenin. It has a stable, cross-linked structure and an aromatic character that contains carbon, hydrogen, and oxygen with a stoichiometry of $C_{90}H_{144}O_{27}$. The modification of sporopollenin was achieved via chemical immobilization of the appropriate organic groups onto sporopollenin surface [4-5].

MATERIALS AND METHODS

Materials

Sporopollenin (Merck Darmstadt Co.) was used as adsorbent in this study. $Cu(NO_3)_2$, $Co(NO_3)_2$ and $Ni(NO_3)_2$, (3-Chloropropyl)trimethoxysilane [CPTS] and (E)-2-((2-hydroxynaphthalen-1-yl)methyleneamino)pyridin-3-ol (HNMAP) were purchased from Merck. All chemicals used in the study were analytical graded. Ultra pure water was used in experimental study.

Instruments

Infrared spectra were measured in the range of 650–4,000 cm^{-1} by a Perkin Elmer 100 FT-IR spectrometer (KBr pellets, 21 ℃ temperature, 39% moisture, 1 atm pressure). The pH values were monitored with pH meter. The thermostatic shaker was used for the sorption experiments. The heavy metal concentrations were determined by UV-Visible Spectroscopy. All aqueous solutions were prepared with ultra pure distilled water obtained from a water purification system (Millipore Milli-Q Plus).

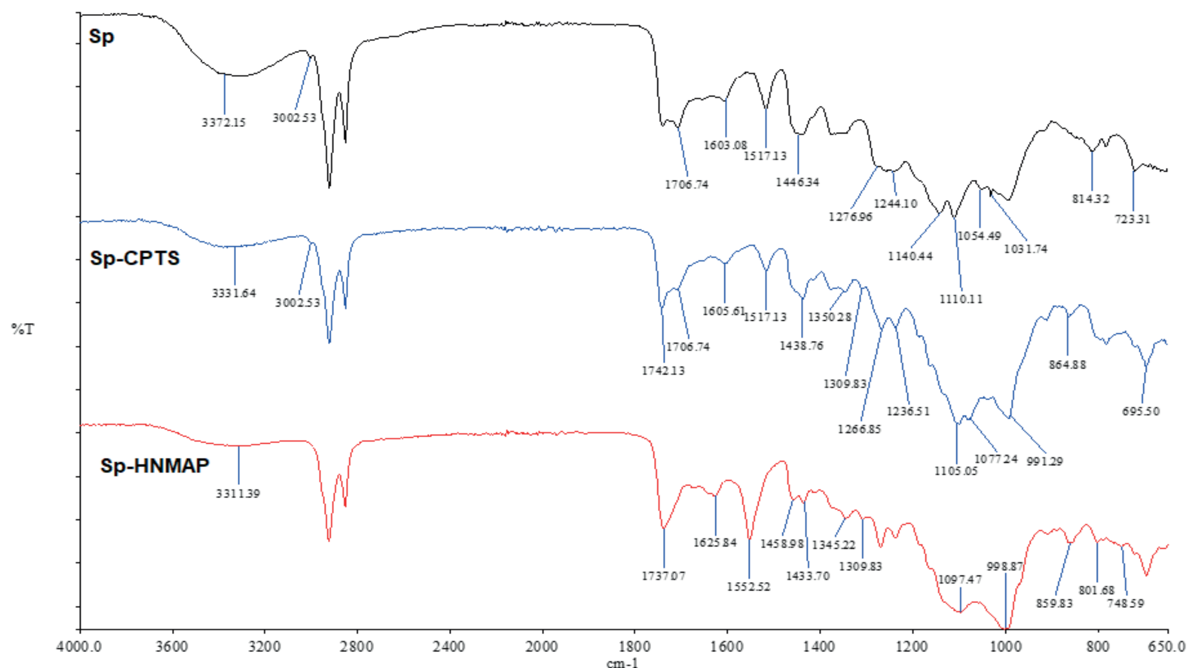
RESULT AND DISCUSSION

Characterization

The immobilized sporopollenin was characterized by FTIR and SEM. The infrared spectra of the prepared surface were shown in Fig.1.

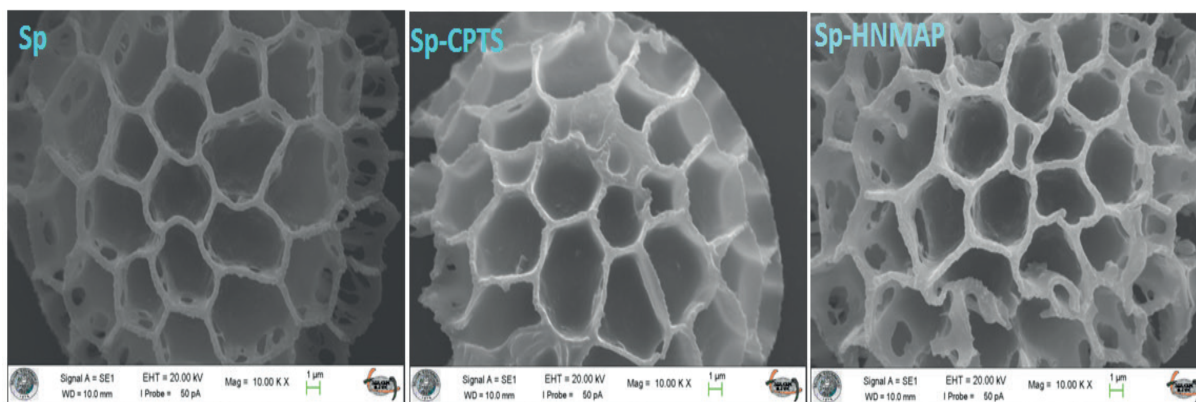
It is observed between 3400 and 3200 cm^{-1} of the purely sporopollenin peak. CH_2 stretching vibration in Sp-CPTS was shifted from 2920 cm^{-1} to 2840 cm^{-1} (Sp). Spectra of Sp-HNMAP have broad –OH peak at 3100 cm^{-1} because of the –OH groups (Fig.1)[6]. Stretching of C=N group observed at 1644 cm^{-1} indicated primer amine group in Sp-CPTS and Sp-HNMAP and organic substances. The peak at 1204 -1150 cm^{-1} was bending of–C-OH. The peaks at 1479-1500 and 2860 cm^{-1} were interpreted as stretching of C=C and C-H in the benzene ring respectively [7].

Figure 1. FTIR spectra of Sp, Sp-CPTS and Sp-HNMAP.



SEM images of pure sporopollenin are shown in Figure 2(a). The structure of the Sp- CPTS compound is shown in Figure 2(b). Figure 2(c) shows NHMAP showing a very smooth morphology after immobilization to the Sp-CPTS surface. The surface of the sporopollenin coated with NHMAP shows an irregular morphology. The presence of NHMAP on the surface of sporopollenin confirms immobilization.

Figure 2. SEM images of Sp (a), Si- CPTS (b) and Sp-HNMAP (c).



CONCLUSION

According to the results obtained from experimental studies, the best chromium removal was carried out at pH = 5.0, with 0.075 g adsorbent, at 323.15 ^{0}C temperature, in concentration of 40 mg/L in 180 min. FT-IR spectroscopy and SEM techniques confirmed the immobilization of SP-HNMAP. Adsorption is mostly found to be pH dependent. Since the results are consistent with the Langmuir isotherm model, adsorption mechanism is explained by this model.

The calculated thermodynamic parameters [8] have shown that the reactions are endothermic and spontaneous. For spontaneous reactions, ΔG^{0} values were negative at 25-50 ^{0}C. ΔG^{o} value decreased with increase in temperature. The adsorption of heavy metal ions on Sp-NHMAP increased at higher temperatures [8]. The Sp-NHMAP has high capacity to adsorb Cu (II), Co(II) and Ni(II) ions in the wastewater.

This work is very important in terms of characterization, calculation of the thermodynamic properties and the synthesis of Sp-NHMAP and its effective use in the removal of heavy metals in wastewater.

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Identification of Sainfoin Species (*Onobrychis* spp.) from Morphologic Characters and Flower Colour by L*a*b*, RGB and HSV Colours by Principle Component Analysis

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Abstract

The aim of this study, to determine similarities/dissimilarities of *Onobrychis* species for morphologic characters (flower diameter, days to flowering, main stem length and width, number of stem, leaflet length and width), and colour values (L*, a*, b*, R, G, B, H, S and V) by using principle component analysis. Plant materials, belonging to *Onobrychis* species; *Onobrychis hypargyrea* Boiss. (O_1), *Onobrychis viciifolia* Scop. (O_2), *Onobrychis montana* subsp. *cadmea* P.W.Ball (O_3), *Onobrychis armena* Boiss. (O_4), *Onobrychis gracilis* Besser (O_5), *Onobrychis hajastana* Grossh. (O_6), *Onobrychis lasiostachya* Boiss. (O_7) *Onobrychis oxyodonta* Boiss. (O_8) and *Onobrychis podperae Širj.* (O_9) were collected from different locations (grassland, arable area and forest area) in Eskişehir province. According to these explanations; morphologic characters, main stem length, leaflet width and days to flowering; Lab colours, L and b; RGB colours, R and B, HSV colours, H and S were determined as best contributors. *Onobrychis* species, O_3, O_4 and O_9; morphologic characters, main stem length and width leaflet length and width; colours; L, b, R, B S and V were determined as stabile species/characters. *Onobrychis viciifolia* Scop. (O_2) with *Onobrychis hajastana* Grossh. (O_6) and *Onobrychis montana* subsp. *cadmea* P.W.Ball (O_3) with *Onobrychis lasiostachya* Boiss. (O_7) were found as similar species.

Keywords: *Onobrychis* spp., fodder crops, morphologic characters, Lab, RGB, HSV colours, principal component analysis.

INTRODUCTION

Sainfoin (*Onobrychis sp.*), consisting of the combination of the French words sain and foin, comes to mean as healthy food [36]. Sainfoin (*Onobrychis sp.*), cultivated since ancient times, is a common crop in the Near East flora including Turkey that is also one of the micro gene centres for sainfoin, alfalfa, lentil, vetch etc [9; 17; 18; 20; 23; 24; 29]. Sainfoin with large number of erect or semi-erect plant structure, is known perennial crop, and grows a length of 30-60 cm under normal conditions and 90 cm in good soil conditions. The sainfoin flower as a typical leguminous flower is raceme, having pink colours of the petals. There are dark pink shorts on the flag corolla leaf; the corolla came from five crown leaves with bright rose-red colour and dark coloured lines. Because of foreign pollination, flowers attract bugs [1; 16; 29; 39-41]. These genetic resources in sainfoin consisting of a number of species, and adapted to various environmental conditions such as temperature, precipitation, drought, salinity, diseases and pests, are very rich in genetic diversity [2; 19; 37]. Sainfoin is an important plant for the evaluation of calcareous and arid soils, drought conditions [22; 38]. There are almost 900 leguminous plants in Turkey and 46 of them belong to *Onobrychis* species. There are many sainfoin species comprising commercially used cultivars and natural species having high potential potency. In sainfoin species; *Onobrychis hajastana* Grossh., *Onobrychis hypargyrea* Boiss., *Onobrychis oxyodonta* Boiss., *Onobrychis viciifolia* Scop., *Onobrychis podperae* Širj., *Onobrychis lasiostachya* Boiss., *Onobrychis montana* subsp. *cadmea* P.W.Ball, *Onobrychis armena* Boiss., *Onobrychis gracilis* Besser are natural plants of vegetation grow in most parts of areas in Turkey [10; 17].

The species and variety diagnosis in plants is very important in plant breeding and different methods are used for species identification. In flowering plants, especially in forage plants, flower colour is very important in terms of its structure and its content representing the plant. Therefore, variety or plant identification from flowers is very important in plant identification [1; 13; 30]. It is possible to make plant identification from the flower, and the flower colour gives an important clue to this. Many studies have revealed that flower colour, fleshy shape and structure have an important place in defining the plant [3; 6; 29]. In the same way, our study was based on diagnosis of sainfoin species with flower colours by using different colour methods. There are a lot of methods in the world about colours, and Lab, RGB and HSV colour measurement methods are the three of the most used methods. Lab colour medium is the system, comprising a 3-axis colour system, L for lightness and a and b for the colour dimensions. It consists of all colours in the spectrum. The Lab colour space shows means of colour spectrum and mostly used in different activities including agriculture. L assigns lightness from zero (no lightness) and 100 (maximum lightness). Other a shows positive values +60 red colour and negative values -60 green colour. The other b shows positive value positive values +60 yellow colour negative values -60 blue colour. RGB colour system creates all of colours from the combination of the red, green and blue colours. RGB is the image model revealing colour combination model. Colour densities vary from zero (black) to 255 (white) for each RGB (red, green, blue) component. 255 denotes pure white, and zero is pure black. The image is defined on the red, green, blue colour codes r, g, b. Each pixel receives intermediate values according to these colour codes. The HSV (Hue, Saturation, Value) colour space defines colours

as colour essence, saturation and brightness, respectively. S, saturation, determines the "vitality" of the colour. High saturation results in vibrant colours, while low likelihood causes the colour to approach grey shades. It changes within 0-100. V, the brightness, determines the lightness of the colour, that is, the white ratio within it. It varies from 0-100. H, hue, colour essence determines the dominant wavelength of the colour, for example yellow, blue, green, etc. It ranges from 0Â° to 360 Â°. The brightness determines the lightness of the colour, that is, the white ratio within it. Both Lab, RGB and HSV methods can be used safely and reliably in determining the colour characteristics of an object [7; 25; 44]. On the other side, flower colour and flower characteristics are the way of recognizing plants in forage plants [1; 3; 6; 13; 29; 30]. From here, plant identification can be made according to flower colours using these methods. The aim of this study, to determine similarities/dissimilarities of *Onobrychis* species for morphologic characters (flower diameter (cm), days to flowering, main stem length and width (cm), number of stem, leaflet length and width (cm)), and colour values (L, a, b, R, G, B, H, S and V) by using principle component analysis in sainfoin species.

MATERIALS AND METHOD

The study was carried out in the field of Transitional Zone Agricultural Research Institute in Eskişehir province in 2015-1016. Plant materials, belonging to *Onobrychis* species; *Onobrychis hypargyrea* Boiss. (O_1), *Onobrychis viciifolia* Scop. (O_2), *Onobrychis montana* subsp. *cadmea* P.W.Ball (O_3), *Onobrychis armena* Boiss. (O_4), *Onobrychis gracilis* Besser (O_5), *Onobrychis hajastana* Grossh (O_6), *Onobrychis lasiostachya* Boiss. (O_7), *Onobrychis oxyodonta* Boiss. (O_8) and *Onobrychis podperae Širj.* (O_9) were collected from different locations (grassland, arable area and forest area) in Eskişehir province. The seeds of total 387 plants were germinated in pots in glasshouse, seedlings of sainfoin species were transferred to field as a single plant in 1 m x 1 m plot size (10 plants in each row) and allowed to grow. Soil characteristics of experimental area were; 7,16 pH, 1,12 EC (dS/m), 0,075 total salt (%), 2,03 lime (%), 1,62 Organic Matter (%), 97,21 Phosphorus (P_2O_5, kg/ha) and 742,23 (Potassium, K_2O). Fertilizers as 30 kg/ha and 60kg/da P_2O_5 were applied before plants were transferred to field to allow plant growth. Precipitations and average temperature were 328,4 mm and 8,2 °C in 2015-2016 and 421,3 mm and 8,9 °C in long-term years (1970-2016). The map, showing *Onobrychis*-genotype-gather locations was given in Figure 1.

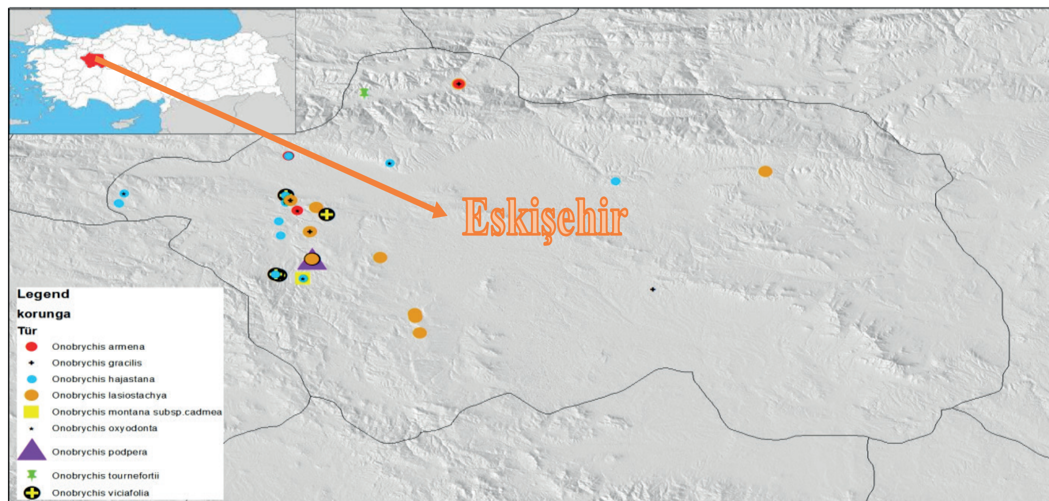

Figure 1. Locations related to gathering areas of *Onobrychis* species in Eskişehir province.

Flower diameter (cm), days to flowering, main stem length and width (cm), number of stem, leaflet length and width (cm) as morphologic characters [5; 6]; L, a, b, R, G, B, H, S and V as colour values [12; 32; 43; 45] were measured. Both morphologic characters and colour values were taken when plants reached flowering stages. Colour values were measured from corollas of flowers on medium with light intensity of 550-600 mmol / m^2 in *Onobrychis* species by Mobile Colour Grab Program for R, G, B, H, S and V colour measurements. Konica Minolta Cr-5 Model Colour Measurement Device for L, a b was used. Colour values and morphologic parameters were taken with two and five replicates, and means of replicates were evaluated and analysed. Analyses of the data obtained to reveal the similarities/differences of *Onobrychis* species were evaluated in the MINITAB 17 package program.

RESULTS AND DISCUSSION

Sainfoin is a perennial fodder crop in the leguminous family and could be cultivated for 5-6 years. It shows well grow and adaptation to arid conditions/climates. It is also very promising plant to help the meeting the animal feed demand in arid conditions. Protein is rich and the quality of the bait is good, the sapwood is rich in calcium, phosphorus and other mineral substances. It can be used as a good pasture plant because it is resistant to grassing. Sainfoin can also be fed to the animals with fresh, dry grass or silage. When dairy cattle are given a certain amount of sainfoin every day, digestive system disorders are rarely encountered. It ensures the growth of all animals, increases their yield. Besides, sainfoin is a very good honey-making plant in the bees because it gives plenty of flowers [19; 37; 39; 40]. Demand in animal foods geometrically increases, so has world population. To help to meet demands in animal food, it is vital to increase the use of high yielding fodder crops including sainfoin. This phenomenon could only be accomplished by

successful breeding programs in which vast and rich genotypic variations are used. Definition of plants, determination of their distinguishing characteristics will help to determine different plant genotypes in breeding programs [5; 6]. The flower type and colour are different for each sainfoin type. By using this feature, it is possible to distinguish the sainfoin species and the sainfoin can be successfully distinguished from the flower type and colour characteristics [37]. The flower type and colour characteristics of *Onobrychis* species were given in Figure 2.

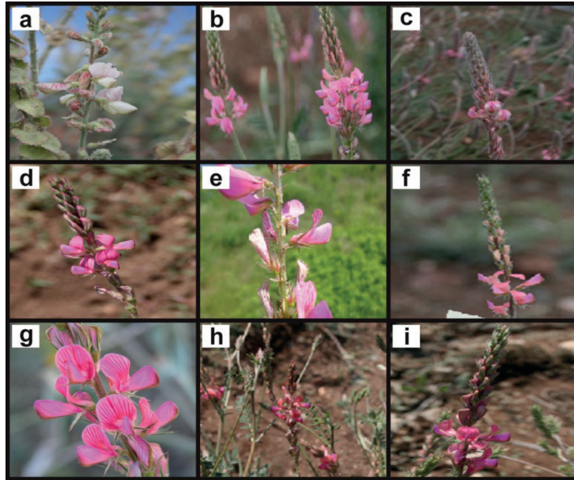

Figure 2. The flower type and colour characteristics of *Onobrychis* species (**a:** *O. hypargyrea* Boiss., **b:** *O. viciifolia* Scop., **c:** *O. montana* subsp. *cadmea* P.W.Ball, **d:** *O. armena* Boiss., **e:** *O. gracilis* Besser, **f:** *O. hajastana* Grossh., **g:** *O. lasiostachya* Boiss., **h:** *O. oxyodonta* Boiss., **i:** *O. podperae Širj.*).

Onobrychis hypargyrea **Boiss. (O_1)** is a multi-year herbaceous crop, growing environment and altitude: rocky and calcareous slopes, grassland, step forest clearance, resistant to cold and drought. It could be used as green fertilizer because it fixes nitrogen to the soil. In deserted areas and in hilly areas such as slopes, seeding enriches the soil by preventing desertification and erosion. Flowers attract bouquets and birds and increase biodiversity [14; 35; 42]. ***Onobrychis viciifolia*** **Scop. (O_2)** is perennial, erect and the most cultivated crop in the world. This crop could be recognized with bright pink flowers, 50-50 cm crop height and 5-8 paired leaflets. Though it has lots of cultivated and released types, native types could be seen in fallow areas, roadsides, sandy, clay soils. It grows in 200-2000 altitude in most parts of Anatolia [1; 6; 26]. If sainfoin seeds are contaminated with bacteria nitrogen is added to the soil by nodosity bacteria. Sainfoin could be fed to the animals green or dry. It is an important fodder crop in terms of its high mineral content [4]. ***Onobrychis montana*** **subsp. cadmea P. W. Ball (O_3)** is a herbaceous multi-year-old plant known as hemicryptophyte, the majority of which are dead buds, and whose body surface is located on the soil, so that it is not damaged in the cold cycle. Sainfoin is ideal plant for ruined mine sites in terms of soil amelioration etc. It binds nitrogen to the soil to provide nitrogen reserves, it can be used as green fertilizer because it fixes nitrogen. Sainfoin enriches the soil by preventing desertification and erosion. Flowers attract bees and birds and increase biodiversity [42]. **O*nobrychis armena*** **Boiss. (O_4)** is a species widely found in the arid and salty areas of Anatolia where rainfall is low. In these adverse climatic and geographic conditions. *Onobrychis armena* Boiss., winter resistant, is a preferred plant in arid conditions compared to other plants. *Onobrychis armena*, winter resistant, is a preferred plant in arid conditions compared to other plants in meadows of Central Anatolia and Eastern Anatolia.

Onobrychis gracilis **Besser (O_5)** is perennial plant with herbaceous body that is vertical or raised, 40-70 cm hairless. It has 5-8 pairs of leaves and leaflets are linear. Having pink colour, the flower is narrow and long. This plant is found in temperate areas and up to 1500 altitude, and could not show well growth over this altitude. *O. gracilis* is not as resistant as other *Onobrychis* species against drought, cold and over grazing [1; 35; 42]. ***Onobrychis hajastana*** **Grossh. (O_6)** is well adapted to grows stony and limy-clay soils, in steppes or sloppy arid pastures of Eastern Anatolia having more than 1500 m altitude. It is also perennial and endangered crop having prostrate, semi prostrate and semi erect types, almost 60 cm stem height, 6-8 paired leaflets, it is resistant to crushing, drought grazing and flowers from June to August [31]. ***Onobrychis lasiostachya*** **Boiss. (O_7)** is a perennial with 80-120 cm plant height and a pale pink form of flower. This plant has a wide adaptation ability including Central and Eastern Europe, Turkey, the Caucasus and the southern territory of Siberia. This plant, which is very resistant to drought, cold and frost, can grow in sandy and clayey soils, rocky regions, shortly all kinds of soil types. [1; 8; 11; 26]. ***Onobrychis oxyodonta*** **Boiss. (O_8)** is perennial crop with erect, semi erect and medium crop types, 50-60 cm crop height, 5-8 paired leaflets. It grows lime steppes, fallow areas, sandy soils with fallow fields, Cedrus-Pinus woods, sandy slopes, 400-2000 m altitude in many parts of Anatolia. It flowers July, august with pink, dark pink flower [4; 33]. ***Onobrychis podperae*** *Širj.* **(O_9)** is perennial plant with subterranean substructure. The body of the vertical structure can be extended up to 20-60 cm and has a clear light green line. The base leaves are 5-10 pairs and the upper leaves 3-9 pairs. With pink colour, the flower state is sparse, very flowering and lengthening in the fruit period. This plant grows in altitudes of 300-1300 m consisting of limestone rocks, slopes and steppes. It is resistant to drought and could be seen in Turkey, Iran, Russia etc. [21; 46].

Correlation is a statistical method used to determine if there is a linear relationship between two numerical measurements, and if so, what the direction and severity of this relationship is. If the correlation coefficient is negative, there is an inverse relationship between the two variables, that is, one of the variables is increasing while the other is decreasing [15; 27]. Maximum, minimum values and means in Morphologic parameters and colour values of *Onobrychis* species were given in Table 1.

Table 1. Maximum, minimum values and means in Morphologic parameters and colour values of *Onobrychis* species.

	Flower Colour	Variable	Mean	Minimum	Maximum
***Onobrychis hypargyrea* Boiss. (O_1)**	Careys pink	**Flower Dia.**	102,6±31,80	35,01	122,91
***Onobrychis viciifolia* Scop. (O_2)**	Pink flare	**Days to Flow.**	140,91±2,51	138,0	147,22
***Onobrychis montana* subsp. *cadmea* P. W. Ball (O_3)**	Pink flare	**Main St.Len.**	57,67±5,07	44,60	60,84
***Onobrychis armena* Boiss. (O_4)**	Pale violet red	**Main St.Wİd.**	0,77±0,21	0,40	1,06
***Onobrychis gracilis* Besser (O_5)**	Vanilla ice	**No.Stem**	34,63±1,16	32,32	36,37
***Onobrychis hajastana* Grossh. (O_6)**	Pink	**Leaflet Le.**	7,22±0,28	6,70	7,58
***Onobrychis lasiostachya* Boiss. (O_7)**	Pale violet	**Leaflet Wid.**	2,88±0,98	1,20	4,21
***Onobrychis oxyodonta* Boiss. (O_8)**	Indian red	**L**	49,50±7,68	44,64	69,46
***Onobrychis podperae* Širj. (O_9)**	Pale violet red				

Variable	Mean	Minimum	Maximum	Variable	Mean	Minimum	Maximum
a	17,86±6,80	1,76	23,32	**b**	163,34±44,92	96,00	232,00
b	9,47±5,60	3,29	20,64	**H**	294,36±109,64	4,0	348,0
R	221,67±13,12	194,00	240,00	**S**	38,33±18,55	14,00	62,00
G	123,6±38,60	72,0	187,0	**V**	85,89±6,85	70,00	94,00

Correlations between morphologic characters and colour values in *Onobrychis* species were given in Table 2. Negative/positive significant correlations between characters are determined as; **negative and significant at 5 %/1 %:** between days to flowering and a, days to flowering and H, number of stem and a, L and a, L and H, a and b, b and H, G and S, B and S.

Table 2. Correlations between morphologic characters and colour values in *Onobrychis* species.

	Flower Dia.	Days to Flow.	Main St.Len.	Main St.Wİd.
Days to Flow.	-0,185ns			
Main St.Len.	0,731*	0,518ns		
Main St.Wİd.	0,608ns	0,231ns	0,776*	
No.Stem	0,282ns	0,751*	0,709*	0,390ns
Leaflet Le.	0,688*	0,172ns	0,747*	0,663*
Leaflet Wid.	0,655*	0,130ns	0,701*	0,784*
L	-0,489ns	0,923**	0,210ns	0,026ns
a	0,313ns	-0,908**	-0,347ns	-0,135ns
b	-0,086ns	0,838**	0,494ns	0,331ns
R	0,125ns	-0,195ns	-0,020ns	0,123ns
G	-0,063ns	0,393ns	0,242ns	0,097ns
B	0,461ns	0,153ns	0,514ns	0,437ns
H	0,473ns	-0,947**	-0,246ns	-0,074ns
S	-0,134ns	-0,338ns	-0,398ns	-0,403ns
V	0,031ns	-0,150ns	-0,087ns	0,002ns

	No.Stem	Leaflet Le.	Leaflet Wid.	L
Leaflet Le.	0,202ns			
Leaflet Wid.	0,315ns	0,887**		
L	0,517ns	0,019ns	-0,001ns	
a	-0,686*	-0,088ns	-0,071ns	-0,916**
b	0,697*	0,285ns	0,307ns	0,788*
R	-0,038ns	-0,119ns	-0,021ns	-0,123ns
G	0,122ns	0,025ns	-0,117ns	0,399ns
B	0,202ns	0,473ns	0,367ns	0,089ns
H	-0,556ns	0,016ns	0,036ns	-0,989**
S	-0,080ns	-0,471ns	-0,362ns	-0,393ns
V	-0,018ns	-0,207ns	-0,120ns	-0,056ns

	a	b	R	G
b	-0,929**			
R	0,124ns	-0,148ns		
G	-0,257ns	0,018ns	0,492ns	
B	-0,197ns	0,259ns	0,724*	0,532ns
H	0,919**	-0,797*	0,160ns	-0,392ns
S	0,384ns	-0,335ns	-0,584ns	-0,706*
V	0,060ns	-0,110ns	0,989**	0,494ns

	B	H	S
H	-0,057ns		
S	-0,862**	0,340ns	
V	0,692*	0,098ns	-0,556ns

Positive and significant at 5 %/1 %: between flower diameter and main stem length, flower diameter and leaflet length, flower diameter and leaflet width, days to flowering and number of stem, days to flowering and L, days to flowering and b, main stem length and main stem width, main stem length and number of stem, main stem length and leaflet length, main stem length and leaflet width, main stem width and leaflet length, main stem width and leaflet width, number of stem and b, leaflet length and leaflet width, L and b, a and H, R and B, R and V, B and V. Yield components are important in investigating high yielding genotypes and increase the success of breeding programs. Determining correlations between yield components helps to understand relationship between components and what effects yield components in *Onobrychis sativa* L. [1; 28; 34]. Moreover, relationship between plant characters could be explained by different statistical methods and principle component analysis analyse mutual effects of characters and reduce these relations in characters to lesser components and determines the factor structure formed by variables. This method determines the variance explained by the sum of each of the factors and their factors. In other word, this method provides recognition, classification, size reduction and interpretation. Table 3 showed eigen values and cumulative variances of morphologic characters and colour values by principal component analysis in *Onobrychis* species.

Table 3. Eigen values and cumulative variances of morphologic characters and colour values by principal component analysis in *Onobrychis* species.

Eigen analysis of the Correlation Matrix for Morphologic Characters					
	PC_1	**PC_2**	**Days to Flowering**	**PC_1**	**PC_2**
Eigenvalue	4,2482	1,6672	**Main Stem Length**	0,189	0,679
Proportion	0,607	0,238	**Main Stem Width**	0,464	0,138
Cumulative	0,607	0,845	**Number of Stem**	0,416	-0,087
Variable	**PC_1**	**PC_2**	**Leaflet Length**	0,414	0,542
Flower Diameter	0,371	-0,342	**Leaflet Width**	0,425	-0,217
Eigen analysis of the Correlation Matrix for Lab Colour					
	PC_1	**PC_2**	**Variables**	**PC_1**	**PC_2**
Eigenvalue	2,7567	0,2119	**L**	0,566	0,725
Proportion	0,919	0,071	**a**	-0,596	0,032
Cumulative	0,919	0,990	**b**	0,569	-0,687
Eigen analysis of the Correlation Matrix for RGB Colour					
	PC_1	**PC_2**	**Variables**	**PC_1**	**PC_2**
Eigenvalue	2,1719	0,5549	**R**	0,596	-0,433
Proportion	0,724	0,185	**G**	0,526	0,847
Cumulative	0,724	0,909	**B**	0,607	-0,309
Eigen analysis of the Correlation Matrix for HSV Colour					
	PC_1	**PC_2**	**Variables**	**PC_1**	**PC_2**
Eigenvalue	1,6112	1,0870	**H**	0,307	0,856
Proportion	0,537	0,362	**S**	0,728	0,071
Cumulative	0,537	0,899	**V**	-0,613	0,512

In morphologic characters and Lab colour; first factor showing the highest eigenvalues, covered almost 4,2482-60,7 % and 2,7567- 91,9 % of the total variance, respectively. The second factor explained eigenvalues of 1,6672-23,8 % and 0,2119-7,1 %, respectively. In morphologic characters, main stem length (0,464), leaflet width (0,425) in Factor 1, days to flowering (0,679) in Factor 2 had higher contribution. In Lab colour, L (0,566) and b (0,569) in Factor 1 gave higher contribution. In RGB and HSV colours; the first factor showed the highest eigenvalues as 2,1719-72,4 % and 1,6112-53,7 % of the total variance, respectively. The second factor assigned eigenvalues of 0,5549-18,5 % and 1,0870-36,2 %, respectively. In RGB colour, R (0,596) and B (0,607) in Factor 1 showed higher contribution. In HSV colour, S (0,728) in Factor 1 and H (0,856) in Factor 2 gave higher contribution. Main stem length, leaflet width and days to flowering in morphologic characters; L and b in Lab colour were found as the best contributors. R and B in RGB colour, H and S in HSV colour were found as best contributors (Table 3). A biplot graph representing a part of the principle component analysis was given in Figure 3.

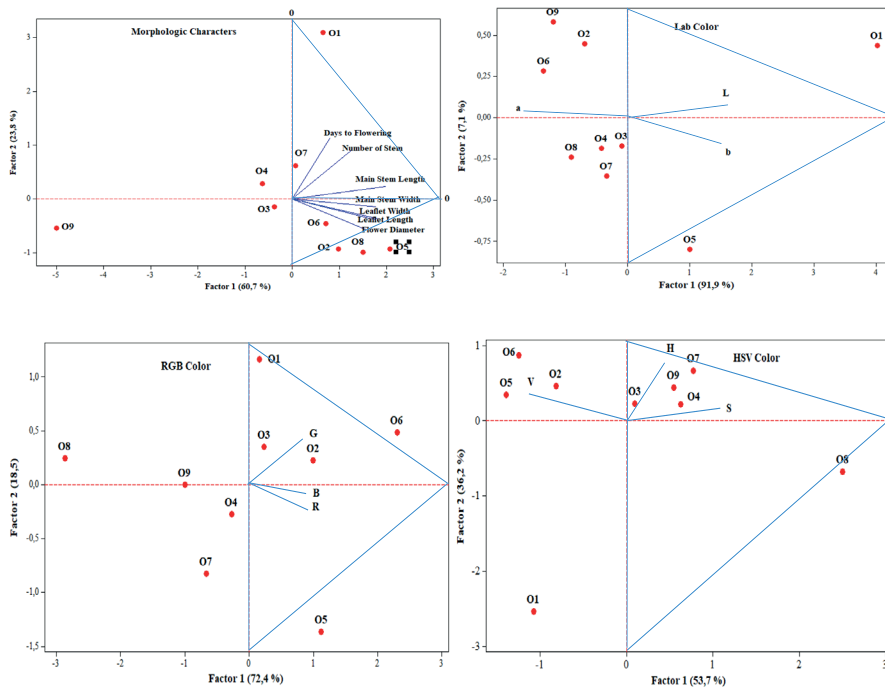

Figure 3. Biplot graph representing relationship between *Onobrychis* species, morphologic characters and colour values.

In morphologic characters, days to flowering and number of stem created in one group, while flower diameter, main stem length and width, leaflet length and width participated same group. O_3, O_4 and O_7 were in same group; O_2, O_6, O_5 and O_8 joined in same group. O_1, O_5 and O_9 have formed separate, singular groups (Figure 3). In lab colour, two groups occurred; L an b in one group and a in the other. O_3, O_4, O_7 and O_8 had same group, while O_2, O_6 and O_9 created one group. O_1 and O_5 were alone. *Onobrychis* species and R, G, B, H, S, V colours drew different behaviours in RGB and HSV analyses. B and R were in same group, while G was alone. O_1, O_2, O_3 and O_6 participated in same group, were O_4, O_7 and O_9 were found in one group. O_5 and O_8 were alone in RGB colour. In HSV colour, O_3, O_4, O_7 and O_9 joined in same group; while O_2, O_5, and O_6 created one group. O_1 and O_8 were alone (Figure 3).

According to these explanations; morphologic characters, main stem length, leaflet width and days to flowering; Lab colours, L and b; RGB colours, R and B, HSV colours, H and S were determined as best contributors (Table 3). *Onobrychis* species, O_3, O_4 and O_9; morphologic characters, main stem length and width leaflet length and width; colours; L, b, R, B, S and V were determined as stabile species/characters (Figure 3). A model denoting similarities/dissimilarities of *Onobrychis* species for morphologic characters and colour values was shown in Figure 4.

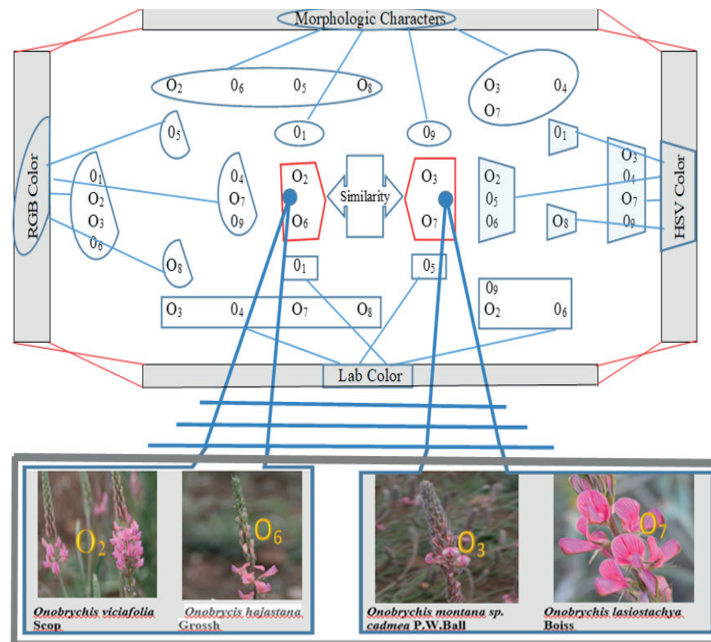

Figure 4. A model denoting similarities/dissimilarities of *Onobrychis* species for morphologic characters and colour values.

Similarities/dissimilarities of *Onobrychis* species; *Onobrychis* species; *Onobrychis hypargyrea* Boiss. (O_1), *Onobrychis viciifolia* Scop. (O_2), *Onobrychis montana* subsp. *cadmea* P.W.Ball (O_3), *Onobrychis armena* Boiss. (O_4), *Onobrychis gracilis* Besser (O_5), *Onobrychis hajastana* Grossh. (O_6), *Onobrychis lasiostachya* Boiss. (O_7), *Onobrychis oxyodonta* Boiss. (O_8) and *Onobrychis podperae Širj.* (O_9) were evaluated for morphologic characters and colour values in the model. *Onobrychis viciifolia* Scop. (O_2) with *Onobrychis hajastana* Grossh. (O_6) and *Onobrychis montana* subsp. *cadmea* P.W.Ball (O_3) with *Onobrychis lasiostachya* Boiss. (O_7) were found as similar species.

Sainfoin (*Onobrychis* spp.) is one of the most valuable plants among leguminous fodder crops. With its high protein content and durability to drought, the sainfoin with its wide range of richness has an important potential to close off the increasing animal feed need in the future. Therefore, revealing the similarities/dissimilarities of the *Onobrychis* species in terms of different characteristics will enhance the success of sainfoin breeding programs. The use of different light reflectance values in addition to the morphological properties will contribute to this distinction of *Onobrychis* species.

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