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Discovering Thoughts, Inventing Future

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Role of Hepatitis B Virus Surface Antigen Quantification in E Antigen Negative Chronic Hepatitis B Infection

By R. M. Mukherjee, Jyothi Arava, P. Balkumar Reddy, P. N. Rao, R. Gupta & D. N. Reddy

Asian Health Care Foundation, India

Abstract- *Background/Aim:* Hepatitis B surface antigen(HBsAg) is routinely detected qualitatively in Hepatitis B Virus(HBV) infection where its persistence beyond 6 months defines chronic hepatitis B(CHB) infection. Hepatitis B e antigen (HBeAg) usually indicates active HBV replication and risk of transmission of infection. In spite of the emerging use of chemiluminescence based quantitative HBsAg assays for therapeutic monitoring of the patients, we measured HBsAg concentration by sandwich ELISA as a cost effective alternative to ascertain the role of HBsAg levels as surrogate marker of untreated HBeAg negative subjects having CHB infection.

Keywords: *HBsAg, quantification, ELISA, viral load, HBeAg negative, CHB infection.*

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Role of Hepatitis B Virus Surface Antigen Quantification in E Antigen Negative Chronic Hepatitis B Infection

R. M. Mukherjee [α], Jyothi Arava [σ], P. Balkumar Reddy [ρ], P. N. Rao [ω], R. Gupta [¥] & D. N. Reddy [§]

Abstract- *Background/Aim:* Hepatitis B surface antigen (HBsAg) is routinely detected qualitatively in Hepatitis B Virus (HBV) infection where its persistence beyond 6 months defines chronic hepatitis B(CHB) infection. Hepatitis B e antigen (HBeAg) usually indicates active HBV replication and risk of transmission of infection. In spite of the emerging use of chemiluminescence based quantitative HBsAg assays for therapeutic monitoring of the patients, we measured HBsAg concentration by sandwich ELISA as a cost effective alternative to ascertain the role of HBsAg levels as surrogate marker of untreated HBeAg negative subjects having CHB infection.

Methods : Sixty two subjects were evaluated for serum HBeAg,anti HBe and ALT status by standard ELISA and biochemical procedures.The amount of serum HBV DNA determined by real time TaqMan PCR assay(Roche Diagnostics,USA).Serum HBsAg level(ng/ml) was ascertained by a third generation sandwich ELISA kit(Alpha Diagnostics International,USA)and results expressed upon conversion to IU/ml.

*Results :*The median age of the subjects was 40.5 years(IQR=18;90% male) of which 92% were HBeAg negative and anti-HBe positive.Median ALT value was 35.5 IU/ml(IQR=37).Median viral load(Log copies/ml)and HBsAg(IU/ml) were 4.57(IQR=2.84)and 38002.3 (IQR=23736.8) respectively. When grouped on the basis of viral load(<2000 IU/ml>),the median HBsAg concentration seen is 35439.3 (IQR=43093.3) and 44712.7(IQR=25082.4) while median ALT values were 29(IQR=21.2)and 47(IQR=73.2) in lower(<2000 IU/ml) and higher(>2000 IU/ml)viral load groups respectively.

Conclusion : Serum HBsAg concentration showed no correlation with serum HBV DNA level in our study subjects which is at par with other studies on CHB patients. Based on the reported correlation between serum HBsAg level and intrahepatic ccc DNA, higher amount of HBsAg in subjects having lower viral load is indicative of the presence of higher amount of intrahepatic HBV DNA in these subjects which warrant further study. Quantitation of HBsAg by ELISA can be a cheaper alternative to chemiluminescence assay to be used as a surrogate marker differentiating certain phases of CHB infection.

Keywords: HBsAg, quantification, ELISA, viral load, HBeAg negative, CHB infection.

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I. Introduction

The prevalence of Hepatitis B virus (HBV) infection in terms of the prevalence of Hepatitis B surface antigen (HBsAg) in the general population of Asia, Africa, Southern Europe and South America ranges from 2 to 20%.(1).While the persistence of HBsAg for more than 6 months defines chronic HBV state,its clearance from serum ,is considered the nearest-to-cure outcome of HBV infection. Hepatitis B e antigen (HBeAg) usually indicates active HBV replication and risk of transmission of infection. Although seroconversion from HBeAg to anti-HBe is usually associated with remission of liver disease, a certain proportion of anti-HBe-positive patients being harboring precore/core promoter mutations, continue to have viral replication with ongoing progression of the disease. Thus, studies regarding the prevalence and clinical significance of HBeAg-negative chronic hepatitis B (CHB) have been increasingly gained importance. Despite the usefulness of the routine qualitative detection of HBsAg as the first serological marker of the disease, recent studies indicate the importance of quantitative kinetic measurement of HBsAg for therapeutic monitoring particularly for the patients who are negative for HBeAg (2,3). HBsAg quantification is easy, cheap and may provide a mean to establish the prognosis of antiviral therapy in the future. moreover, HBsAg quantification appears to be a surrogate marker of the amount of covalently closed circular DNA (cccDNA), the persistent intrahepatic form of HBV DNA in the hepatocytes and a predictor of a sustained virological response to antiviral treatment off therapy(4,5). Despite the added clinical importance of HBsAg clearance, this has not been included as a primary endpoint in treatment trials due to the low frequency of its occurrence. The majority of nucleoside analogue studies, even with prolonged therapy, have demonstrated rates of HBsAg clearance comparable to those observed naturally i.e. between 1%-2% and 0.5%-1% annually for HBV carriers in western and Asian countries respectively (6, 7).Based on the above fact, one can envision scenarios where measuring HBsAg concentration could be an important, additive tool to currently used HBV DNA testing. The development of commercial assay systems particularly

the assay based on chemiluminescence (Architect, Abbott Diagnostics, USA) which has been used so far to quantify HBsAg concentration, provided an opportunity to compare the kinetics of HBsAg decline during interferon and nucleoside analogue therapy(2,3,5).In the present study, in order to ascertain the role of quantitative measurement of serum HBsAg for use as surrogate marker to viral load and ALT levels ,we attempted to evaluate HBsAg level in treatment naïve CHB patients with special reference to HBeAg negative anti HBe subjects. Due to non availability of a chemiluminescence platform (Architect, Abbott Diagnostics) for the said assay, we used a third generation quantitative sandwich ELISA kit(Alpha Diagnostics International, USA) as a cheaper alternative to measure HBsAg concentrations in our study subjects representing inactive HBsAg carrier state and CHB infections (8).

II. Materials and Methods

a) Subjects

A total of 62 subjects who attended the out patient's department of the Asian Institute of Gastroenterology, Hyderabad, India and were positive for HBsAg for more than six months were included in this study. Apart from the recording of demographic patterns and respective clinical investigations, five milliliter of blood was taken from each study subject to assess the various biochemical, serological anad virological parameters. Informed consent was taken from each study subjects prior commencement of the study.

b) Serological and Biochemical parameters

Serum HBeAg and anti HBe and status of the study subjects were determined by commercial ELISA kits(Amar-EASE, Taiwan) as per manufacturer's instructions. ALT value was ascertained by automated biochemistry analysis system (RandoxUSA).

c) Quantitation of serum HBV DNA

Extraction of viral DNA from patient's sera was brought out using High Pure System Viral Nucleic Acid kit(Roche Molecular Systems, NJ, USA) as per the manufacturer's instructions. Amplification and subsequent quantification of extracted DNA were performed by the COBAS® TaqMan® HBV Test using a COBAS® TaqMan® 48 Analyzer(Roche Molecular Systems, NJ, USA) which has a lower limit detection of 6 IU(35 copies) of HBV DNA/ml.

d) Quantitation of serum HBsAg

Serum concentration of HbsAg was measured by a sandwich ELISA kit(Alpha Diagnostic International, USA)as per manufacturer's instructions. The aforesaid kit meets the requirements for a 3rd generation test upon testing against the FDA reference panel. Performance of this kit compared with a commercial test kit (Abbot Auszyme II) and supposed to detect all possible combinations of HBsAg subtypes(adw, adr, ayw, ayr)having a lower limit of sensitivity of 0.3 ng/ml as per manufacturer's declarations..For convenience, HBsAg concentrations measured in ng/ml was converted to IU/ml considering 0.15ng/ml of HBsAg is equivalent to 350IU/ml(9).

e) Statitical analysis

Descriptive statistics (mean,median,standard deviations and interquartile ranges), Student's t test and Fisher's exact tests were performed as and where applicable using SPSS software ,version 13.0 (SPSS, Inc., Chicago, IL). A value of $P<0.05$ was considered statistically significant.

III. Results

Baseline features of the 62 study subjects are depicted in Table 1. The median age of the subjects was 40.5 years(IQR=18).Majority(90%)were male and most of them(92%)were already seroconverted from HBeAg positive to HBeAg negative status with concomittant anti-HBe positivity. The median ALT value noted was 35.5 IU/ml(IQR=37) while the median viral load(Log copies/ml) and HBsAg(IU/ml) concentrations were 4.57(IQR=2.84)and 38002.3 (IQR=23736.8) respectively. When grouped on the basis of viral load (<2000 IU/ml>),the median HBsAg concentration seen is 35439.3 (Range 815.5-111141;IQR=43093.3) and 44712.7(Range 723-111141; IQR=25082.4) while median ALT values were 29 (Range 10-184;IQR=21.2) and 47(Range 12-372;IQR=73.2) in lower (Group 1≤2000 IU/ml,n=32) and higher viral load (Group 2 ≥2000 IU/ml,,n=30) groups respectively(Fig.1).Higher ALT values observed with increasing viral load as evident from significant differences(p=0.017) of ALT values between the higher and lower viral load groups which was not reflected by the corresponding levels of HBsAg between these groups. In group 1(≤ 2000 IU/ml viral load),21.8% subjects had elevated ALT values (≥ 40 IU/ml, median=65.0) having median HBsAg level of 31757.9(log 4.50) IU/ml while in group 2(≥2000 IU/ml viral load), 36.6% subjects showed lower ALT values (≤ 40 IU/ml; median=32.0) with a median HBsAg concentration of 36441.2(log 4.56) IU/ml thus showing serum HBsAg concentration is independent of serum HBV DNA and ALT levels in these subjects.

IV. Discussion

Apart from its use as a routine diagnostic marker for HBV induced liver disease,the predominantly spherical pleomorphic particles of HBsAg are suggested to be more prevalent than complete virions of HBV(10) and this excess surface protein might have implications towards chronicity of the disease(11).The

estimation of HBsAg concentration in sera of HBV infected subjects started long back(12) and prior to the advent of nucleic acid testing, quantitation of HBsAg was found to be a significant tool for predicting the outcome of an acute HBV infection(13). Despite its reported usefulness in predicting therapeutic response to interferon(14),the labour intensive procedures of such quantitative measurements(15) hampered the wide spread application of such assays. Quantitative measurement of serum HBsAg based on ELISA and chemiluminescence microparticle assay(CIMA)has been used (16,17) which recently gained importance providing level of HBsAg as a potential marker for monitoring therapeutic responses(2,3).Furthermore, the role of serum HBsAg quantification in distinguishing inactive carriers from the subjects having active form of the disease along with observations on significant differences in median baseline serum HBsAg titres across the different phases of CHB has also been implicated (18,19). The serum level of HBV DNA is thus being revisited as a dynamic parameter in chronic HBV and now becoming a vital part of the pre-treatment evaluation and assessment of the efficacy of antiviral treatment. Measurement of serum HBV DNA is the common tool to monitor treatment response. While the level of circulating HBV DNA testing has been shown to be the strongest predictor of the development of cirrhosis and hepatocellular carcinoma (HCC) (20,21), non association of serum HBV DNA with the histologic activity in patients with HBV-related decompensated cirrhosis has also been reported(22). In spite of the conflicting reports (5,16,17) regarding the correlation between serum HBV DNA and serum HBsAg concentrations, we tried to evaluate the same in treatment naïve CHB patients. In our study,correlation between serum HBV DNA and serum concentration of HBsAg was not evident, a similar observation noted in other studies(16,17) while such correlation(canonical correlation)in small number of subjects being reported by others(5).Since the majority(90%)of our study subjects already seroconverted from HBeAg positive to negative status with associated anti HBe positivity, the differentiating level of HBsAg between HBeAg positive and negative group of patients could not be ascertained in this study. Genotype related variation of HBsAg levels have been documented where median values of 4.11(log10) IU/ml and 3.85(log10)IU/ml measured by CIMA were shown to be present in patients infected with genotypes A and D respectively(2). In our study, the median HBsAg concentration measured by ELISA was 4.63(log10) IU/ml in a setting where genotype D is predominant(23).Interestingly, quantitative measurement of serum HBsAg gained importance as a surrogate marker for viral covalently closed circular DNA (cccDNA) and intrahepatic HBV DNA which are belived to maintain the replicative form of HBV DNA hence maintain the chronic form of the disease. The occurrence of viral reactivation observed in a significant proportion of patients who appear negative for serum HBV DNA after combined treatment course of peginterferon and lamivudine(24) seem to reflect the inadequacy of serum HBV DNA to represent the level of covalently closed circular DNA (cccDNA) and total intrahepatic HBV DNA as ultimate predictor of sustained virological response (25). Changes in ccc DNA were correlated with a similar reduction in serum HBsAg titre measured by ELISA during adefovir treatment (26). Furthermore, serum HBsAg levels shown to correlate well with the cccDNA and intrahepatic HBV DNA where patients with lower baseline cccDNA, intrahepatic HBV DNA, and HBsAg level but not serum HBV DNA level tend to develop sustained virologic response to peginterferon and lamivudine combination therapy(4).In light of the above mentioned fact,and based on our data of HBsAg levels in lower and higher viral load groups,it can be stated that the subjects in our study, having higher levels of HBsAg though differ significantly by their serum HBV DNA concentrations but might harbor similar amount of ccc DNA/intrahepatic HBV DNA pool in their liver. Considering a serum viral load of > 10000 copies/mL (2000 IU/mL) is a strong risk predictor of HCC, independent of HBeAg status, ALT level and liver cirrhosis (21,27),monitoring of our patients having higher level of HBsAg but comparatively lesser amount of serum HBV DNA is warranted. Our data showed a number of patients having higher levels of serum HBsAg and trend of elevated ALT but lesser amount of serum HBV DNA, thus, support a potential role of quantitative estimation of serum HBsAg in differentiating consecutive phases of CHB (18) that reflect the natural course of HBV infection. Finally, in corroboration to our previous report (28),our study demonstrated that serum HBsAg quantification assay is unable to substitute serum HBV DNA estimation in treatment naïve CHB patients but can be used as a surrogate marker of disease manifestations. Furthermore, in comparison to the chemiluminescence based assays, this ELISA based quantitation of serum HBsAg can be used as a cheaper alternative to monitor the therapeutic responses in developing countries like India.

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Table 1 : Baseline Feature of the Study Subjects

Charasteristics	All patients(n=62)
Age(years);Mean(Range)	38.2± 13.9(14-78)
Sex(% male)	90.3
Serum ALT(IU/ml);Mean±SD (Range)	57.41± 60.7(10-372)
Serum HBV DNA(Log Copies/ml);Mean±SD (Range)	4.45± 2.13(1.54-8.80)
HBeAg –Ve/anti HBe +Ve (%)	92.0
HBeAg +Ve/anti HBe –Ve (%)	8.0
Serum HBsAg(IU/ml);Mean±SD (Range)	43032.81±32848.02 (723-111141)

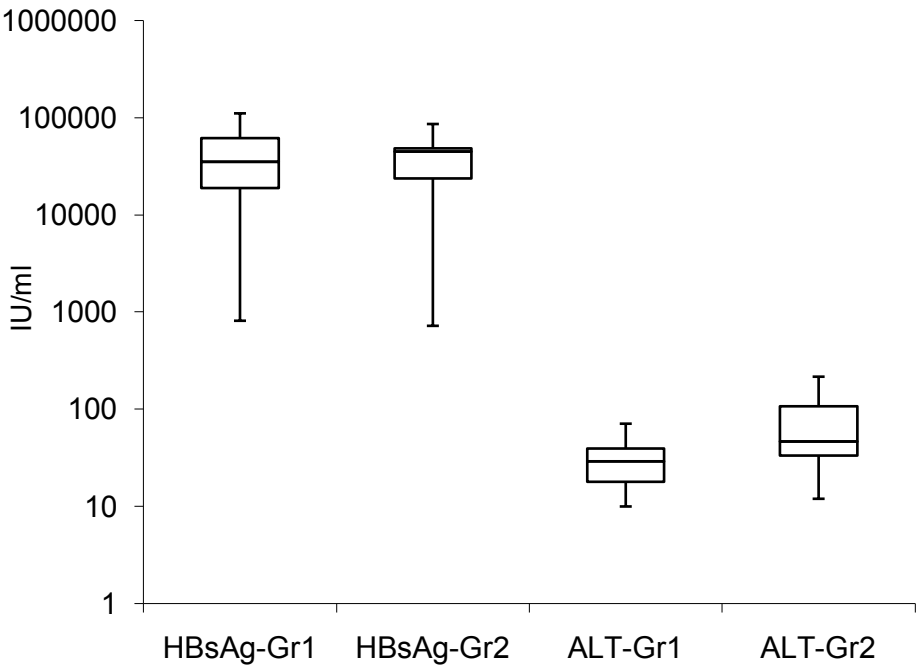

Figure 1 : HBsAg and ALT Values in Gr-1(n=32,HBV DNA ≤ 2000IU/ml) and Gr-2(n=30,HBV DNA ≥ 2000 IU/ml) Subjects(The boxes represent the interquartile ranges(IQR), the whiskers indicate the ranges and the center line in the box indicate median).

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The Relationship between Tumour Necrosis Factor -Alpha and Zinc/Copper Ratio in Iraqi Patients with Allergic Rhinitis

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Abstract- *Background:* Allergi rhinitis(AR) is a significant cause of widespread morbidity, high medical treatment costs,reduced work productivity,lowquality of life and can be associated with conditions such as fatigue, headache, cognitive impairment, and sleep disturbance.

Aim of the study: To find out the relation between tumour necrosis factor alpha (TNF-**α**) and Zn/Cu ratioin the patients with mild or moderate/severe cases.

Patients and Methods: Ninety subject were enrolled in this study .Fifty patients with AR were subdivided into two groups i.e. mild (comprising 20 patients) with AR , moderate/ severe (comprising 30 patients) with AR . Forty subjects who are apparently healthy were taken as a control group. Serum TNF-**α** was determind by using enzyme- linked immunosorbent assay (ELISA). Zn and Cu were determined by using colorimetric method.

Results: Serum levels of TNF-**α** was significantly higher in mild and moderate/severe groups compared with control group ($p<0.01$).A significant positive correlation between TNF-**α** and copper. A significant negative correlation between TNF-**α** and zinc in AR patients in mild and moderate/severe cases.

Conclusion: Zn/Cu ratio affect TNF-**α** level in patients with AR.

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The Relationship between Tumour Necrosis Factor -Alpha and Zinc/Copper Ratio in Iraqi Patients with Allergic Rhinitis

Tarek H. Abed Tawfik Al-Khyat [α], SafaaHussainAlturaihy [σ] & ZinahAbbass Ali [ρ]

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I. Introduction

Allergic rhinitis (AR) according to the document (Allergic Rhinitis and Its Impact on Asthma) ARIA is defined as a symptomatic disorder of the nose, induced after allergen exposure due to an immunoglobulin E (IgE)-mediated inflammation of the membranes lining the nose [1]. The classic symptoms of allergic rhinitis are rhinorrhoea, sneezing, nasal congestion and nasal itching [2]. ARIA classification of AR is according to, duration of symptoms: persistent and intermittent, severity of symptoms: mild, moderate/severe [3]. Mild AR (no impairment of, sleep, daily activities, leisure or sport, school or work and symptoms not troublesome). In Moderate/severe AR, One or more of the following criteria are present(sleep disturbance, impairment of daily activities, leisure and sport, impairment of school of work troublesome symptoms) [4]. An etiology of allergic rhinitis develop as a result of an IgE- mediated immune response to an inhaled allergen (allergens are antigens that induce and react with specific IgE antibodies). The allergic inflammatory cascade has three phases [5]. First (sensitization) in a susceptible person, initial contact with an allergen leads to the production of IgE antibodies against the allergen. These IgE antibodies bind to high-affinity receptors on mast cells and basophile [6]. Second (early-phase response)on further exposure to the allergen, sensitized mast cells are activated when two molecules of boundIgE are cross-linked by the allergen(antigen) [7]. Third (late-phase response) over the next few hours the nasal mucosa is infiltrated by other inflammatory cells (e.g. eosinophils, neutrophils, basophils, T-cells). These release further inflammatory mediators, producing a sustained inflammatory reaction which may persist for hours or days. The predominant late-phase symptom is nasal congestion [8] Allergic rhinitis can be caused by (Common causes) house dust mite, Pollens, animals Moulds (Less common) [9]. Previous studiefound tumor necrosis factor-alpha and interferon gamma, soluble inter cellular -1 in bronchia asthma and allergic rhinitis: relation with disease severity[10]. In this study, the relationship between TNF-alpha and Zn/Cu ratio was investigated in mild and moderate/severe cases of the disease.

II. Patients and Methods

This study was conducted in Babylon Maternity and PediatricTeaching Hospital and in the laboratory of Biochemistry Department, College of Medicine, University of Babylon in the period starting from December 2012 to May 2013.Fifty patients with AR. These selected patients were divided into two groups according to severity of disease.

The first: Mild group included 20 patients with mild AR, their age ranged between (20-25) years. Second moderate/severe group included 30 patients with AR. Full history was taken from all patient which include: age, residence, smoking, family history of allergic rhinitis, medical history drug history and surgical history, No drugs were prescribed to those patients thatmay interfere with the measured parameters. Fourty apparently healthy subject (who are age and sex-matched with the patients group were selected as a

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control group in the study. All control subject have no history of chronic disease (as diabetes mellitus, hypertension inflammatory disease such as rheumatoid arthritis) and not smoking. The statistical analysis was performed by using SPSS version 18 for windows. Data were expressed as Mean ± SD. The normality of the distribution of all variables was assessed by the Student's F-test and Pearson correlation analysis that have been used to determine the significant difference between the two groups. P values less than 0.05 is considered significant.

III. Results and Discussion

The results in (table-1) reveals a highly significant increase in the sera level of TNF-α in patients with allergic rhinitis in both mild and moderate/severe cases compared with those of control group(P1<0.01 and P2<0.01) as well as a highly significant increasebetween the mild and moderate/severe cases (P3<0.01). Such increase may be attributed to the activation of Th cellsleads to the production of various cytokines, such as interleukin IL-4, IL-5, interferon (IFN), and TNF-α (is a major cytokine produced by mast cells that upregulates endothelial and epithelial adhesion molecules). During the late phase, all these cytokines were recruited; with transendothelial migration and infiltration of activated T-cells, eosinophils, basophils, neutrophils and macrophages into the nasal mucosa[11]. TNF-α causes changes in the ionized calcium flux within smooth muscle. which lead consequently to the promotion of IgE production. IgE triggers the release of mediators that are responsible for arteriolar dilation, increased vascular permeability, itching, rhinorrhea and mucous secretions [10].Serum zinc showed a highly significant (p<0.01) decrease in moderate/severe AR compared with those of healthy subjects and highly significant (p<0.01)decrease observed between mild cases and control group.There was a significant increase between the mild and moderate/severe cases(p<0.05). Decreased zinc level in the sera of AR may be attributed to its depletion throughout its antioxidant action and its involvement immune response. Zinc plays a central role in the immune system affecting a number of aspects of cellular and humoral immunity [12]. Zinc was also shown to play an essential part in anti-apoptotic effect for respiratory epithelium besides its involvement in the structure of some antioxidant enzyme[13].Oxidative stress may lead to membrane instability causing the destructive events and histamine secretion from mastocytes that lead consequently to the pathogenesis of AR[14]. Anandaetal found that zinc supplementation prevents pulmonary pathology due to hyperoxia[15].

Membrane zinc concentrations are strongly influenced by dietary zinc status where zinc plays an important role in preserving membrane integrity through its binding to thiolate groups. Megha and Ratnesh reported that approximately twice as much zinc is absorbed from a non vegetarian or high meat diet than from a diet based on rice and wheat (which is the typical diet in developing countries) where usually a high phytate content that decrease zinc absorption[17].

A significant increase in copper level was observed in the sera of moderate/severe patients in comparison with control group (p<0.05) while a highly significant (p<0.01) increment in copper was seen in mild cases of AR patients compared with those of control group, and significant increase in mild comparison with moderate/severe cases (p<0.05). the highly significant increase in copper levels in the sera of patients with AR may be attributed to the reciprocal relationship between zinc and copper in normal physiology where zinc affect the absorption of Cu and vice versa. In addition the increase in oxidative process as reported by some researchers[18].From the above results, we can conclude that zinc supplementation is quiet essential for Iraqi people infected with AR in order to alleviate TNF-α levels which was shown to be a good marker in patients with AR especially when the latter was shown to increase with disease severity and a high significant correlation was found to exist between these parameter and TNF –α levels.

Table 1 : Biochemical parametrers of allergic rhinitis and controlGroups.

Parameter	Mild n=20	Moderate/ Severe n= 30	Control n=40	P values
TNF –αpg/ml Mean± SD Range	37.70±4.15 (34.7-50.1)	100.84±12.55 (90.2-124.1)	17.88±3.97 (13.70-25.2)	P1 < 0.01** P2< 0.01** P3< 0.01**
Copper µg/dL Mean± SD Range	132.54±6.70 (125.2-143.1)	165.6±13.04 (150.2-185.02)	96.56±7.79 80.02-101.4)	P1 < 0.01** P2< 0.05* P3< 0.05*
Zinc µg/dl Mean± SD Range	75.8±3.04 (70.01-78.40)	62.15±5.07 (45.1-66.40)	100.19±6.23 (90.70-110.1)	P1< 0.01** P2< 0.01** P3< 0.05*

Zn/Cu Ratio Mean± SD Range	0.67±0.098 (0.56-0.84)	0.36±0.028 (0.31-0.40)	0.95±0.03 (0.92-1.03)	P1 < 0.01** P2< 0.05* P3< 0.01**

P1= Mild VsControl P2 = moderate/severe VsControl P3 = Mild Vs Moderate/Severe

***Highly significant * Significant*

Table 2 : Pearson's correlation between the levels of copper, zinc, zn/cu Ratio and TNF in different groups (n= 90)

parameres	mild		Moderate/severe		control	
	r	p	r	p	r	p
Cu vs TNF-α	0.5	0.01	0.7	0.05	0.24	0.05
Zn vs TNF-α	-0.56	0.01	-0.51	0.01	0.3	0.1
Zn/Cu Ratio vs TNF-α	-0.51	0.01	-0.8	0.05	0.03	0.1

Significant = P< 0.05 high significant = P< 0.01

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Biochemical Changes in Proximate and Body Composition of Tor Tor (Hamilton, 1822) Fed with Various Concentrations of Protein

By Lone Akram & Lone Swapna

Abstract- The present study was aimed to assess the proximate biochemical composition of body and faecal matter of Tor tor fed with feed combination containing containing 25% CP (T-1), 30% CP (T-2), 40% CP (T-3) and control group (T-4) containing 20% CP. The results of the proximate composition of the faeces of Tor tor revealed the higher CP% of 18.64±0.03 in fishes fed on feed containing 40±0.01 CP in the basal diet. The whole body carcass analysis revealed higher concentrations of accumulated CP% as high as 65.34±34 (T- 3), fed with 40% basal crude protein. The overall study revealed that inclusion of 40% CP in mahaseer diet could lead to better growth and feed efficiency.

Keywords: *crude protein, tor tor, carcass composition, biochemistry.*

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I. Introduction

Tor tor is the most common Himalayan mahseer and a very attractive sport fish with excellent food value. Tor tor is a highly nutritious fish with good economic value. It shows a steady decline in abundance in reservoirs in India. It inhabits riverine pools and lakes and also in streams with good flows and a rocky bottom where they attain the best growth. They are benthopelagic, potamodromous and occur in tropical freshwaters (15–30 oC) at depths of upto 15 m. Adults have an omnivorous feeding habits and feed on small fish, insects, molluscs, zooplankton, debris, sand, mud, fish scales and bones, fruits, chironomid larvae, water beetles, crustaceans, filamentous algae and macrophytes. Juveniles mainly consume insects (Desai, 2003). Among Indian mahseers, Tor tor (Hamilton, 1822) is the most important food and game fish of India after Tor putitora (Hamilton, 1822).

Studies on the nutritional aspects from culture viewpoint related to conservation and propagation, though important, are very limited particularly for Narmada mahseer. Attempts have been made to raise fry of putitora mahseer on formulated diets at NRCCWF, while in case of khudree mahseer more systematic studies have been conducted to evaluate the optimum protein requirement (Murthy and Keshavanath, 1986), protein sources (Keshavanath et al., 1986) and protein sparing effect of sardine oil (Bazaz and Keshavanath, 1993). During different stages of mahseer development, the protein requirement by this species needs to be understood for modifying/formulating the feed in order to make it more balanced and nutritive.

The present study has aimed to generate baseline data on nutrient requirements so as to develop appropriate feeds to enhance growth of mahseer in captivity under aquaculture conditions so as to increase its production. At present there is only limited knowledge on the nutritional requirement of mahseer, Tor tor when cultured in captivity in tropical waters. Information on the nutritional requirement of fish and its availability from different sources is essential for formulation of complete feed. The present work describes the nutritional requirement for the development of fisheries and aquaculture of Tor tor. The main aim of the study is to find out which formulated feed with optimum protein level is more beneficial to obtain fast and better growth rate of this particular fish species when cultured in captivity in tropical climatic conditions. It will help to evaluate dietary requirement of fry to adult stage of Tor tor, so as to formulate nutritionally balanced diets.

II. Materials and Methods

Treatments

T-1: Fishes fed with feed containing 25% protein level
T-2: Fishes fed with feed containing 35% protein level
T-3: Fishes fed with feed containing 40% protein level
T-4: Fishes fed with the available commercial feed (ACF) containing 20% CP

a) Proximate analysis of feed ingredients and feeds

Proximate analysis of all the formulated feeds was performed according to AOAC (1995) methods. The feeds were analyzed before formulation of different diets.

i. *Estimation of moisture content*

Moisture content was estimated by the following formula

$$\% \text{ moisture} = \frac{\text{Sample wt. (g)} - \text{Dried sample wt. (g)}}{\text{Sample wt. (g)}} \times 100$$

ii. *Estimation of Ash content*

Ash estimation was calculated as under:

$$\% \text{ Ash} = \frac{\text{Ash weight (g)}}{\text{Sample weight (g)}} \times 100$$

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iii. *Estimation of Crude Protein content*

The estimation of crude protein content involved three phases i.e, digestion, distillation and titration. The crude protein was calculated by the formula:

$$\% \text{ Crude protein} = \frac{V \times 0.00014 \times D \times 100}{W \times A} \times 6.2$$

Where, V = Volume of N/100 H2SO4
D = Dilution factor
W = Weight (g) of sample
A = Aliquot taken

iv. *Estimation of Crude Lipid content*

Crude lipid content was calculated by the formulla

Calculation:

$$\% \text{ Lipid} = \frac{\text{Cup weight after extraction (g)} - \text{Empty cup weight (g)} \times 100}{\text{Sample weight}}$$

v. *Estimation of Calcium content*

0.5 g of ash sample was taken in a Kjeldahl tube and 20 ml of concentrated HNO3 was added to it. The solution was then digested in a fume cupboard at 110oC till it became clear. Simultaneously 3 numbers of blank (digestion tube + acid) were digested. The volume was made to 50 ml by adding distilled water and stored in polythene bottles till analysis was done by atomic absorption spectrophotometer following Factar's instructions.

vi. *Determination of Gross energy*

Gross energy was calculated by the formula:

Calculation: $\frac{(\text{Rise in temperature} \times 3147) - 23}{\text{Sample weight (g)} \times 1000}$

(Combined energy value of nickel wire + cotton = 23 Cal).

vii. *Percentage of feed*

The experiment had four dietary treatments, three prepared formulated feeds and one commercial feed. The fry's kept in three tanks (aquarium) during July 2005 to June 2006 (first trial) were fed on feeds having 25%, 35% and 40% protein levels and the fourth tank (aquarium) fry's were fed on commercial feed having 20% protein content. The feeding was done daily @ 5% body weight. The feed was given twice daily morning and evening.

viii. *Collection of wasted feed (Wf) and faecal matter (Fm) for proximate analysis*

To determine the proximate composition of faeces, it was collected at different times from the tanks fed with different diets. This was done for a number of days till the quantity was sufficient for analysis. The faecal and wasted feed was collected by siphon pipe and dried in an oven in a petridish. The collected matter from respective tanks were pooled together and stored in air tight bottles until analysis. Same methods were used for proximate analysis.

ix. *Proximate analysis of body carcass*

At the start and end of both the experiments the fishes were taken and their total length and body weights were recorded. After evisceration, the fishes were cut into pieces. These collected pieces were weighed and put into hot air oven at 60oC for 72 hours for drying to determine the moisture content. The dried samples in aluminium foil were stored in dessicator for proximate analyses following the procedure as described for feed.

III. Results

The present study was aimed at the assessment of the biochemical constituents of fish food containing 25% CP (T-1), 30% CP (T-2), 40% CP (T-3) and control group 20% CP, fed to Tor tor fry and assessment its effect on the biochemistry of faecal matter and carcass at the end of the experimental period. The study shall be a valuable information tool for assessment of the protein inclusions into the feed and its subsequent effect on biochemistry of body composition of the fish. The percentage composition of four feed treatments is presented in table 1. The biochemical constitution of the formulated feed showed that the CP concentration was higher in T-3 (40.0±0.01). The treatment group had the appropriate energy content as higher levels of crude lipid (19.14±0.12) and carbohydrate (38.78±1.01). The gross energy (Kj/g) for the treatment group was enumerated to be 17.28±1.21. In comparison to other groups which included less P:E ratio showed gross energy as 16.48±0.34 (T-3; CP = 30%) and 15.82±0.21 (T-2; CP = 25%). The routine feed (ACF) which is fed to Mahaseer (Tor tor) contained CP (%), lipid (%) and energy content (Kj/g) of 20.0±0.01, 17.23±0.10 and 13.51±0.12 respectively.

Table 2 demonstrates the proximate composition of waste feed and faecal matter of Tor tor. The waste feed and faecal matter was collected on routine basis daily and subjected to biochemical analysis. The results are the mean±SD of the results. Among four treatments, the Cp content was highest in T-3 (5.25±0.11), as compared to the ACF, which contain CP (%) as less as 2.14±0.07. The lipid content was recorded as 3.16±0.06 for T-3, with as less as 1.14±0.04 (ACF) and as high as 5.37±0.01 (T-1). The gross energy (Kj/g) analysis of the feed and waste material revealed minor differences among the treatment groups, with T-1 having the highest gross energy (7.61±0.02) and T-4 (ACF) exhibiting the lowest GE of 5.9±0.04. The initial and final biochemical components of the body carcass of fish subjected to various dietary protein levels ate

presented in table 3. The initial CP (%) value (59.67±0.08) increased to the value of 65.34±0.10 in T-3, which was the highest among the other treatment groups. In case of ACF, the CP (%) showed a minor increase with a value of 58.78±0.10. The proximate analysis of crude lipid (%) in T-3 showed an increase to the tone of 25.27±0.01 from the initial value of 17.57±0.02. Besides other components like ash (%) and dry matter (%), gross energy (Kj/g) showed a remarkable increase to the tone of 20.78±0.01, as compared to the initial value of 16.32±0.01. Overall the biochemistry of feed, unfed feed, faecal matter and the body carcass revealed an enormous change subjected to the basic feed formula which mainly utilized the inclusion of 40% CP, added by the adequate sources of energy.

IV. Discussion

The present research work is an effort put with an intention to upgrade the aquaculture nutrition science, with respect to commercially important fish (Mahaseer), by using different protein, lipid and carbohydrate ratios for determination of a feed formulation, which would reduce the FCR value and makes the fish growth economical. Although there has been so many attempts to formulate the diets as per the requirement of the fishes, present work is no exception to that, except it encompasses a featured work on all the aspects and feed formulation, feeding and its impact on various physio-biochemical aspects of the test species. The work can be used as an engender for the future researchers and will be a commendable contribution to aquaculture nutrition.

The biochemical composition of various food ingredients used as food for fish and shrimp has been thoroughly authenticated by FAO. In order to get an appropriate idea regarding the performance of a feed at all metabolic levels, the biochemical composition of the formulated diet needs to be charted out. It is better to change the feed composition for Mahaseer, taking its omnivorous feeding habit into consideration, the main constituent being crude protein. In our results, the treatment 3 (CP = 40%) performed well at all biochemical levels. An attempt to determine the effects of four rations on growth, chemical composition and digestibility of the Rainbow trout (Oncorhynchus mykiss Walbaum, 1792) was done by Gumus and Ikis (2009). Four test diets were formulated to have the same levels of protein (40%) and energy (gross energy: around 4800 cal/g), however, these test diets were composed of basic diet with different carbohydrate (0, 3, 12 and 18%) to lipid (0, 6, 15 and 18%) ratios (Diet 1: 0/18; Diet 2: 3/15; Diet 3: 12/6 and Diet 4: 18/0). The authors observed that the percentage of water, protein and ash of fish flesh did not show any ($P>0.05$) change. However, the muscle lipid content of fish significantly ($P<0.05$) decreased as carbohydrate level increased. The authors hypothesis supports our findings, which revealed protein assimilation while enumerating biochemistry of carcass.

Dietary protein and energy requirements of juvenile freshwater angelfish (Pterophyllum scalare) were evaluated by Zuanon, et al. (2009). The authors used 3 × 2 factorial design with three dietary crude protein levels being tested (26, 30, and 34% of CP) combined with two digestible energy levels (3,100 and 3,300 kcal DE/kg of diet) in three replicates. The authors observed that the fish fed diets with 26% CP showed greater protein efficiency values when compared to those fed diets with 34% CP. Diets with 26% of CP and 3100 kcal DE/kg could meet the nutritional requirements of juvenile freshwater angelfish. The dietary protein requirement for optimal growth performance and body composition of juvenile sole fish was evaluated by Abdel et al. (2011). Four diets were formulated with different protein levels (D40, D45, D50 and D55% crude protein). The authors used silverside fish (Atherina boyeri) as additive to the diets as local animal protein source and to enhance palatability of the test diets. Their results showed significant differences ($P<0.05$) in growth performance and feed efficiency between diets. No significance difference ($P<0.05$) in whole body chemical composition (dry matter, crude protein, crude lipid and ash) were found between fish fed all experimental diets. However, slight increase in whole body lipid contents were recorded with D50% CP and D55% CP levels. The results of the present study indicated that, D55% crude protein level can be optimal for meeting the requirement of juvenile sole, Solea aegyptiaca without adverse effects on growth performance and feed efficiency. The hypothesis of the above authors lends complete support to our findings that inclusion of higher concentrations of crude protein could lead to better feeding strategy

The present research findings of the inclusion of 40% CP in Mahaseer diets is completely supported by the work of Muzaffar et al. (2012 a,b) who used 40% CP in carp diet. The authrors reported the highest carcass dry matter, crude protein, crude lipid, ash and energy content, lowest moisture content and carbohydrate content in fingerlings fed with Feed B which contained 40 ± 0.21% protein, 9.31 ± 0.25% lipid and 10.08 ± 0.10% carbohydrate, which supports our research findings. The effects of diets, containing different dietary protein levels (32, 36, 40, and 44%), on growth, feed conversion rate and survival of slender walking catfish (Clarias nieuhofii) fingerlings were studied by Suphada and Anut (2012). Fishes were fed with isocaloric test diets containing 32, 36, 40 and 44% protein, for 12 weeks. Carcass composition analysis indicated a positive correlation between dietary protein level and fish body protein content, but moisture, lipid level and ash content in the fish body were not significantly different among treatments. In conclusion, the 40% protein diet

gave the maximum growth performance, lowest feed conversion ratio and high body protein content in slender walking catfish fingerling during the 12 weeks of the feeding trial. The findings of Suphada and Anut (2012) lend complete support to our findings.

V. Conclusion

The present study revealed that the mahaseer culture in captivity, if fed on a diet containing optimum levels of CP and appropriate level of DE, boosts the growth of the fish and makes it economically viable. The results of the proximate composition of the faeces of Tor tor revealed the higher CP% of 18.64±0.03 in fishes fed on feed containing 40±0.01 CP in the basal diet. The whole body carcass analysis revealed higher concentrations of accumulated CP% as high as 65.34±34 (T-3), fed with 40% basal crude protein. The overall study revealed that inclusion of 40% CP in mahaseer diet could lead to better growth and feed efficiency

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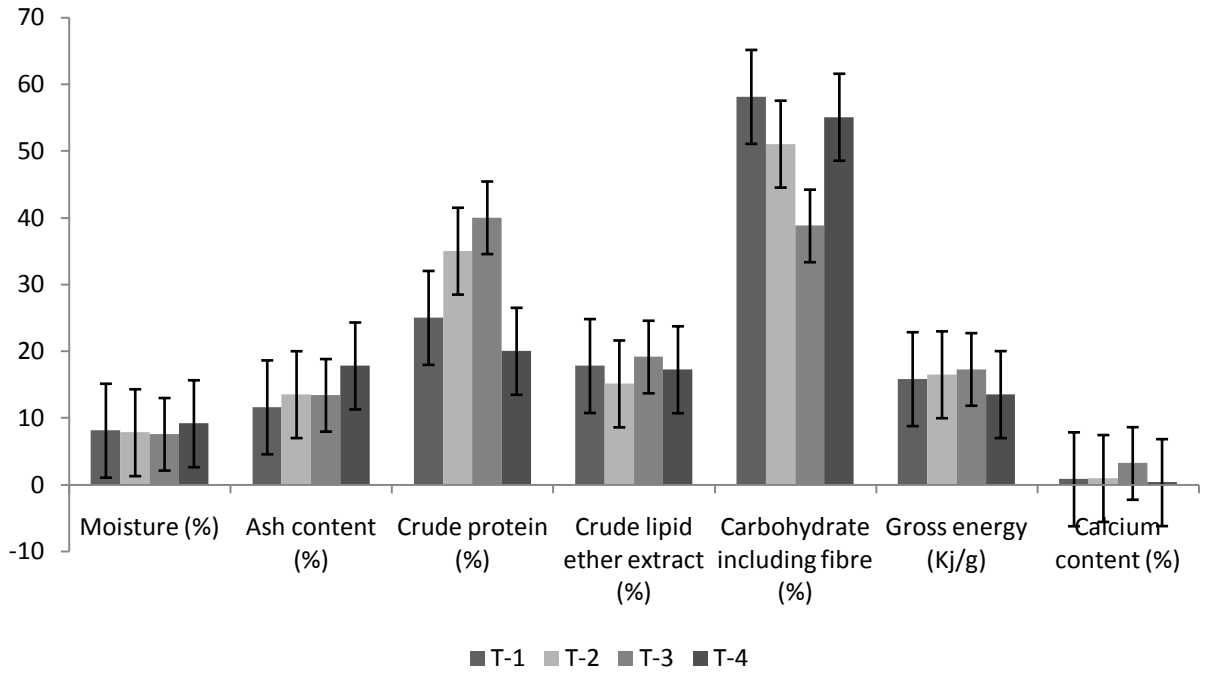

Figure 1: Percentage proximate composition of feed compositions T-1 (CP = 25%); T-2 (CP = 30%); T-3 (CP = 40%) and T-4 (ACF; CP = 20%)

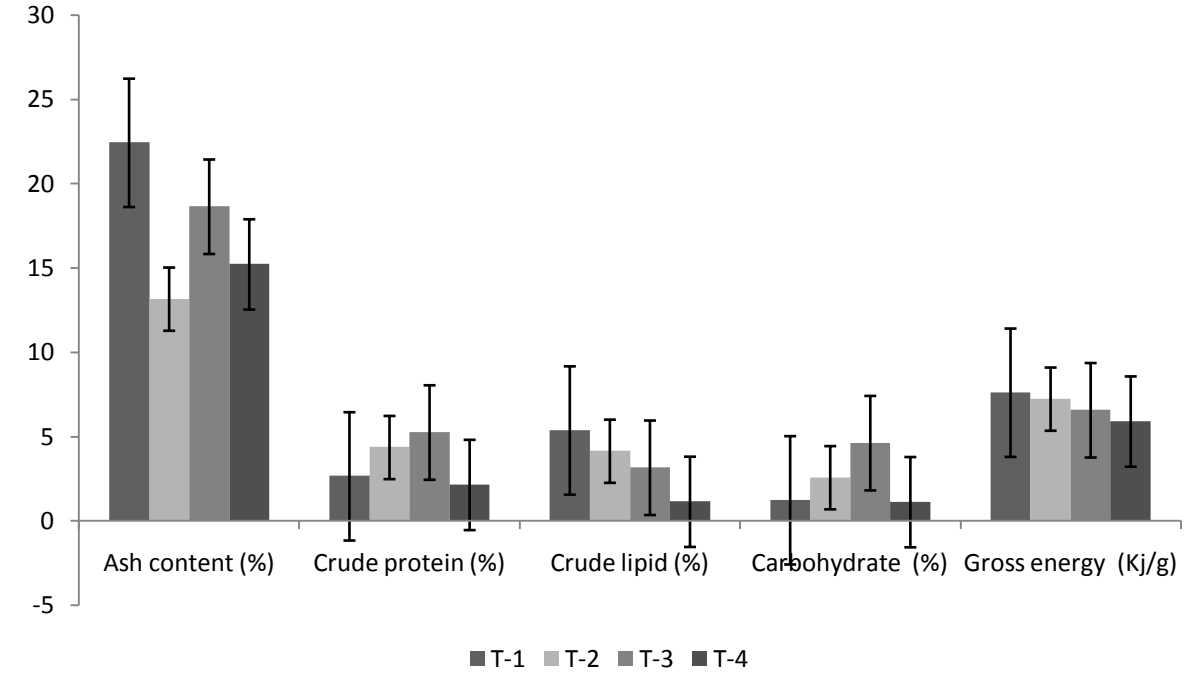

Figure 2 : Percentage proximate composition of faecal matter for four feed compositions T-1 (CP = 25%); T-2 (CP = 30%); T-3 (CP = 40%) and T-4 (ACF; CP = 20%)

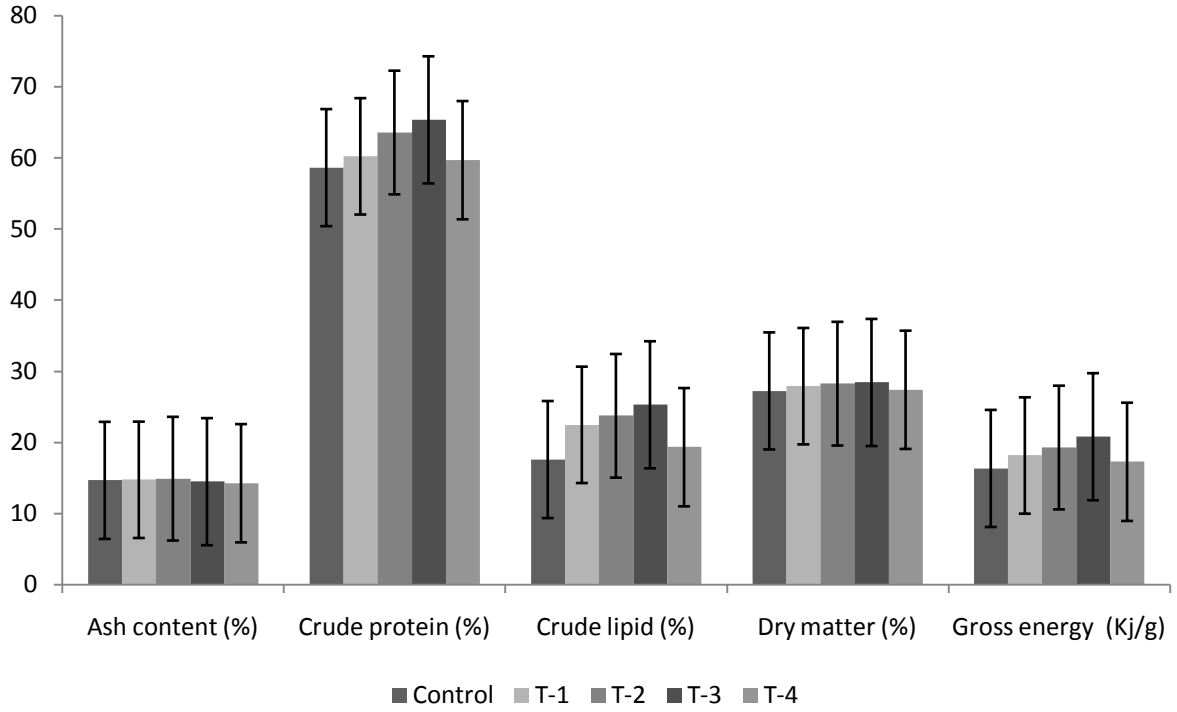

Figure 3 : Percentage proximate body carcass composition of *Tor tor* fed with four feed compositions T-1 (CP = 25%); T-2 (CP = 30%); T-3 (CP = 40%) and T-4 (ACF; CP = 20%)

Table 1 : Percentage proximate composition of Feed I, II, III and Available Commercial feed during rearing of *Tor tor*

Composition (%)	Formulated Feed			Feed IV
	Feed I	Feed II	Feed III	
Moisture (%)	8.18 (± 0.12)	7.8 (± 0.10)	7.56 (± 0.08)	9.14 (± 0.14)
Ash content (%)	11.6 (± 0.17)	13.5 (± 0.12)	13.4 (± 0.02)	17.8 (±)
Crude protein (%)	25.0 (± 0.01)	35.0 (± 0.02)	40.0 (± 0.01)	20.0 (± 0.01)
Crude lipid ether extract (%)	17.79 (± 0.16)	15.11 (± 15)	19.14 (± 0.12)	17.23 (± 0.10)
Carbohydrate including fibre (%)	58.12 (± 0.06)	51.04 (± 0.38)	38.78 (± 1.01)	55.07 (± 0.14)
Gross energy (Kj/g)	15.82 (± 0.21)	16.48 (± 0.34)	17.28 (± 1.21)	13.51 (± 0.12)
Calcium content (%)	0.82 (± 0.002)	0.94 (± 0.002)	3.19 (± 0.004)	0.32 (± 0.001)

ACF= Available commercial Feed and ±= Standard deviation.

Table 2 : Percentage Proximate Composition of wasted feed material & Faecal matter ejected during rearing of *Tor tor* (on dry weight basis)

Composition (%)	T-1	T-II	T-III	T-IV
Crude protein (%)	2.63 (± 0.03)	4.36 (± 0.09)	5.25 (± 0.11)	2.14 (± 0.07)
Crude Lipid (%)	5.37 (± 0.01)	4.14 (± 0.02)	3.16 (± 0.06)	1.14 (± 0.04)
Carbohydrate (%)	1.23 (± 0.05)	2.57 (± 0.04)	4.62 (± 0.07)	1.12 (± 0.02)
Ash content (%)	22.43 (± 0.14)	13.16 (± 0.06)	18.64 (± 0.03)	15.22 (± 0.07)
Gross energy (KJ/g)	7.61 (± 0.02)	7.23 (± 0.02)	6.57 (± 0.07)	5.9 (± 0.04)

T-IV= Available commercial Feed and ±= Standard deviation.

Table 3 : Initial and Final Carracas Percentage Composition in experimental tanks I, II, III and ACF during the rearing of *Tor tor*

Composition (%)	Initial	Final			
		Tank I	Tank II	Tank III	Tank IV
Crude protein (%)	58.62 (± 0.01)	60.21 (± 0.07)	63.56 (± 0.06)	65.34 (± 0.10)	59.67 (± 0.08)
Crude lipid (%)	17.57 (± 0.02)	22.45 (± 0.01)	23.72 (± 0.02)	25.27 (± 0.01)	19.31 (± 0.01)
Ash (%)	14.64 (± 0.01)	14.72 (± 0.02)	14.88 (± 0.03)	14.46 (± 0.02)	14.24 (± 0.01)
Dry matter (%)	27.22 (± 0.06)	27.89 (± 0.06)	28.24 (± 0.01)	28.41 (± 0.04)	27.38 (± 0.10)
Gross energy (kj/g)	16.32 (± 0.01)	18.14 (± 0.04)	19.26 (± 0.02)	20.78 (± 0.01)	17.25 (± 0.03)

±= Standard deviation.

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Mutagenic Strength of Ethanol Tolerant Saccharomyces Species Isolated from Palmwine Sap *(Raphia Sudanica)*

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Keywords: *saccharomyces species, mutagenic agents, fermentation, acridine orange.*

GJSFR-G Classification : *FOR Code: 100402p*

Strictly as per the compliance and regulations of :

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I. Introduction

Nowadays, manufacturers have begun to show unprecedented interest in innovations by introducing benefits from re-engineering and genetic manipulation. These innovations were ushered in because of certain constraints intrinsic to the organisms being used for the respective manufacturing processes. These include the need to improve yeast resistance to ethanol, mutagens, temperature, carbon dioxide as well as eliminating production of other compounds which may contaminate the product and of course, improve both yield and product recovery (Aunstrup,2007). In order to obtain strains showing more suitable properties, genetic manipulation methods have been used (Dubey, 2009). However due to the euploid, diploid or polyploid nature of most strains of yeast used in ethanol fermentation, traditional crossing techniques have not been very useful. This made the use of other technologies such as classical mutagenesis, protoplast fusion and transformation necessary (Arnord and Jose, 2008).

However, classical mutagenesis which involves the production of mutants by the exposure of microbial strains to mutagenic chemicals or ultraviolet rays to induce changes in their genomes leading to production of Improved strains would be the main focused of this research work. Seven days (168 hours) ethanol tolerance Saccharomyces species isolated from palmwine sap would be subjected to mutagenic effect of acridine orange (a basic fluorescent dye that selectively binds to genetic material and can differentiate between deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)), Such mutants may, however, show undesirable secondary mutations which can influence the behaviour of cultures during fermentation. Saccharomyces appear microscopically as globose or ovoidal cells with multilateral budding and possibly pseudohyphae. The yeast forms one to four ascospores, which are smooth and ellipsoidal. Colonies appear smooth, usually flat, and occasionally raised and opaque (Martini and Martini, 1998). The two primary species found in wines, S. bayanus and S. cerevisiae (anamorph: Candida robusta), ferment glucose, sucrose, and raffinose and assimilate glucose, sucrose, maltose, raffinose, and ethanol but not nitrate. Saccharomyces cannot utilize five carbon sugars (e.g., pentoses) (Kudo et al., 1988). On a dry weight basis, Saccharomyces contains 3% to 5% phosphate, 2.5% potassium, 0.3% to 0.4% magnesium, 0.5% sulfur, and trace amounts of calcium, chlorine, copper, iron, zinc, and manganese (Monk, 1994; Walker, 1998). Yeast must be supplied with a source of phosphate, which is incorporated into nucleic acids, phospholipids, adenosine-5′-triphosphate (ATP), and other compounds. Potassium is necessary for uptake of phosphate, and a deficiency may be linked to sluggish alcoholic fermentations (Kudo et al., 1988). Other minerals needed by Saccharomyces during fermentation have a variety of functions but are used primarily as enzyme activators. Besides minerals, yeasts require various vitamins such as thiamin, riboflavin, pantothenic acid, pyridoxine, nicotinamide, biotin, and inositol depending on species and specific growing conditions (Monk, 1994; Ough et al., 1989). In general, practically all strains of Saccharomyces require biotin

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and pantothenic acid while some also need inositol and/or thiamin for synthesis of nucleic acid, protein and fatty acids (Walker, 1998).

Palm wine is a traditional wine extracted from palm trees Elaeis guineensis and Raphia hookeri. Palm wine is consumed for its nutritional effect because of its probiotic content (Ezeronye 2004; Lourens-Hattingh and Wiljoen 2001; Heller 2001). Palm wine is consumed throughout the tropics and appears as a whitish liquid produced by natural fermentation of the sap of Elaeis guineensis and Raphia hookeri (Uzogara et al., 1990; Uzochukwu et al., 1991). The unfermented sap is clean, sweet, colourless syrup containing about 10 - 12% sugar, which is mainly sucrose (Bassir, 1962; Okafor, 1975a). Upon fermentation by the natural microbial flora, the sugar level decreases rapidly as it is converted to alcohol and other products (Obire, 2005) whereas, the sap becomes milky-white due to the increased microbial suspension resulting from the prolific growth of the fermenting organism (Okafor, 1975).

Palm wine is characterized by an effervescence of gas resulting from the fermentation of the sucrose content, by the fermenting organisms (Bassir, 1962). Previous studies on the microbiology of E guineensis and R. hookeri have incriminated several bacterial and yeast flora to be involved in the fermentation process (Fapa-runsi and Bassir, 1972; Okafor, 1972; Okafor, 1975; Eze and Ogan, 1987; Amanchukuru et al., 1989; Ejiofor, 1994; Orimaiye, 1997; Nester et al., 2004). These organisms have also been reported to originate from several sources, which include tapping equipment, containers, the environment, etc (Faparunsi and Bassir, 1972; Eapen, 1979).

George et al. (2009) described the enhancement of the recombinaenic and mutagenic activities of bleomycin in yeast by intercalation of acridine compounds into DNA. Strain D7 of Saccharomyces cerevisiae isolated from palmwine was used to measure the induction by bleomycin (BLM) of mitotic recombination at the trp5 locus and point mutations at ilv1 in the presence and absence of acridine compounds. BLM is a potent mutagen and recombinagen in the D7 assay. The acridines vary, some being mutagenic or recombinagenic and others not. Combined treatments were used to distinguish whether a genetically inactive acridine has no effect on the genetic activity of BLM or modulates its action. When an acridine is itself genetically active, combined treatments were used to determine whether its effects are additive with those of BLM or whether there is interaction between the two compounds. Acridine compounds that share the ability to intercalate between the base pairs of DNA but differ in their mutagenic specificity owing to the presence of different substituent groups were analysed. Clear potentiation and synergistic interactions were detected in combined treatments with BLM and aminoacridines, nitroacridines or an acridine mustard. Potentiation and synergy were also observed in sequential exposures in which the yeast were grown in the presence of acridine compounds and then treated with BLM in the absence of free acridine. The results are consistent with an increase in BLM susceptibility conferred by acridine intercalation. It is likely that the intercalating agents increase the access of BLM to the minor groove of DNA, where it abstracts a hydrogen from the 4' position of deoxyribose, creating a free radical that is processed.

The main focus of this paper is to mutagenically alter the genome (using acridine orange) from the reference Saccharomyces species isolated from palmwine sap (incubated for 168 hours), that coded for metabolic/enzymatic synthesis in fermentation and reassess the mutated yeast strains for fermentative potential to determine the true genetically stable and the degree of persistence of the species in fermentation processes with the objective of targeting the selection and improvement of Saccharomyces species in other to improve the overall process control, yields and efficiency as well as the quality, safety and consistency of bioprocesses products.

II. Materials and Method

a) Sample Collection

Fresh palm wine samples from Raphia palm (Raphia sudanica) was collected from main traditional palm wine trappers in Akur village (Kwara state polytechnic), Ilorin, Kwara state whittin 30 minutes of tapping. The freshly tapped samples was collected using pre-sterilized 200ml capacity sample bottle with perforated screw cap. The perforated screw caps were plugged with sterile non-absorbent cotton-wool. The samples were transported to the laboratory in a cooler equipped with packs of freezing mixture of salt and ice-block for analysis within 1 hour of collection.

b) Laboratory ageing of palmwine sample

The freshly tapped Palmwine sample collected was stored in opaque 250 ml conical flask plugged with cotton wool for 168 hours (7 days) at room temperature (28±2˚C) on an orbital shaker at 100 rpm to prevent sedimentation. Determination of pH and percentage ethanol content (% v/v) of palm wine sample was carried out aseptically at 0, 24, 36, 48, 72, 96, 120, 144, and 168 hours of fermentation (Ogbulie et al., 2007 and Nwachukwu et al., 2006a and b).

c) Isolation and identification of ethanol tolerant Saccharomyces spp. from aged palmwine

Five milliliters of thoroughly mixed palmwine was centrifuged in sterile centrifuge bottles at low speed for 5 minutes. One milliliter of the sediment was transferred using sterile pipette onto sterile Glucose Yeast-extract Agar (GYA), spread using a sterile bent glass rod and incubated at room temperature (28±2˚c)

for 24 – 48 hours (Okafor, 2008). Chlorophenicol was added to the GYA at 0.05 mg/ml to suppress the growth of bacteria (Nwachukwu , 2008, Bechem et al., 2007). Vaughan-Martini and Martini, (1998) Barnett et al. (1990), Kregger Van-Rij (1987) for yeast identification were employed. The test include morphology, surface characteristics, presence of pseudohyphae (pseudomycellium), ascospore formation and vegetative reproduction, nitrate utilization, growth in 10% NaCl + N50% glucose in yeast extract, growth in°C37and 30° C and growth in 50% w/w glucose yeast extract . Fermentation test include sugars such as lactose, maltose, saccharose, galactose and raffinose.

d) Fermentation Assessment Test

Batch fermentation was carried out on each of the saccharomyces isolate, by transferring 1ml of the isolate starter culture contanning about 107 cells ml-1 into 100ml of the fermentation medium in 250ml conical flasks. The flasks were incubated at room temperature. Determination of percentage ethanol content (% v/v) produced by each isolate was carried out aseptically at 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 days of fermentation period (Nwachukwu and Ibekwe, 2004; Wellala et al., 2004)

e) Determination of Minimum Inhibitory Concentration (MIC) of Acridine Orange

The minimum inhibitory concentration of the acridine orange was determine according to the method described by Crosa et al. (1994), Akiotoh-E and Reed (1981)

Yeast extract glucose medium was prepared and sterilized using autoclave. One milliliter (1ml) of the prepared broth was dispensed into the test tubes numbered 2 to 12 using sterile pipette. A stock solution containing a concentration of 0.2g of acridine orange in 10ml of deionized water was prepared and 1ml of the solution was dispensed into each of tube number 1 and 2. Subsequently, from tube 2, serial dilution was carried out using 1ml transfer up to tube number 10 and 1ml from tube 10 was discarded.

Also an overnight culture (starter culture) of each of the test isolates were prepared and 1:100 (10^2) dillution of broth were made and from this dillution, 1ml of the inoculum was transferred into each tube 1 to 12 with exception of tube 11, to which another yeast extract glucose medium would be added.

The final volume in each tube was 2ml with exception of tube number 1 that had only 1ml of acridine orange (served as control for acridine orange), Tube 12, which contained 1ml of culture and acridine orange, served for viability of the culture in use and tube 11 served as control for the sterility of the sterile broth.

The final concentration of acridine orange in each of the tube numbered 1 to 10 after dillution were; 20,000, 10,000, 5,000, 2,500, 1,200, 625, 312.5, 156.25, 75.125, and 37.06 ug/ml respectively.

The set-up was incubated at 28±2° C for 24 -48 hours after which the tubes were examined for growth. The last tubes in which growth failed to occur for each isolated species were taken to be the minimum inhibitory concentration (MIC) tube. The concentrations of the MIC and sub-MIC tubes were noted and SUB-MIC concentrations were used for the determination of genetic strength.

The above procedures were repeated for each of the isolated Saccharomyces species to determine the genetic strength and stability of the species.

f) Determination of mutagenic strength in isolated Saccharomyces species

Determination of genetic strength from isolated Saccharomyces species using mutagenic method (acridine orange) was performed according to the method of George et al. (2008), Grinsted and Bennet (1988), Akiotoh-e and Reed (1981).

Sub-MIC concentrations for each species were chosen against such isolate in the determination procedure.

The test Saccharomyces species was inoculated into a sterile yeast glucose medium and allowed to stand for 24 hours. This broth served as inoculum. Another sterile medium broth was prepared and distributed in 1ml volume into four different test tubes and sterilized by autoclaving. A stock solution of acridine orange in sterile deionized water (sub-MIC concentration) was prepared and 1ml of the solution was dispensed into the test tubes containing sterile broth. The content of the tube was vortexed for seconds and 20µl of overnight-incubated inoculum was added with the help of micropipette (each tubes represented different isolates of the Saccharomyces species). The tubes were incubated at room temperature (28±2°C) for 24-48 hours after which the growth obtained were inoculated into the Petri-dishes containing yeast-extract glucose agar + chlorophenicol and incubated under the same conditions.

The colonies appeared were then subjected to fermentative re-assessment as described to determine the genetic strength and stability

III. Result and Discussion

The two most important physiochemical parameters determined in this research work clearly show that the percentage content of ethanol of freshly tapped palmwine was 2.0%v/v with pH of 6.0. The ethanol content increased with time of ageing to a peak of 5.1% v/v at 72hours , after which it diminish gradually to a level of 3.0% v/v after seven (7) days of ageing (figure 1). The decline in the ethanol level of the sample with respect to age is an indication of preference of the microorganisms for metabolism especially the yeast for fermentation (Priest and Campell, 1996). This result could be as result of some of the alcohol being

converted into more organic acids by the fermenting yeasts and bacteria (Ogbulie et al., 2007; Kenneth and Charles, 2007; Robert, 2006; Nwachukwu et al., 2006a; Ogbonna, 1984; Okafor, 1972). The steadily reduction of pH of the wine sample from 6.0 to 1.5 after 7 days of fermentation is probably due to the accumulation of acidity, because of formation of organic acid with time. This result is correlated with the finding of Nwachukwu et al. (2006a), Oyagade (2004), Okafor (1972).

Microscopic examination of the 7-days aged palm wine sample showed that palm wine serves as a good medium for the growth of numerous microorganisms especially yeast cells. However, the following ethanol tolerant Saccharomyces species; S. cerevisiae, S. uvarun, S. lactis, S. flagilis and Hansenulla species were isolated and identified . All Saccharomyces species isolates showed resistance to chloramphenicol. The chloramphenicol inhibits the growth of most bacteria while allowing the yeast to grow. The ethanol tolerance results indicated that with no exogenous ethanol added, the saccharomyces yeast isolated after 7-days clearly tolerated the physico-chemical conditions imposed by the palm wine (Nwachukwu et al., 2006c). This indicated that they were better adapted to the conditions in the wines than some of the other organisms involved in palm wine fermentation. The occurrence of these ethanol tolerant saccharomyces isolates in the palm wine samples, however, supports the reports made by Amoa-Awua (2007), Nwachukwu et al., (2006a), Oyagade (2004), Ejiofor (1994) Ikenebomeh and Omayuli (1988), Casey and Igledew (1989), Ogbonna (1984), Okafor, (1980), Faparunsi and Bassir (1971) and Okafor (1975).

Nine (9) days Fermentation assessment test indicating the rate of production of ethanol on 15% glucose culture medium by the isolated Saccharomyces species as presented in figure 2 indicated the initial rate of ethanol production was higher in S. cerevisiae (4.1% v/v) than S. uvarum, and S. flagilis (1.4% v/v) While S. lactis shown zero (0% v/v) production until after 48 hours. However, the percentage of ethanol produced after 9 days of assessment was highest in S. uvarum, and S. flagilis (10.8%v/v) and lowest in S. lactis (8.1%v/v) while S. cerevisiae recorded 10.4% v/v.

A clear relationship in the fermentation pattern was evidence between the four isolates, where the initial rate of fermentation was the highest in S. cerevisiae followed by S. uvarum S. flagilis and S. lactis. However, the percentage of ethanol by each isolate on 15% glucose medium after reaching the contant specific gravity varies from 10.4 and 10.8 to 8.1 indicating that the final ethanol yield was not significant difference among the four Saccharomyces species. These result was in agreements with the finding of Wellalla et al. (2004) Tabera et al. (1985) and Ogbonna (1984) in which one Saccharomyces species that showed a higher rate of fermentation than another at one stage of fermentation was not always found to be the highest ethanol producer. However, the increased production and tolerance of ethanol could be as a result of duplication/mutation of the genes responsible for both ethanol production and tolerance, these characters have been known to be polygenic (Nwachukwu, 2008).

The determination of the minimum inhibitory concentration (MIC) of the mutagenic agent, acridine orange (BDH Chemical Ltd, Poole, England) was to provide a minimum concentration (sub-MIC) of the agent that would selectively bind to the yeast chromosomal DNA molecule for easier mutational changes (George et al., 2009; Grinsted and Bennet, 1988). The minimum inhibitory concentration (MIC) of the acridine orange (mutagenic agent) to the reference Saccharomyces species was determine after 48 hours of specific incubation (figure 3). After three (3) successive experimental trials for each isolate, both S. lactis and S. uvarum had the highest (MIC) value of 4166.67μg and 3333.33μg while S. cerevisiae and S. fragilis recorded 2083.33μg and 1250μg respectively. Therefore, the sub-MIC values recorded are 1666.67, 2083.33, 1041.67 and 625 for S. uvarum, S. lactis, S. cerevisiae and S. fragilis respectively.

Fermentation re-assessment test conducted after mutagenic experiment to determine the genetic strength of the isolated species using sub-MIC values are presented in figure 4. The result show that S. lactis had zero percentage (0% v/v) ethanol content after 9 days of fermentation while S. flagilis produced 2.6 %v/v ethanol concentration after 4 days of fermentation period and 2.8 %v/v was observed in S. uvarum after 3 days of fermentation. The highest rate of ethanol concentration was detected in S. cerevisiae with 10.3 %v/v ethanol content recorded after 5 days of fermentation period. However, amount of ethanol produced was terminated in S. cerevisiae, S. flagilis and S. uvarum from 5, 4 and 3 days and constant throughout the duration of fermentation period as shown in figure 4.

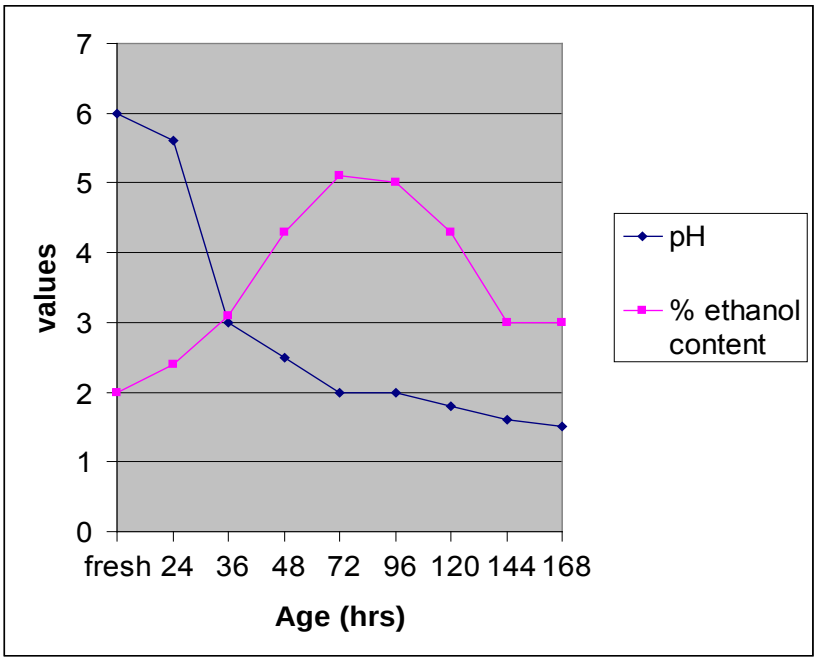

Figure 1 : Changes in the physiological parameters of aged palmwine

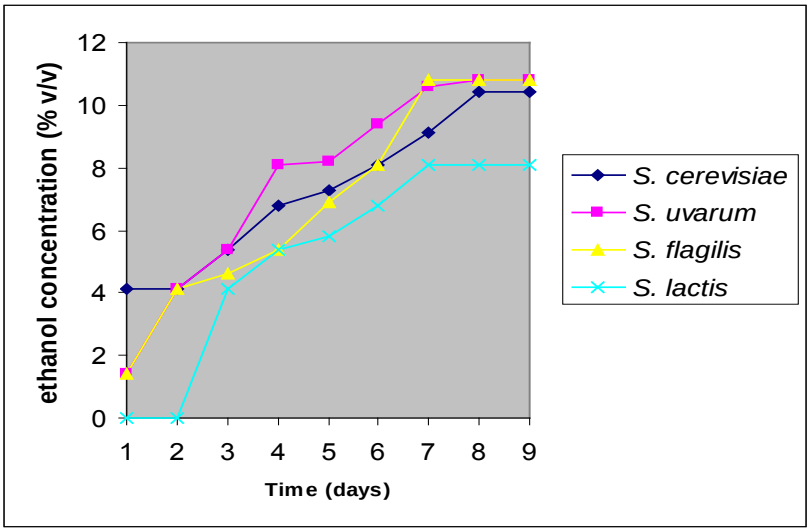

Figure 2 : Rate of ethanol production by isolated Saccharomyces species on 15% glucose culture medium (Fermentation assessment test).

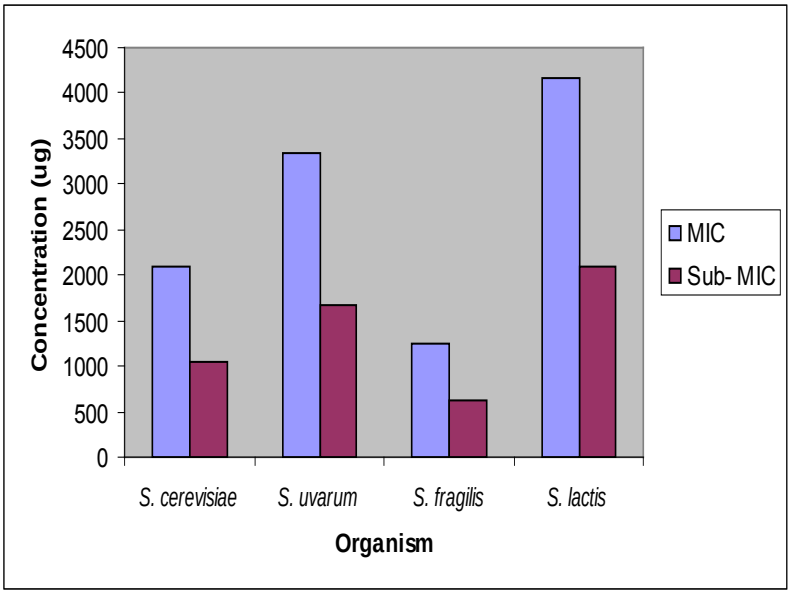

Figure 3 : Minimum and sub-Minimum inhibitory concentration of ACRIDINE ORANGE (mutagens) on isolated Saccharomyces species

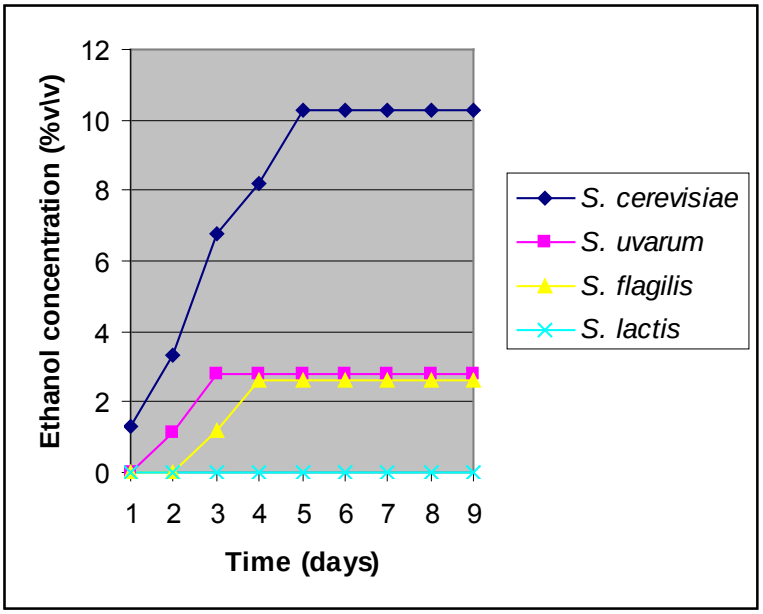

Figure 4 : Ethanol production by mutagenic treated saccharomyces isolates on 15 % glucose culture medium. (fermentation re-assessement test)

IV. Conclusion

Mutagenesis experiment conducted indicated that the four isolated Saccharomyces species actually harbours genetic materials encoded for fermentative property, that is, the fermentative ability of the isolates are partially and completely control by genetic properties embedded in the nuclear material. This was confirmed after fermentation re-assessment test at which all isolates recorded negative values compared to earlier fermentation assessment test except S. cerevisiae that show relative genetic stability.

This research work has provided ideas about the mutagenic capability and effectiveness of fermentative abilities of the Saccharomyces species and raises a very strong hope for the industries ethanol fermentation industries of reduced production costs. The application of their stability is that, these yeast mutants could be promising as industrial organisms for use in ethanol and other fermentation industries.

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- Center on shortening results - bound background information to a verdict or two, if completely necessary
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	A-B	**C-D**	**E-F**
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Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
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References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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