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Hyper-production of Glucose Oxidase (GOX) from *Aspergillus niger* by using Chemical Mutagenesis

Tanzila Sahar[1]*, Muhammad Anjum Zia[1]*, Munir Ahmad Sheikh[1] and Shaukat Ali[2]

[1]Department of Biochemistry, University of Agriculture Faisalabad-38040, Pakistan.
[2]Department of Chemistry, University of Agriculture Faisalabad-38040, Pakistan.

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Glucose oxidase have get pronounced attention due to its wide range of uses in different industries including chemical, pharmaceutical, food, beverage, biotechnology and clinical analysis. Present investigation deals with N-nitroso-N-methylurea (NMU) chemical that was used as mutagen for enhanced glucose oxidase (GOX) production from the wild strain of *Aspergillus niger*. After mutagenesis and screening methods, TS-NMU-100 mutant derived isolate of *Aspergillus niger* screened out as best positive mutants with the 260.10% increase in glucose oxidase activity corresponding to its wild strain. Production of GOX from wild and NMU mutant derived strains of *Aspergillus niger* was carried out by using pre-optimized media conditions using corn steep liquor (CSL) as substrate. It was found that N-nitroso-N-methylurea, chemical mutagen could be efficiently used for the hyper-production of GOX from *A. niger*. It was also revealed that NMU mutant strains of *Aspergillus niger (A. niger)* having a strong capability of GOX production and could be valuable for further advance research investigations and commercial scale production of GOX especially in food and pharmaceutical preparations.

Key words: *Aspergillus niger (A. niger)*, Glucose oxidase (GOX), N-nitroso-N-methylurea (NMU), mutagenesis, Corn steep liquor (CSL).

Nowadays, enzymes are extensively used as environment-friendly biocatalysts in diverse industries (Feng, 2005). Microorganisms are natural and major sources of enzymes, have been used in the manufacturing of different products since ancient times (Kirk *et al.,* 2002). Currently, microbial glucose oxidase (Gox) have got pronounced attention due to its wide range of usages in different industries including chemical, pharmaceutical, clinical analysis, food & beverage, biotechnology and in some other industries and new areas of its practical uses are constantly expanding.

* To whom all correspondence should be addressed.
Tel.: 041-9200161; Fax: 041-9200764;
E-mail: tanzilasahar@yahoo.com

Fungi are well-described microorganisms due to their capability to yield a large number of different extracellular and intracellular products. Glucose oxidase is produced from a variety of fungal sources but most commonly produced from *Aspergillus* (Witteveen *et al.,* 1992; Zia *et al.,* 2010) and *Penicillium* (Eremin*et al.,* 2006; Sukhachev*aet al.,* 2004) species, from which *Aspergillus niger* is the most frequently and successfully used for glucose oxidase production at laboratory and industrial level because of the metabolic versatility of this strain (Pluschkell *et al.,* 1996; Khurshid, 2008).

Today, studies have been more devoted on the improvedenzymes production by employingvarious strains improvement/ developmentmethodsi.e mutagenesis and enzyme engineering approaches (Khattab and Bazaraa,

2005). Industrial scale production of glucose oxidase is improved with the mutagenesis of microbes that may results inimproved-production of glucose oxidase to meet its growing uses in upcoming future. Mutagenesis is used to produce heritable deliberate variations in genome by using diverse kind of mutagens. Lots of efforts have been exercised for enhanced glucose oxidase production by using traditional and conventional selection methods like mutagenesis using different microbes as test organism (Witteveen*et al.*,1990; Zia *et al.*, 2010).Major advantage of random mutagenesis and screening method is the simplicity and easiness of this technique, there is no need of any prior knowledge of microbiology and physiology of desired microorganism that was manipulated (Stanbury*et al.*, 1995).

Hence, mutagenesis approach can be enriched in terms of both type of mutagen and dose rate. However, genetic engineering methods made it possible to attain highly potential glucose oxidase producers but the conventional methods of mutagenesis still effectively and successfully employed for this purpose by using numerous mutagens.Improving biosynthetic ability of microbial enzyme producers is a significant phase in enzyme biotechnology (Semashko*et al*. 2000).Mutant stability of *Aspergillus niger* is comparatively high, thus the risk of revert-ness of mutant is less pronounced (Khurshid, 2008).

This research study has been made to evaluate the mutagenic effect of N-nitroso-N-methylurea (NMU) on wild *Aspergillus niger* strain in order to achieve its mutant strains having improved glucose oxidase producing capability that could be used for GOX production at commercialscale using pre-optimized and economical medium conditions.

MATERIALS AND METHODS

Strain Selection

Pure culture of *Aspergillus niger* was obtained from First Fungal Culture Bank, University of the Punjab, Lahore, Pakistan. The stock culture of wild strain of *Aspergillus niger* was maintained on PDA (potato dextrose agar) medium (that iscomposed of 2 g glucose, 2 g starch, , 0.3 g Urea, 0.008 g potassium dihydrogen phosphate, 0.15 g potassium chloride, 0.05 g magnesium sulfate heptahydrate, 0.001 g zinc sulphateheptahydrateand 2 g agar in g/100mL dis. H_2O, pH of medium was maintained at 4 and microbial culture incubated at 30°C) and preserved in refrigerator at 4°C (Rasul*et al.*, 2011; Zia *et al.*, 2012a).

Inoculum Preparation

*Aspergillus niger*spores from PDA medium were aseptically transferred in laminar air flow to Vogel's media that was used as inoculum medium (Zia *et al.*, 2010). Vogel's medium composed of 2g glucose, 0.5g potassium dihydrogen phosphate, 0.2g yeast extract, 0.1g peptone, 0.5g trisodium citrate, 0.4g diammoniumsulphate, 0.02g magnesium sulfate heptahydrate and 0.2g ammonium nitrate in 100ml dist. H_2O (pH was maintained at 5.5). For homogeneous microbial growth, small marble beads were added in inoculum flask and autoclaved for sterilization for 15 min at 15 psi pressure and 121°C temperature. Then inoculum medium incubated for 36 hours at 30°C on an orbital shaker (150 rpm) that was then used for further mutagenesis experiments (Rasul*et al.*, 2011).

Strain Improvement/Mutagenesis

Chemical mutagen, N-nitroso-N-methylurea (NMU) was used to induce mutagenesis in *Aspergillus niger* for the improved GOX production.Spore suspension of*Aspergillus niger* was treated with NMU (0.1%) solution for selected time intervals that was 0, 20, 40, 60, 80, 100 and 120 minutes at 37°C under continuous/ constant stirring.Afterwards, all the fractions of spores suspension of *Aspergillus niger* collected were centrifuged at 13,000 rpm for 15 minutes to gather spores, then washed these microbial spores thrice with sterilized distilled water to eliminate mutagen from microbial spores, and then these spores re-dissolved in sterilized distilled water (Semashko*et al.*, 2000; Zia *et al.*, 2010). These re-suspended spore suspension was spread on the petri plates containing PDA supplemented with 1% triton X-100 (colony restrictor) with the help of sterilized spreader in laminar air flow and these plates were kept at 30°C in incubator till the colonies appeared.All experimentsconducted in sterilizedenvironment.Then efficiency of mutagen was assessed by drawing three-log kill curve. kill/ survival curve was drawn for the

selection of best mutation time for the hyper production of glucose oxidase enzyme (Petruccioli*et al.,* 1999; Zia *et al.,* 2010).

Screening and SelectionProcedures for Isolation of Optimistic Mutants

Optimal exposure time of NMU mutagen was screened by drawing three-log-kill curve (Zia *et al.,* 2010). At this phase almost ninety percent decrease in viability was determined.In this study Triton X-100 (at 1-2% vol/vol) was used as microbial colony restrictor (Khattab and Bazaraa, 2005) that limited the microbial growth by permitting the highly resistant colonies to grow.Microbial colonies present on optimal exposure time were then again subject to grow on triton X-100 (1%) for further screening. For precise screening, microbial colonies confirmed on colony restrictor were then subjected to selective marker, 2-Deoxy-D-glucos (1mg/mL) that is an analogue of glucose for further screening (Gromada and Fiedurek, 1997, Zia *et al.,* 2012b). Afterwards,microbial colonies that showed potential growth on 2-Deoxy-D-glucos comprising PDA medium were selected for further screening and verified further by enzyme diffusion zone test for optical and spectrophotometrically quantifiableinvestigation. Above selected potential mutant fungal colonies were then examined on 2% agar comprising petriplates by using peroxidase (225 U/mL) and O-dianisidine (0.1 g/L) for glucose oxidase (GOX) production.Clear color (brown) zone was seenround the fungal coloniesdue to the existence and production of GOX that was clearly measured and perceived (El-Enshasy, 1998). Fungal colonies that are capable of makingutmostzone diameter with better enzyme activity corresponding toits parental strain were selected for additionalscrutinization for GOX production. Results of enzyme diffusion zone test re-conformed by well test (Fiedurek*et al.,* 1986; Gromada and Fiedurek, 1997).

In well test, wells (approximately 0.3 mm) are formed in agar (2%) petriplates by pasteurized glass pipettes. Fungal spores kept in these wells and 0.1 mL reaction mixture (peroxidase + phosphate buffer + O-dianisidine) was added in these wells. After few minutes, clear brown zone formedround the fungal colonies, measure the zone diameter. Colonies having greater zone size have more ability to produce enzyme.The above screened colonies producing larger zone were measured and scratched, liquefied in (0.1 M) phosphate buffer (pH 6), filtered and then enzyme activity was quantitatively scrutinized on spectrophotometically at 460 nm (Zia *et al.,* 2010).

Enzyme production

Liquid state fermentation was used for glucose oxidase production from wild and mutant isolates of *Aspergillus niger*using pre-optimized medium conditions. Corn steep liquor (two percent) was employed as cheap substrate in this medium. Production medium is composed of glucose 4g, urea 0.3g, potassium dihydrogen phosphate 0.6g, calcium carbonate 0.04g and that was inoculated by inoculum (5%) in 100mL distilled water. The pH of production medium was maintained at 5 and media flasks were kept on rotary shaker for 36 hours at 150 rpm in triplicate and incubation temperature was adjusted at 30ºC (Zia *et al.,* 2010; Zia *et al.,* 2012c). Resultant crude product kept in cell homogenizer for 15 min for homogenization, and thenconsequent suspension was subjected to high speed centrifugation for the further removal of biomass at 10,000 rpm for 15 minutes(4ºC),subsequent suspension then clarifiedby Whatman filter paper (Gromada and Fiedurek, 1997;Rasul*et al.,* 2011).

Glucose oxidaseAssay

Enzyme activity of glucose oxidase was measured quantitatively in clarified suspension by spectrophotometric analysis (Worthington, 1988) at 460 nm for 3 to 5 minutes. Glucose oxidase activity was defined as quantity of enzyme needed to oxidize one unit of glucose in micromole per minute at 30ºC. In glucose oxidase enzyme assay, enzyme was analyzed by using1% orthodianisidinesoln, peroxidase enzyme and 18% D-glucose solnas a substrate.All the data given in this study is the average of measurements.

RESULTS AND DISCUSSION

The area of microbial enzyme applications are constantly increasing in various industries and it considers as a vitalphase in enzyme biotechnology field. Various fields of genetic engineering have worked together to achieve exceedingly dynamic enzyme producers. However, customaryproceduresi.e mutagenesis are

still successfully used for the improved production of different enzymes (Semashko*et al.*, 2000) because of its simplicity and cost effectiveness.*Aspergillus niger*is successfully used for the production of various commercially important enzymes because of its effective production ability. Previously *Aspergillus niger*had been genotypically upgraded by using different phys-chemical mutagens for the improved production of various industrially important microbial products (Rasul*et al.*, 2011; Zia *et al.*, 2012b).

Nitrosomethylurea inhibits synthesis of DNA and degraded cellular DNA (Rosenkranz*et al*. 1969), cause devitalization (Rosenkranz and Howard, 1970) and also known as primarily carcinogenic compound. In their study, they found that the DNA of treated E.coli was disrupted into the acid-soluble fragments/products. This type of solubilization may occurre due to the reaction between the nitrosomethylurea and cellular DNA or specific enzymatic excision of chemically modified cellular DNA. Biological action of nitrosomethylurea is influenced by the temperature and time duration of the exposure.

In this study all experiments carried out in triplicate. Awild fungal spore suspension (*Aspergillus niger)*exposed to NMU (0.1 percent concentration of N-nitroso-N-methylurea) that was used as chemical mutagen at different exposure time ranging from 0, 20, 40, 60, 80, 100 and 120 minutes (Fig.1).Previous investigations revealed that mutagenesis is aneconomical method to yieldupgraded enzyme producers that can be used for industrial enzyme production.

Positive NMU mutants of *Aspergillus niger*havingimproved GOX producing capability were screened out using various screening and selection methods. Firstly, three-log-kill curve was used to evaluate the optimal exposure time of NMU mutagen at which almost 90% fungal spores killing was occurred. From three-log-kill curve, it was revealed that optimal exposure time was hundred minutes, named as TS-NMU-100 and at this exposure time survival rate of fungal spores was 36.90% and killing rate was 63.1% (Fig.2). Semashko*et al*. (2000) found optimum exposure time at 90 minutes using NMU as mutagen and they also documented that effect of N-nitroso-N-methylurea depends on the NMU dose concentration and its exposure timeto the microbial spore.

Khattab and Bazarra (2005) documented that number of colonies decreased with the increase in the exposure time while quantity of resistant colonies rise with the growing exposure time and then afterward sharp dropwas found. Khattab and Bazarra(2005) used ethyl methane sulfonate (EMS) as chemical mutagen for enhanced

Table 1. Screening analysis of wild and mutant derived strains of *Aspergillusniger*

S.No	Strain	Diffusion Zone test(cm)	Well test (cm)	Enzyme activity/ Analytical test (U/ml)	%age increase in Enzyme activity
	N-nitroso-N-methylurea (NMU) Mutagenesis				
1.	Wild/control	0.9	1.1	11.01	100
2.	TS-NMU-2	1.2	1.4	13.91	126.34
3.	TS-NMU-5	1.2	1.5	14.34	130.25
4.	TS-NMU-7	1.1	1.15	12.21	110.90
5.	TS-NMU-9	1	1.2	13.02	118.26
6.	TS-NMU-13	1.4	1.8	16.2	165.3

Table 2. Glucose oxidase activity of NMU mutant strains of *Aspergillusniger* produced by liquid stat fermentation

Type	Enzyme activity (U ml^{-1})	%age increase in Enzyme activity
N-nitroso-N-methylurea (NMU) Mutagenesis		
Wild/control	12.43	100
TS-NMU-2	16.72	134.51
TS-NMU-5	22.47	180.77
TS-NMU-7	13.53	108.85
TS-NMU-9	14.80	119.07
TS-NMU-13	32.33	260.10

production of glucose oxidase. Zia, (2007) investigated the effect of MNNG and EB and found 82% and 76.135% killing of microbial spores respectively using kill curve determination.Haq*et al*. (2014) investigated the improved production of glucose oxidase from *Aspergillus niger* IIB-31 using EMS and nitrous acid as chemical mutagens via random mutagenesis and optimization of media components.

Coloniespresent on the optimal exposure time subjected to a review by the colony restrictor, triton X-100. It is a synthetic/chemical detergent that minimizes the growth of microorganisms. It causes a delay in microbial growth at logarithmic phase (Sun *et al.*, 2008). By three-log kill curve, 100 minutes was selected as optimum exposure time for NMU mutagen that comprises 13 colonies. When theseselected colonies subjected to PDA mediumcontaining triton X-100 (1%), out of these 13colonies, 7 colonies namely TS-NMU-1, TS-NMU-2, TS-NMU-5, TS-NMU-7, TS-NMU-9, TS-NMU- 12 and TS-NMU-13displayed much more growth resistance corresponding to rest of wild and mutant colonies of *A. niger* against the colony restrictor (Fig.3).

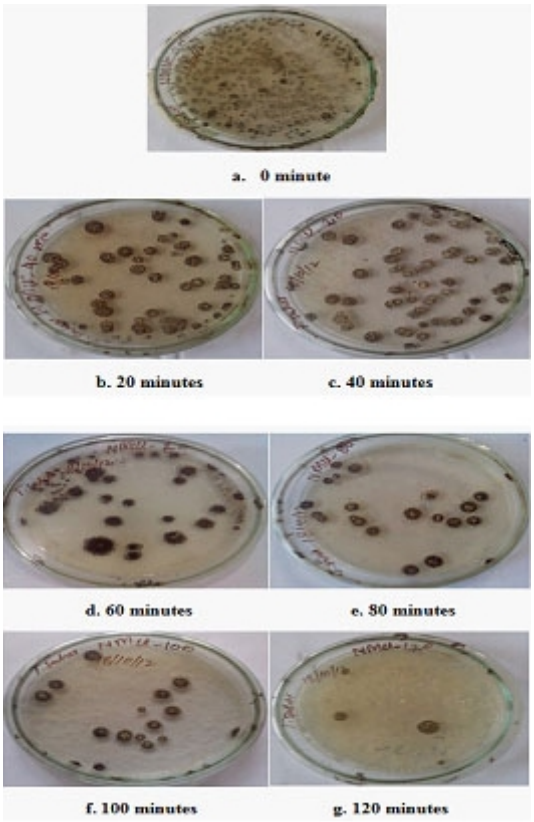

Fig.1. Colony restricted growth of *Aspergillusniger* at various exposure times on triton X-100 containing PDA plates for chemical mutagenesis (a. 0 minute (control), b. 20 minutes, c. 40 minutes, d. 60 minutes, e. 80 minutes, f. 100 minutes and g. 120 minutes of NMU exposure)

It restricts the growth of microbial colonies and produce yellowish color zone around the colonies by limiting the extent and sporulation of microbial colonies (Khattab and Bazaraa, 2005, Zia, 2007) and it used for the screening of highly potential mutant colonies having the overproducing capacity of glucose oxide by hindering the growth of low potential *Aspergillus niger* mutant colonies.

Coloniesthat verified on colony restrictor then subjected to 2-Deoxy-D-glucose (that is glucose analogue and has the 2-hydroxyl group replaced by hydrogen) at 1mg/mL levelwhich is used as selective marker for precise selection and screening. In case of chemical mutagenesis, 5 colonies out of seven selected colonies named as TS-NMU-2, TS-NMU-5, TS-NMU-7, TS-NMU-9 and TS-NMU-13 showed stronger/potential growth than the rest of others colonies onselective marker containing PDA plates (Fig.4). Microbial growth resistance against lethal2-deoxy-D-glucoseused as a criterion to isolatepositive mutantsfrom the rest of mutants (Azin and Noroozi, 2001).

Khattab and Bazaraa (2005) and Rasul*et al.* (2011) also investigated the role of 2-deoxy-D-glucoseas selective marker and screened out the potentially resistance UV mutants having improved capability of GOX production. Selective

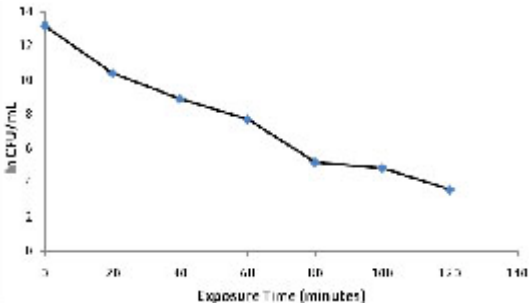

Fig. 2. Effect of N-nitroso-N-methylurea to formulate 3-log kill curve for chemical mutagenesis

marker is used in place of glucose thatis taken up by cell glucose transporters but 2-Deoxy-D-glucose cannotundergoglycolysis because of its 2-deoxy group unlike glucose (Pelicano*et al*., 2006).

Mutant colony isolates that displayedbettergrowth potential against selective marker further screened by enzyme diffusion zone test, well test andanalytical test for visual and quantifiableinvestigations. These methods are specific for the identification of positive mutants established on enzymatic reaction (Park *et al*., 2000 and Melherbe*et al*., 2003).

On the basis of enzyme diffusion zone test, it was found that*Aspergillus niger*TS-NMU-13 are finest positive mutant colony with enhanced

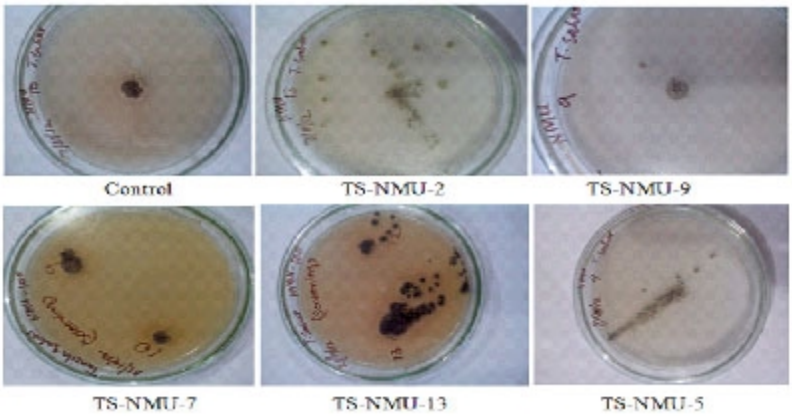

Fig. 3. Plate screening of selected dose by N-nitroso-N-methylurea mutagenesis

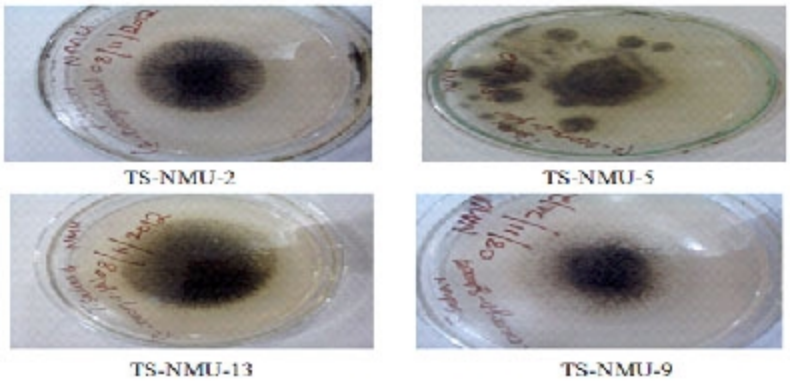

Fig. 4. Selection of positive mutants by selective marker for N-nitroso-N-methylurea mutagenesis

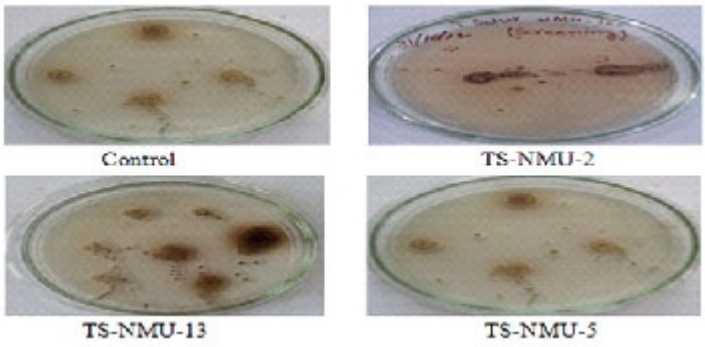

Fig. 5. Enzyme diffusion zone test for the selection of best mutant of N-nitroso-N-methylurea mutagenesis

diffused zones of GOX with 1.56 fold rise in enzyme activity corresponding to its parent wild strain (Fig. 5). While results obtained by enzyme diffusion zone test was re-conformed by employing well test. From well test, it was conformed that *Aspergillus niger*TS-NMU-13mutant colony have great potential for glucose oxidase production with 1.64 fold increase in enzyme activity as compared to wild strain(Fig. 6). Well test has some advantages as compared to diffusion zone test i.e. in case of diffusion zone test, there is a mixture of microbial spores or colonies may be present and reaction mixture may not completely absorbed by the microbial spores due to solid agar medium while in case of well test, only desired microbial colony spores are placed in well and reaction mixture is easily absorbed by the spores in the well.

Above selected and screened mutant colony isolates was further analyzed by analytical test for optical and quantifiable investigation using spectrophotometer.Results showed that TS-NMU-13(Table 1)havebetter capacity to yield GOX enzymeas compared to other mutant isolates and its parent wild strain that was used as control. Afterwards,selected mutant strain used for additionalinvestigation and it was found that TS-NMU-13 having 165.3% increase in glucose oxidase(GOX) activity corresponding to its parent wild strain as shown in Table (1).

Semashko*et al.* (2000) adopted a diffusion zone method to examine the optimistic UV and NMU mutant isolates. Park *et al.* (2000) also selected potential GOX producing mutants by using the enzyme diffusion zone analysis. Khattab and Bazaraa (2005) and Rasul*et al.* (2011) also exhibited an enzyme diffusion zone test for isolation and screening of potentially overproducing glucose oxidase mutants by measuring the size of dark brown zone formed around the colony. Fiedurek*et al.* (1986) reported that after UV rays treatment, GOX producing ability of *Aspergillus niger* increased almost 1.5-1.8 percent that was assessed diffusion zone test corresponding to its parent wild strain.

Production of glucose oxidase carried out from selected mutants colony isolates and wild stain of *Aspergillus niger* as control using pre-optimized fermentation medium conditions.Corn steep liquor (CSL)was used as acost-effectivesubstrate. The industrial by-products or wastes which usually manufactured in large amount and create problems for product safe removal thatare utilized in fermentation process. The industrialists and environmentalists also found troubles in recycling of some industrial waste products (Swain and Padhi,

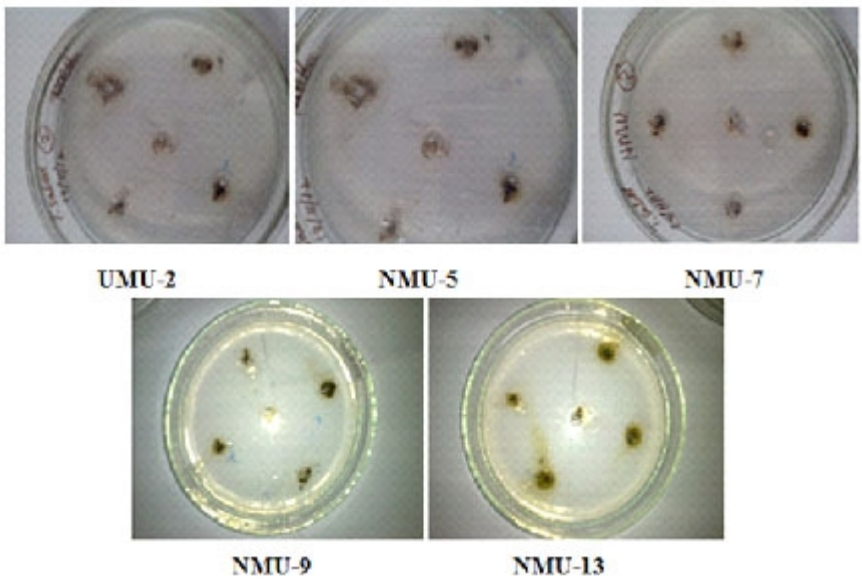

Fig. 6. Well test for the selection of positive mutant of N-nitroso-N-methylurea mutagenesis (central well represents the control)

2012),one of such industrial waste is CSL. CSL could be reprocessed by microbial fermentation because certain microorganismsutilizedCSL astheir nutrient source. All fermentation experimentswere conducted in triplicate using corn steep liquor at 2% concentration for 36 h of incubation (at 150 rpm rotation)at 30°Ctemperature.

Glucose oxidase activity of wild and mutants colonies examination (Table 2) clearly described that TS-NMU-13 mutant colony have potentially high enzyme activity corresponding to other mutant isolates and parental *Aspergillus niger* strain and this positive mutant isolate then named as TS-NMU-100, where 100 represents the optimum exposure time of mutagen. Mutant namely TS-NMU-100 had been selected in the present study displayed that there was almost 260.10% increase in GOX activity as compared to its parent wild strain (Table2).

Previously CSL had been studied by numerous investigators as economical source of carbon for commercial and laboratory scale production of various enzymes (Nascimento*et al.*, 2008). Our findings can be compared with the Semashko*et al.,* (2000), who found that NMU can be proficiently used for *Penicillium funiculosum* mutagenesisto enhance the production of glucose oxidase. Gromada and Fiedurek (1997)also recorded greater than 125% increase in the glucose oxidase production after mutagenesis of *Aspergillus niger*. Khattab and Bazaraa (2005) found that UV rays and ethyl methane was most effective mutagens and all fusants showed increase in activity from 285.5 to 394.2% as compared to the original strain. Gothoskar and Dharmadhikari, (2013) reported that EB as chemical mutagen is more affective for improved production of GOX from *A.niger* as compared to UV rays. UV rays produced 47% increase while EB produced 54% increase in glucose oxidase production from mutant isolates of A. niger over the parental strain. Previously, various studied revealed that glucose oxidase producing potential of *Aspergillus niger* and *Penicillium funiculosum* was considerably increased after mutagenesis (Semashko*et al.*, 2000; Zia *et al.*, 2012b and Haq*et al.*, 2014).

CONCLUSION

Based on the above results, it isdetermined that N-nitroso-N-methylurea (NMU) can be efficiently used to produced positive mutations in *A. niger* for the hyperproduction of GOX. It is also evaluated that mutant derived*Aspergillus niger*TS-NMU-100has a highly remarkablecapability for GOX production by consuming pre-optimized media nutrients and cultivation conditions corresponding to its parent strain of *A. niger*and it could be valuable for further advanceinvestigation and commercial scale production of glucose oxidase (GOX).

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