

Original Article

## Comparative Analysis of *Mucor Indicus* Against *Aspergillus Niger* and *Aspergillus Fumigatus* for Wheat Straw Fermentation to Produce Efficient, Inexpensive and Eco-Friendly Bioethanol

Sana Zahra Naqvi<sup>1,2\*</sup>, Syed Azhar Abbas<sup>3</sup>, Muhammad Ali-ul-Husnain Naqvi<sup>4</sup>, Nayab Batool<sup>1</sup>, Tayyaba Younas<sup>1,5\*</sup>

<sup>1</sup>Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan

<sup>2</sup>College of Preventive Veterinary Medicine, Nanjing Agricultural University, Nanjing, China

<sup>3</sup>School of Environment, Nanjing Normal University, Nanjing, China

<sup>4</sup>Department of Parasitology, University of Agriculture, Faisalabad, Pakistan

<sup>5</sup>Department of Chemical Engineering, Monash University, Australia

**\*Corresponding Author:** Sana Zahra Naqvi, Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan, College of Preventive Veterinary Medicine, Nanjing Agricultural University, Nanjing, China, Tel: +923066483672, E-mail: [2018207077@njau.edu.cn](mailto:2018207077@njau.edu.cn); Tayyaba Younas, Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan, Department of Chemical Engineering, Monash University, Australia, Tel: +61412084360, E-mail: [tayyaba.younas@monash.edu](mailto:tayyaba.younas@monash.edu)

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### Abstract

Increasing fuel prices and depleting hydrocarbon resources have compelled scientists to accelerate their efforts in finding reliable petroleum alternatives. Bioethanol has emerged as one of the best solutions

to fit in the situation. Therefore, this colorless, biodegradable, eco-friendly liquid by all means is capable of replacing petrol. Its unique characteristics appealed modern scientists to conduct researches in

this dimension more seriously. Among various production ways, bioethanol can easily be produced by fermenting lignocellulosic materials through fungus. This study compared and analyzed its production by fermenting pretreated wheat straw using three fungi; *Mucor indicus*, *Aspergillus niger* and *Aspergillus fumigatus* separately. At first, samples collected from soil (n=111) and rotten/decomposed vegetables (n=14) were grown on yeast extract peptone dextrose (YPD) agar at 37°C for 5 days. From a total of 34 isolated colonies, three most abundant fungal species were selected and identified while others were left unknown. Morphological and microscopic characterization of these fungi was done by lacto-phenol cotton blue staining. To minimize limiting factors of production, fed batch process was opted for fungal fermentation of sugars. Bioethanol was extracted by rotary evaporator while estimation was done through colorimetric method. UV induced mutagenesis enhanced ethanol production potential of these isolates. For both wild and mutant isolates, this potential was compared and analyzed graphically. At 3% sugar concentration, bioethanol production from *Mucor indicus*, *Aspergillus niger* and *Aspergillus fumigatus* was optimum i.e. 8.2%, 6.1% and 6.4% respectively and it became 8.4% for *Mucor indicus*, 6.4% for *Aspergillus niger* and 6.4% for *Aspergillus fumigatus* after 15 minutes UV exposure. Hence, under same circumstances *Mucor indicus*, in comparison with other two strains came out as the most competent one to produce efficient bioethanol from wheat straw fermentation.

**Keywords:** Second generation bioethanol; Wheat straw; Fermentation media; Pretreatment; *Mucor indicus*; *Aspergillus niger*; *Aspergillus fumigatus*

## Introduction

Global energy dependency has drastically increased in couple of decades. Accelerating population and modernized lifestyle are thought to be the two main factors behind [1]. Fossil fuel largely remained the source to fulfill the unceasing energy demands. At present, transportation sector alone consumes nearly 60% of the oil produced internationally, which as a consequence, contributes enormously to global air pollution, hence, jeopardizes the situation further. Its share in emitting hazardous gases like, Carbon Monoxide(CO) and Carbon Dioxide (CO<sub>2</sub>) is alarmingly high, i.e. 70% and 19% respectively. One-gallon of gasoline emits 8 kg CO<sub>2</sub>. Therefore, more vehicles moving on road means more the world is getting polluted. Taking into account 806 million vehicles in 2007 worldwide, statistic suggests that this number will surpass 1.3 billion by 2030 and 2 billion by 2050. Apart from rapid depletion of natural resources, it is clearly an environmental catastrophe which is around the corner. Consequently, it has signified the urgency of environment friendly energy alternatives. To serve the purpose, some alternates are already available for instance; bioethanol, liquefied petroleum gas (LPG), natural gas, methanol, biodiesel, boron, hydrogen, electricity, and solar fuels [2]. Among them, 'Bioethanol' exhibits several pluses over fossil fuels, therefore, largely known as a favorable source of renewable energy now-a-days. It is economical, ecofriendly and above all has characterized CO<sub>2</sub>-cycle in ignition [2,3]. According to the Energy Information Agency (EIA) report, there will be 60% more requirement of energy than today in 2030. If this requirement fashion of energy will prevail then world will encounter an energy crisis because universal petroleum reserves will be diminished in less than 45 years. Other important problems are global warming and environment

pollution. If the normal world temperature rises further up to 2<sup>0</sup>C, there will be possibly no life on earth and results into extinction of many species. So this is need of hour to produce bioethanol for wellbeing of life as prevailing place for bioethanol in fuel market [4]. Moreover, bioethanol utilizes non-edible parts of sugar cane and cereal grains like: aforesaid feed stock which is agricultural residues. Though it is the second generation of bioethanol feed stock, but it is helpful in tackling food and feed insecurity [5-7]. Researches show that wheat straw is the most probable residue in this regard, its availability and cost further supports the selection [8-11]. 1.3-1.4 kg of wheat straw are normally obtained from 1 kg wheat grain, whereas worldwide production of wheat remained nearly 680 Mt in 2011. Wheat straw; which comprises of 15% lignin, 20-30% hemicelluloses and 35–45% cellulose makes it striking feedstock for ethanol production [1].

Almost all agricultural residues produce ‘Lignocellulosic Compounds’ and these compounds could be best utilized in producing bioethanol which would have been wasted otherwise. To serve the purpose, acids are used to delignify them [12-14] then selected microbes ferment the already delignified substrate [15]. Bioconversion of various organic compounds into ethanol through fermentation is mostly carried out by fungus [7]. Lignocellulose, the main constituent of wheat straw, is an abundant resource for ethanol production and it could be hydrolyzed into a range of pentoses and hexoses like glucose, mannose, galactose, xylose and arabinose [16]. For optimum utilization of available sugars during fermentation, selection of a feasible fungus is very crucial. Similarly, features like; survival under adverse environment, substrate (moist & solid) utilization and abundant growth further elaborate the

role of particular fungus selected for conversion reaction. Considering these characteristics and available data, *Mucor indicus*, *Aspergillus niger* and *Aspergillus fumigatus* were shortlisted to undergo initial trials for this study. *Aspergillus* fermentation limitation makes it less effective for bioconversion as it can only ferment a few types of hexoses for instance; glucose, however, hexoses like xylose remain unfermented [3,17]. Normally, fungi are sensitive towards high temperatures; nonetheless, *Mucor* is capable of fermenting pentoses and hexoses which supports its growth at temperatures where other fungi usually fail to survive. High temperature bearing potential reduces the chance of contamination as well [18]. Therefore, *Mucor* is economical and outstandingly suitable for fermentation in tropical regions where temperatures are normally higher [19].

Wheat straw; an abundant yet unfortunate agricultural residue which sometimes possesses zero to low commercial value has actually very high potential to be turned into a valuable bio-fuel. In present work, we have adopted simple but smart techniques to facilitate this conversion; these techniques and methodologies, due to their simple nature are easy to be implemented elsewhere. The optimum conditions discovered as a result of comparative study could be lucrative to produce bioethanol commercially. Besides, this research did not involve any laborious, expensive or hazardous activity at all.

## Materials and Methods

### Microorganisms

Decomposed vegetables [onion & potato (n=14)] and 111 soil samples were collected to isolate fungal strains. Collected samples were kept in labeled zip

lock plastic bags until further processing. Soil samples were serially diluted and then shifted to agar medium while vegetable samples were shifted directly to the plates by tapping them over the agar plate followed by incubation at 37°C for 5 days. Samples were primarily cultured on Potato Dextrose Agar (PDA) first and then sub-cultured on Sabouraud's (SD) and Yeast-extract Peptone Dextrose (YPD) agar. Morphological and microscopic (lacto-phenol cotton blue staining) characterization was performed to confirm the fungal specie. For lacto-phenol cotton blue staining, a drop of the stain was placed on a clean slide and a portion of the mycelia was placed on the stain using a sterilized needle and forceps. A cover slip was then placed on the wet mount and the slide was examined by a light microscope at various magnifications. The morphology and characteristics of the conidia and conidiophores were then used to classify the different types of fungus according to the standard taxonomic system [20].

Three most abundant colonies were chosen for further processing [16,21]. Confirmed species were streaked over slants of sabouraud's dextrose agar for preservation, incubated and sealed tightly to place at 4°C for further use.

### Wheat Straw

Wheat straw was collected from agricultural farm of 'University of Agriculture, Faisalabad'. To store these straws, washing, cleaning, drying, and moisture removal were carried out. Wheat straw samples were then kept in sterile zip bags.

### Media preparation

Media used were PDA (potato starch-4g, dextrose-20g and agar-15g per liter of distilled water), SDA

(peptone-10g, dextrose-40g and agar-15g per liter of distilled water) and YPD agar (yeast extract-10g, peptone-20g, dextrose-20g and agar-15g per liter of distilled water). Broth media, when required, were prepared following the same concentrations without adding agar. The pH was adjusted as 5.6 for PDA and SDA & for YPD pH corrected at 6.5. All the media were prepared following standard methods and autoclaved at 121°C, 15 lb pressure for 15 minutes.

### Fermentation media

Fermentation media was prepared by minor modification of previously described media. The pH was adjusted at 5.5 [22]. 2% sugar medium was prepared by adding 20ml acid hydrolysate, 5g yeast extract, 7.5g ammonium sulphate, 3.5g dipotassium hydro-phosphate, 1g dihydrate calcium chloride, 0.75g hepta-hydrate, 1ml vitamin solution and 1ml trace elements in 1000ml of distilled water.

Vitamin solution was composed of 0.2g p-amino benzoic acid, 1g nicotinic acid, 1g calcium pentothenate, 1g pyridoxine, 1g thiamine, 1g biotin and 25 gram inositol in 1000ml of distilled water and trace metal solution was composed of 1g EDTA, 0.9g dihydrate calcium chloride, hepta-hydrate zinc sulphate, 0.6g hepta-hydrate ferrous sulphate, 0.2g boric acid, 0.16g dihydrate manganese chloride, 0.08g dehydrate sodium molybdenum oxide, 0.06g dihydrate cobalt chloride, 0.06g penta hydrate copper sulphate and 0.02g potassium iodide in 1000ml of distilled water. Trace element and vitamin solution was added in the media after autoclave because these were sterilized by filtration (0.22µm filter) already.

Fermentation media for 3% and 4% sugar was prepared with the similar approach as it was done previously, the only exception was the volume of

acid hydrolysate, 20ml was replaced by 30ml and 40ml respectively [22,23].

### Pretreatment of substrate

Wheat straw (20g) was soaked in 100ml of 32% HCl and incubated at 32°C for 24 hours at 150rpm. Post incubation, mixture of wheat straw and acid was diluted 10 times then heated at 100°C for 30 minutes. After filtration, solution was subjected to evaporation to get thick sugar syrup of 15 ml [24]. Sugar presence in the solution was confirmed by Benedict's test [25] and quantitative analysis was estimated by Anthrone test [26]. This sugar syrup was used as a carbon source in fermentation media named as acid hydrolysate.

### Fermentation

Fed batch fermentation method was followed. Inoculums were prepared by sterile distilled water (10ml) addition to Petri plates with fungal growth and spore counting was performed through haemocytometer. Cultivation was started with 50ml medium, inoculated with 1ml spore suspension ( $6 \times 10^6$  spore/ml, for purely yeast like growth) in anaerobic condition. Feeding rate was 50ml/hour for 5 hours. Medium was incubated for 37°C at 150 rpm for 5 days. After completion of incubation, medium was filtered to remove fungal biomass. Fermentation was also done with 3% and 4% sugar fermentation media [23].

### Product Recovery

Bioethanol from filtered solution was extracted by usage of rotary evaporator at 78°C and 120 rpm [27].

### Ethanol Analysis

0.115M solution of potassium dichromate ( $K_2Cr_2O_7$ ) was prepared. 11 sterilized test tubes were taken and

divided into two groups, group 1st contained 9 test tubes of standard and group 2nd contained 9 test tubes of samples. Each test tube contained 2ml of 0.115M  $K_2Cr_2O_7$  and 9ml water.

In group 1<sup>st</sup>, 2ml of ethanol 0%, 2%, 4% s.... 16% in 9 test tubes respectively. In group 2<sup>nd</sup>, 2ml of extracted ethanol from samples was added respectively. Then all of these test tubes were heated in boiling water bath for 10 minutes for proper color generation. Optical density was measured at 600 nm [27,28].

### UV Mutagenesis


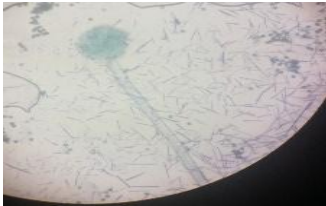

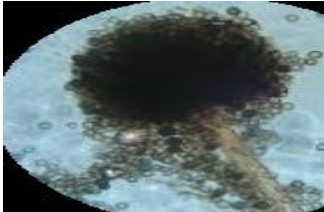
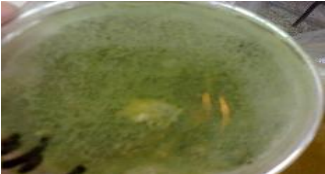

Physical mutagenesis was carried out by UV light exposure. Spore suspension of each isolate was prepared by adding 1ml sterile distilled water to the Petri plates with fungal growth and shifted to sterilize test tubes. Then 100µl suspension from each test tube was taken and spread on YPD agar plates. Plates with inoculation were exposed to 290nm UV light at a distance of 20cm for 15, 20, 25 and 30 minutes and incubated at 37°C for 5 days [29,30]. The survivals were marked as mutants and utilized for fermentation process for all given sugar concentrations of media.

## Results

### Isolation Results

Soil and vegetable samples which were earlier processed on YPD agar in order to grow *M. indicus* anaerobically and *A. niger* & *A. fumigates* aerobically, were confirmed after careful examination of their colony morphology and microscopic features. Key morphological and microscopic characteristics exhibited by these strains are described in Table 1 along with their images.

**Table 1:** Morphological and Microscopic Characteristics of *M. indicus*, *A. niger* and *A. fumigatus*

Sr. No.	Identified Isolates	Colony Morphology	Microscopy
1.	<i>M. indicus</i>	<ul style="list-style-type: none"> <li>White woolly growth</li> <li>Cotton to fluffy</li> </ul> 	<ul style="list-style-type: none"> <li>Hyaline sporangiophore</li> <li>Round columellae</li> <li>Smooth walled sporangiospore</li> </ul> 
2.	<i>A. niger</i>	<ul style="list-style-type: none"> <li>Yellow to white</li> <li>Turning black with formation of conidia</li> </ul> 	<ul style="list-style-type: none"> <li>Hyaline conidiophore turned black towards tip</li> <li>Round conidial head with dark brown color and split into several loose columns</li> </ul> 
3.	<i>A. fumigates</i>	<ul style="list-style-type: none"> <li>Colonies are velvety, yellow to green</li> </ul> 	<ul style="list-style-type: none"> <li>Short conidiophores having conical shape towards tip</li> <li>Typical columnar conidial heads</li> </ul> 

**Acid Hydrolysis**

After strong acid hydrolysis of wheat straw at low temperature, obtained sugar syrup was examined to confirm sugar presence. Appearance of brick red color upon Benedict test, confirmed the existence of high levels of sugar in the syrup whereas, Anthrone test rated its concentration as 0.56g/ml of syrup.

**Ethanol Analysis Result**

Ethanol produced after fermentation underwent quantity assessment through colorimetric method for each set. O.D. measured from Set-I of standards was plotted in a graph shown below (Figure 1).

To estimate ethanol percentages, O.D of Set-II was compared with the standard curve. A graph is plotted

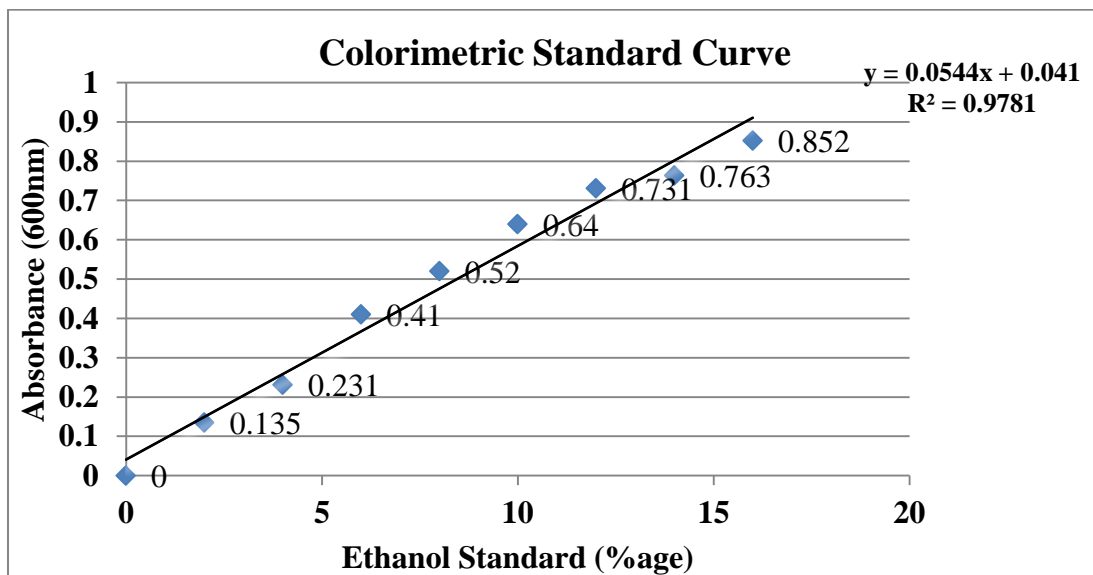
for *M. indicus*, *A. niger* and *A. fumigatus* indicating ethanol percentages along with three sugar concentrations i.e. 2, 3 and 4 percent (Figure 2). 3% sugar concentration was chosen for advanced processes as it gave optimum ethanol production statistics.

### Mutagenesis

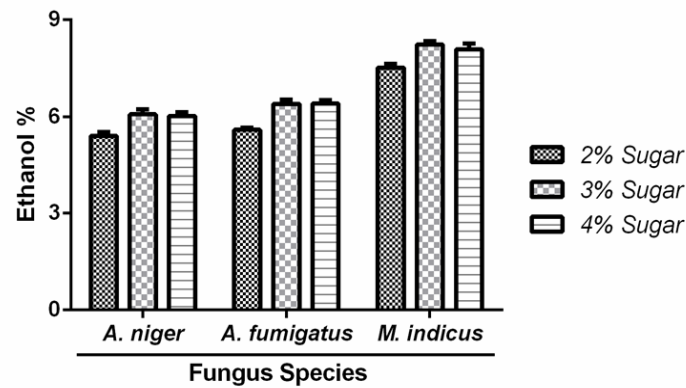
To improve ethanol production, random mutagenesis was performed by exposing *M. indicus*, *A. niger* and *A. fumigatus* to 290nm UV for 15, 20, 25 and 30

minutes time intervals respectively. A handsome colony number was attained at 15 minutes exposure which gradually decreased upon extra exposure (20-25 min) and turned zero at 30 minutes. Figure 3 demonstrates the survival curves of these strains.

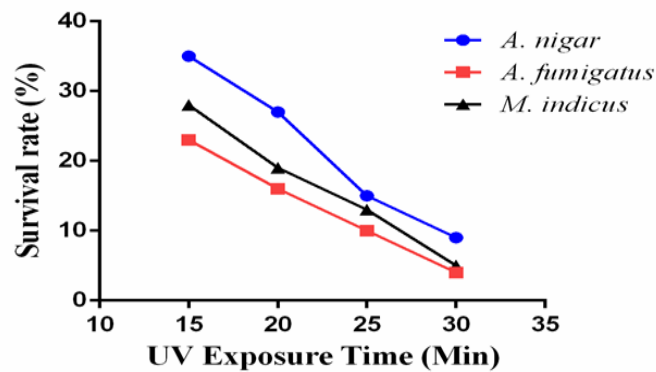
Post UV mutagenesis and fermentation of all fungal isolates with 3% sugar concentration; following results were obtained through colorimetric method (Figure 4).



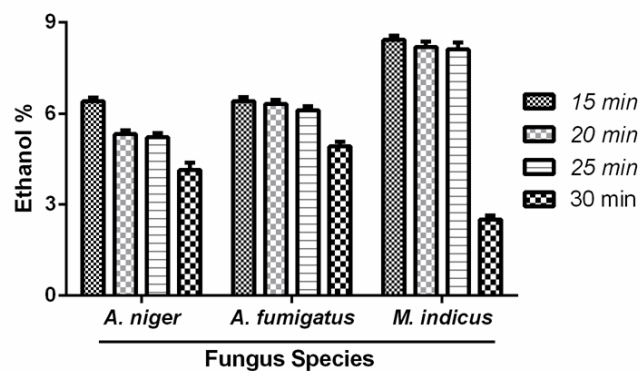
**Figure 1:** Colorimetric method standard curve of concentration versus absorbance. The concentration of ethanol (in % age) was determined by using the equation  $y = 0.0544x + 0.041$  with an  $R^2 = 0.9781$  where  $y$  is absorbance and  $x$  is concentration. In a standard reaction, 2ml of each ethanol concentration was mixed with 2 ml of 0.115M  $K_2Cr_2O_7$  reagent and heated in water bath for 10 min for color generation and then absorbance was measured at 600nm. For unknown samples (purified ethanol), 2ml of 0.115M  $K_2Cr_2O_7$  reagent was mixed with purified ethanol for heating and absorbance measurement. The calibration curve is needed to calculate samples concentration



**Figure 2:** Ethanol Production comparisons of three strains *M. indicus*, *A. niger* and *A. fumigatus* at 2, 3 and 4% sugar



**Figure 3:** Survival curves of *M. indicus*, *A. niger* and *A. fumigatus* at different UV minutes exposure (290nm). Blue circles, black triangles and red rectangles indicate survival rate of *A. niger*, *M. indicus* and *A. fumigatus* respectively. No survivals were observed at 30 min UV exposure



**Figure 4:** Ethanol Production comparisons of three strains *M. indicus*, *A. niger* and *A. fumigatus* after mutagenesis at 3% sugar concentration



## Discussion

*Mucorales* by and large, *M. indicus* in particular, have been known as being amongst the most diverse and thermo-tolerant fungi [31]. Despite the fact, *M. indicus* has been reported to have an essential role in bioethanol production, its suitability for wheat straw fermentation has not been studied yet. Whereas, present study has revealed that *M. indicus* is an extremely suitable fungus for ethanol production from wheat straw. Its performance was observed well ahead of *A. niger* and *A. fumigatus*. Features like faster fermentation rate further single it out as a promising choice to produce ethanol from wheat straw. Therefore, to get faster and efficient yield, sugar from biomass was produced directly after concentrated acid pretreatment at low temperature [32]. As described in previous study, obtained sugar was fed to fermentation media along with vitamins, minerals and trace elements [23]. Like previous study, fed batch fermentation method was adopted to ensure prolonged culture lifetime and higher product recovery [8].

*M. indicus*, *A. niger* and *A. fumigatus* have long been used for ethanol production from variety of agricultural wastes [23,33,34]. While this study, in the first place, focused on their ethanol production efficiencies specifically from wheat straw. Later on, their efficiencies at different sugar concentrations (i.e. 2%, 3%, & 4%) were studied. Results of each concentration varied sharply due to the presence of limiting factors and byproducts. However, for all the three strains, optimum conversion was noted at 3% sugar fermentation media. *A. niger* and *A. fumigatus*, in this study exhibited more or less the same results for ethanol production at 3% sugar both with and without 15 minutes UV exposure. *M. indicus*, however, showed promising results under the same

conditions. Previous studies affirmed that this is due to its capability of absolute fermentation of pentoses and hexoses into ethanol. Moreover, the greater yields along with surprising tolerance behavior against inhibitors present in hydrolysates further support the argument [16,21,35].

Although, there are numerous ethanol analysis methods like; high performance liquid chromatography [36] and gas chromatography [37,38]. However, being economical and easy to handle [27], this study applied colorimetric method. In line with the previous researches, random UV mutagenesis was attempted to enhance ethanol production [29,30]. It is noteworthy that different time intervals for UV exposure did not affect ethanol production much; consequently, our chance to study the exact pathway of mutation at molecular level was confined. Nevertheless, it opened some new avenues for future researches to engage advanced tools and mechanisms in analytic methods.

Second generation bioethanol production was previously assumed uneconomical as it involved high energy consumption during pretreatment and sugar degradation [39-42]. However, wheat straw is naturally a good culture medium for fungal growth which even at low pretreatment temperatures releases high carbohydrate content, making it a smart choice for biofuel production. Moreover, it does not involve any laborious physical pretreatments; washing, drying or vigorous milling, and hence, hydrolyzed directly by adding acid to extract sugars from cellulose [9,11]. The only requirement for proper fermentation of released sugars was; a fungal strain capable of degrading C5 and C6. Therefore, the desperate call for an effective microorganism to ferment C5 and C6 simultaneously was fulfilled

comprehensively by *M. indicus*. Wheat straw fermentation with the help of *M. indicus* gave outstanding ethanol production efficiency. Moreover, their coalition at laboratory scale, turned ethanol production into a cost effective process as well. Hence, further studies are required to scale up its production at commercial levels.

**Authors Contribution:** Sana Zahra Naqvi and Syed Azhar Abbas have contributed equally to this work.

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