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ISOLATION AND GROWTH CHARACTERIZATION OF CHLORATE AND/OR BROMATE RESISTANT MUTANTS GENERATED BY SPONTANEOUS AND INDUCED FOREWORD MUTATIONS AT SEVERAL GENE LOCI IN *ASPERGILLUS NIGER*

Ghassan J. M. Kanan*, Heyam E. Al-Najjar

Department of Biological Sciences, Mu'tah University, Karak – Jordan, P. O. Box (7)

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ABSTRACT

We aimed her mainly to evaluate the contribution of newly employed bromate selection system, in obtaining new *Aspergillus niger* nitrate/nitrite assimilation defective mutants, through Ultraviolet treatment (UV), 1, 2, 7, 8-Diepoxyoctane (DEO), phenols mixture (Phx)) and spontaneous treatments. The newly employed bromate selection system was able to specify only two putative novel mutant types designated *brn* (bromate resistant but chlorate sensitive (RS) strain, which may specify nitrite specific transporter) and *cbrn* mutants (bromate resistant and chlorate resistant strain, which may specify nitrate/nitrite bispecific system). The most relevant and innovative findings of this research work involve the isolation of the RR (*cbrn*) mutants (a new type of nitrate assimilation defective mutants), that could be useful for studying the bispecific nitrate /nitrite transporter system. The majority of obtained bromate resistant mutants (93.3% of the total mutants obtained by all treatments) were of the *brn* type, whereas the remaining percentage (6.76%) was given to *cbrn* strains. The highest percentages of *brn* mutant strains (48% and 58.6% of the total RS strains) were obtained with UA after spontaneous and Phx treatment, whereas Trp has generated 29% and 42% of RS strains after UV and DEO treatments, respectively. The obtained ratios of *cbrn* mutants were higher (i.e. in the range of 8.4%-11.64% of the total bromate mutants) with chemical treatments, especially when U.A or Pro was serving as sole *N*-sources at 25ºC rather than 37ºC. A 69% mutants` yield of *Aspergillus niger* mutant strains representing nine gene loci (*niaD, cnx*-6 loci*, nrt and nirA*) were selected on the bases of chlorate (600 mM) toxicity. All chlorate resistant mutants were completely sensitive to bromate (250 mM). The *niaD* mutants showed the highest percentage (73.97%) of chlorate resistant mutants obtained with all tested treatments. The UV treatment has generated the highest ratio (86.9%) of *niaD* mutants, whereas, the least (61%) was obtained with Phx treatment. The highest percentage of *cnx* mutants (32%) was obtained with Phx treatment. The DEO treatment as compared to other tested treatments was the best to use for obtaining the highest ratios of either *nrt* (13.8%) mutants or *nirA* (1.9%) mutants.

Key words: *Aspergillus niger*. chlorate. bromate. mutagenesis

***Corresponding Author. Mailing address:** Dept of Biological Sciences; Mu'tah University Karak – Jordan; P. O. Box (7).; Tel: +962-032372380 Ext: 4223 Fax: +962-32375540 Mobile: 0795585053.; E-mail: gkanan@mutah.edu.jo

INTRODUCTION

The black mould *Aspergillus niger* has been subjected to various research and industrial uses for several decades since 1919, where its utility comes from strains ability to produce high levels of enzymes (8, 33). However, many mutant strains deficient in nitrate assimilation were isolated on the bases of chlorate toxicity through spontaneous and induced mutagenesis (5, 13, 16). Six phenotypic classes of chlorate resistant mutants (*nrtA, niiA, niaD, cnx, nirA* and *areA*) were characterized and designated as in *Aspergillus nidulans* (9, 13, 20). Genetic studies revealed that *niaD* and *niiA* genes are the structural gene for nitrate and nitrite reductase enzymes respectively, (11, 31). The transcription of both genes depends on two positively acting transcription factors, one is encoded by the *nirA* gene, (mediates nitrate and nitrite induction) and required for efficient transcription of *niaD, niiA*, and *nrtA* (6, 27). The second protein is encoded by the *areA* gene, mediates nitrogen metabolite repression, and is a general transcription factor needed for the efficient transcription of many genes involved in the utilization of different nitrogen sources (12, 20, 21). The *cnx* genes of *A. niger* and *A. nidulans* were found to be required for the biosynthesis of molybdenum cofactor, needed for the activity of both molybdoenzymes (4, 13, 32). Potassium bromate has been classified as a genotoxic carcinogen that induces DNA damage to prokaryotic and eukaryotic mutational test systems (3, 14). In addition, potassium bromate has caused kidney tumor to rodents (23, 36). However, due to its toxilogical effects, there have been a lot of controversies in the use of potassium bromate as a food and beverages additive or as flour improver (24). However, it has been employed here as a new selection system for the isolation of new *A. niger* nitrate assimilation defective mutants. The mutagen 1, 2, 7, 8-Diepoxyoctane (DEO) is one of the most potent mutagens in a wide variety of mutational test systems, including *Salmonella typhimurium* (25), *Saccharomyces cerevisia* (26), *A. nidulans* (17), and human epithelial cells (34). In addition, Ultraviolet light (UV) has widely been used as a mutagen, where it was able to produce different mutant types in *A. niger* and *A. nidulans* (13, 17, 20). However, the phenols Mixture (Phx) which is usually used as a standard solution for water pollution analysis, was reported as a mutagen and a carcinogenic agent, as listed by US Environmental Protection Agency (EPA) (29). To the best of our knowledge no previous mutagenic research methods were conducted for mutants selection (whether in prokaryotic or eukaryotic mutational test systems), using bromate, in addition to the traditional chlorate resistance selection system. Thus, we aimed here mainly to explore the contribution of newly employed bromate selection system for obtaining new *Aspergillus niger* nitrate/nitrite assimilation defective mutants, that might be useful for studying the existence of a bispecific nitrate /nitrite transporter system at the gene level. Furthermore, the current study aimed for elucidating the influence of mutagenic treatments employed (DEO, Phx, Ultraviolet and spontaneous treatment), the ten tested nitrogen sources and the selection temperatures (25º C and 37º C), on the nature of mutants obtained and on mutant yield.

MATERIALS AND METHODS

Wild-Type Fungal Strain and Media

The wild-type strain (with regard to nitrogen regulation) used for mutants isolation was [IMI60286 (ATCC 10864)]. Gene symbols are those in standard use (7). Standard *Aspergillus nidulans* growth media and handling techniques were as described by Clutterbuck (7).

Potassium Bromate and Chlorate Toxicity to *A. niger* Wild-Type Strain

Bromate toxicity (50, 100, 150, 200, 250, 300, 350, 400 and 500 mM) to wild-type strain was investigated by plating conidiospores suspension (approximately $1x10^8$ conidiospores/mL) on glucose supplemented minimal medium, containing a sole *N*-source and a definite bromate concentration as stated above. Similarly, different concentrations of potassium chlorate (100, 200, 300, 400, 500, 600, 700 and 800 mM) were also investigated.

Isolation of *A. niger* Bromate and/or Chlorate Resistant Mutants by Spontaneous and Induced Mutagenesis

Spontaneous Treatment: Mutant strains were isolated on the basis of chlorate (600 mM) and/or bromate (250 mM) resistance with a sole source of nitrogen as described previously (9). Ten nitrogen sources were used and these include: uric acid (UA), proline (Pro), glutamic acid (Glu), histidine (His), aspartic acid (Asp), arginine (Arg), tyrosine (Tyr), tryptophan (Trp), lysine (Lys) and cystine (Cys). Conidiospores were suspended in 5 mL normal saline-Tween 80 (0.05%), then adjusted to a concentration of $1x10^8$ conidiospores /mL. The 5 mL suspension was added to 20 mL of 0.1 M potassium orthophosphate buffer (pH 5.8) and incubated at room temperature for 40 min. Conidia were pelleted, washed twice with sterile distilled water and resuspended in sufficient volume of saline-Tween 80 solution. The suspension was divided into aliquots of 200 µL each, then plated on glucose supplemented minimal medium containing chlorate (600 mM) or bromate (250 mM), a sole *N*-source (10 mM) and sodium deoxycholate (0.08%) for colony size restriction (22). Cultured plates were incubated at either 25 ºC or 37 ºC.

Mutagenesis with 1,2,7,8-Diepoxyoctan (DEO): Mutagenesis with Diepoxyoctane was performed as described previously (1, 2) by adding 5 mL conidia (approximately $1x10^8$ conidiospores /mL) to 20 mL of 0.1 M potassium orthophosphate buffer (pH 5.8) containing 12 mg/mL 1,2,7,8-Diepoxyoctane (Aldrich chemical company). Treatment was for 40 min at 37° C. Conidia were pelleted (4233R Refrigerated Centrifuge) at 6000 rpm for 10 min, washed thrice with sterile distilled water and resuspended in sufficient volume of saline-Tween 80 solution. conidiospores were plated on selective madia and incubated as mentioned above.

Mutagenesis with Phenols Mixture (Phx): Mutagenesis with Phx mixture was performed as described above for DEO treatment, except that 12 mg/mL of phenols mixture were added to the conidiospores suspension. The tested Phx mixture was having the following constituents: 2,4-dinitrophenol, phenol, 4-nitrophenol, 2-chlorophenol, 2-methyl-4,6-dinitrophenol, 2-nitrophenol, 2,4-dimethylphenol, 4-chloro-3-methylphenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, and pentachlorophenol (29).

Mutagenesis with Ultraviolet Light (UV): A 25 mL conidiospores suspension (approximately $1x10^8$ conidiospores /mL) prepared in orthophosphate buffer as mentioned above, was irradiated with ultraviolet light (UV) at an intensity of 20 $J/m^2/s$ (UV-lamp UVKL-6U; 254 nm; Vetter GMBH, Wiesloch, Germany) for 40 min at room temperature. After irradiation conidiospores were processed by plating onto selective media as described for the spontaneous treatment.

Selection of Bromate and/or Chlorate Resistant Mutants

For confirmation of mutants obtained, the putative mutant strains grown on selective media post mutagenesis were further tested at both selection temperatures, on chlorate (600 mM) and bromate (250 mM) with a sole source of nitrogen (10 mM). Assignment of mutations to their gene loci was carried out according to the criteria described previously by Cove (9) and Kanan (17). The assignment of mutations involved replica plating of mutant strains onto the following media types: (i) potassium bromate (250 mM) with uric acid (5 mM) as the best serving sole nitrogen source, (ii) potassium chlorate (600 mM) with proline (10 mM) as a sole nitrogen source, (iii) potassium nitrate (10 mM), (iv) potassium nitrite (5 mM), (v) hypoxanthine (5 mM).

Statistical Analysis

One way ANOVA was carried out to determine the significant effect of tested *N*-sources and selection temperatures on mutants yield. This was followed by independent samples T-test and Post Hoc (Tukey) multiple comparison, to determine the level of significance for tested *N*-sources, selection temperatures and their interactions, on ratios of generated mutants in different gene loci. Multivariate ANOVA and ANCOVA tests, using the SPSS program devised for Windows (version 10) were also used to determine the significant effect of applied treatments and other sources of variance.

RESULTS

Potassium Bromate and Chlorate Toxicity to *A. niger* Wild-Type Strain

Results obtained indicate that bromate and chlorate toxicity to *A. niger* wild-type strain were achieved at a concentration of 250 mM and 600 mM respectively, where complete inhibition of fungal growth was reached.

Conidiospores Viability Assays After Mutagenic Treatments

Viability tests were performed concurrently with mutagenic treatments and the estimated viabilities in term of survivals/mL for spontaneous, DEO, Phx and UV treatments were $8.0x10^4$, $1.5x10^5$, $2.8x10^5$ and $3.3x10,^5$ respectively. However, the percentages of survivals after indicated treatments have reached approximately 100%, 35.4%, 50.32% and 56.63%, respectively.

Effect of Treatments on Proportions of Chlorate Resistant Mutant Classes

Results presented in Table 1 indicate that a sum of 6139 nitrate assimilation defective mutant strains (i.e. 68.98% mutants` yield) in nine different loci (*niaD*, *cnx*-6 loci, *nrt* and *nirA* genes) were isolated on the bases of chlorate (600 mM) toxicity using four mutagenic treatments. However, the obtained mutants` yield (expressed as percentages) after spontaneous, DEO, Phx and UV treatments, were 47.9%; 90%; 68%; and 93.4%, respectively (Table 1). These mutants were chlorate resistant (600 mM) but bromate (250 mM) sensitive strains (designated SR-type). The chlorate resistant mutant strains were selected at two temperature regimes (25º C and 37º C). However, results of T-test indicate that the selection temperature has not significantly influenced the ratios of generated mutants with either spontaneous treatment (*P* values ranged from 0.761-0.942) or UV treatment (*P* values ranged from 0.226-0.398). The selection at these temperatures (25º C and 37º C) did not significantly influence the ratios of generated mutants when Arg, His, Pro, Glu, Asp and Tyr were used as sole *N*-sources with all treatments, where approximately equal ratios of mutants have been obtained at both temperatures. The above mentioned amino acids (except Tyr) were serving as the best sole sources of nitrogen (generated the highest mutants` yield) for mutants selection with all treatments employed (Table 1). The proportions of confirmed mutant classes (at several gene loci) obtained with these amino acids have ranged from 14.5% to 17.4%; 12.9% to 20.3%; 15.6% to 23.4% and 19.5% to 23.4% when spontaneous, DEO, Phx and UV treatments, respectively were employed. The amino acids Lys and Trp did not effectively serve as sole sources of nitrogen for mutants selection at 25º C with all treatments employed (except DEO treatment where, approximately equal ratios of mutants were achieved at both selection temperatures), where no single mutant was selected under these conditions. In contrast, when selection was performed at 37º C with the four mutagenic treatments the same amino acids (Lys and Trp) have generated mutants` yield in the range of 2.9%-7.8% and 3.9%-17.4%, respectively (Table 1). Uric acid was not serving as a sole *N*-source for mutants selection at 37º C with all treatments (except with DEO treatment; equal ratios were obtained at both 25º C and 37º C), where no single mutant was obtained at this temperature. Furthermore, the amino acid Cys was not the *N*-source of choice for mutants selection at either 25º C and 37º C, where no single mutant was obtained with all treatments employed (except with DEO treatment; mutants were obtained in 1:2 ratio at 25º C and 37º C, respectively). Results of Multivariate ANCOVA indicate that the DEO treatment as compared to spontaneous treatment has significantly influenced the ratios of generated mutant types (except *niaD* mutants (*F* value = 1.84)). The calculated *F* values (149.19, 136.7 and 49.53) for *cnx*, *nrt* and *nirA* mutants, respectively were higher than the critical *F* value (4.046) at *df1* and *df49*. Furthermore, the ratios of generated mutants (except for *nrt* mutants; *F* value = 2.05) were significantly influenced by the Phx mutagenic treatment. The calculated *F* values (49.63, 42.70 and 35.86) for ratios of *niaD*, *nirA* and *cnx* mutants, respectively were higher than the tabulated *F* value (4.07) at *df1* and *df49*. The

UV treatment had significantly influenced the ratios of *niaD*, *cnx* and *nirA* mutants (but not that of *nrt* mutants), where the calculated *F* values for these mutants (185.05, 126.95 and 36.17 respectively), were higher than the tabulated *F* value (4.07) at *df1* and *df44*. Results of one way ANOVA indicate that the used *N*-source with all mutagenic treatments had significantly (*P* values ranged from 0.001-0.026) influenced the ratios of obtained mutants in the investigated gene loci (*niaD*, *cnx*, *nrt*) except *nirA* gene (*P* values ranged from 0.098-0.165). The *niaD* mutants as compared to other mutant classes have represented the highest percentage (73.97% of total mutants) of mutants yield obtained with all treatments. The obtained yields of *niaD* mutants were 86.9%; 72.5%; 71.2% and 61% with UV, spontaneous, DEO and Phx treatment, respectively. Presented results (Table 1) indicate that the amino acid Lys was serving as the best sole source of nitrogen for obtaining *niaD* mutants with all mutagenic treatments (except for Pro where, a 93% mutants` yield was obtained with UV treatment), where a percentage of 84% to 90% of the total confirmed mutants was achieved with Lys. However, these percentages of mutants have reflected 3.4% to 17.5% of the total *niaD* mutants produced under indicated conditions. Furthermore, results of Post Hoc (Tukey) multiple comparisons indicate that all *N*-sources (except Cys and UA) which were used with the spontaneous treatment had significantly ($P<0.05$) influenced the ratios of generated *niaD* mutants. The highest percentage of *niaD* mutants (84% of the total confirmed mutants) was obtained with Lys while the least (69%) was generated with Pro. In addition, there were significant differences (*P* values ranged from 0.011-0.033) in *niaD* ratios obtained with His and those obtained with UA, Trp and Tyr after Phx treatment, where the highest percentage of mutants` yield (61%) was obtained with His. Moreover, there were significant differences (*P* values ranged from 0.014-0.045) in *niaD* mutants obtained with Pro (comprising 93%) and ratios generated with UA, Trp or Tyr after UV treatment. The use of Arg, Glu and Pro as sole sources of nitrogen with DEO treatment did not reflect significant difference in obtained ratios of *niaD* muants (*P* values ranged from 0.997-1.000). The obtained *cnx* mutants were ranked the second in terms of mutants yield proportion (17.80%), with all treatments (except DEO treatment). The percentages of *cnx* mutants` yield from the total confirmed mutant classes have reached 32%; 21.7%; 10.8% and 9% with Phx, spontaneous, DEO and UV treatment, respectively. The tested *N*-sources with spontaneous treatment (except Cys) showed no significant differences ($P<0.05$) in ratios of *cnx* and *nirA* mutants which were isolated with the least percentage (Table 1). The amino acid Trp was serving as the best *N*-source for obtaining *cnx* mutants with spontaneous and UV treatments where, the mutants yield has reached 32% and 20% of the total confirmed mutant classes, respectively. However, this reflects 8.5% and 12.6% of the total *cnx* mutants produced under the indicated conditions. Furthermore, UA and Arg have generated 28% and 41% *cnx* mutants` yield with DEO and Phx treatments, respectively. This reflects 9.6% and 19.9% of the total *cnx* mutants generated by the indicated treatments (Table 1). There were significant differences (*P* values ranged from 0.020-0.043) in *cnx* ratios obtained with either Pro or Asp after DEO treatment and that obtained with Lys or Tyr (Table 1), where the highest percentage (12.9%-16.6%) was obtained with the former amino acid. However, the tested *N*-sources with the UV treatment have not reflected significant differences in ratios of *cnx* and *nrt* mutants. The *nrt* mutants were ranked the third in terms of mutants` yield (6.4%) from the total confirmed mutants with all treatments (except DEO treatment; ranked the second (13.76%)), where a range from 4% (with either Phx or UV treatment) to 4.6% (with spontaneous treatment) was reached (Table 1). The highest percentages of *nrt* mutants were achieved with Arg and His in all treatments (except DEO treatment; Cys has generated a 21% yield), where a range of mutants` yield from 5% to 8% of the total mutant classes was achieved (Table 1). Moreover, the used *N*-sources with spontaneous treatment had reflected significant differences in ratios of *nrt* mutants ($P<0.05$) where, the obtained highest percentage (i.e. in the range of 6.8%-8%) was related to the influence of either Arg or His. There were significant differences (*P* values have ranged from 0.001-0.038) in ratios of *nrt* mutants obtained with Arg after DEO

treatment and those obtained with the rest of tested *N*-sources, where the highest proportion from the total confirmed mutants was generated with Arg. In contrast, *nirA* mutants` yield was found to be the least obtained (1.9%) with all tested treatments, where a range from 0.0% (with UV treatment) to 4.4% (with DEO treatment) mutants` yield from the total confirmed mutants generated by the specified treatment was achieved (Table 1). There were siginificant differences (*P* values in the range of 0.006-0.034) in ratios of *nrt* mutants and *nirA* mutants obtained with Arg and those obtained with Lys, UA, Trp and Tyr after Phx treatment. The former amino acid has participated in the generation of the highest mutants` yield for *nrt* mutants (6.5%) and *nirA* mutants (3.5%), while the latter amino acids have generated the least percentages (Table 1).

Table 1. Numbers and percentages of confirmed chlorate (600 mM) resistant *Aspergillus niger* mutant classes generated after spontaneous and induced mutagenic treatments.

N-source [a]	**Spontaneous Treatment**									
	Confirmed mutant classes		***niaD* mutants**		***cnx* mutants**		***nrt* mutants**		***nirA* mutants**	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Arg	300	17.4	216	72	57	19	24	8	3	1
His	250	14.5	193	77	35	14	17	6.8	5	2
Lys	50	2.9	42	84	6	12	2	4	-	-
Pro	250	14.5	172	69	65	26	10	4	3	1
U.A	50	2.9	34	68	15	30	1	2	-	-
Glu	300	17.4	231	77	51	17	12	4	6	2
Asp	300	17.4	210	70	81	27	6	2	3	1
Trp	100	5.8	64	64	32	32	4	4	-	-
Tyr	125	7.2	88	71	33	26	4	3	-	-
Cys	-	-	-	-	-	-	-	-	-	-
Total No of mutants	1725		1250		375		80		20	
N-source	**DEO Treatment**									
	Confirmed mutant classes		**No of *niaD***		**No of *cnx***		**No of *nrt***		**No of *nirA***	
	No	%	No	%	No	%	No	%	No	%
Arg	275	20.3	180	65.5	15	5.5	50	18	5	1.8
His	125	9.2	78	62	14	11	25	20	8	6.4
Lys	50	3.7	42	84	3	6	5	10	-	-
Pro	225	16.6	165	73	29	12.9	18	8	13	5.8
U.A	50	3.7	28	56	14	28	7	14	1	2
Glu	225	16.6	177	78.7	16	7	23	10	9	4
Asp	175	12.9	133	76	29	16.6	9	5	5	3
Trp	125	9.2	84	67	8	6	24	19	9	7
Tyr	50	3.7	33	66	6	12	9	18	2	4
Cys	75	5.5	45	60	12	16	16	21	7	9
Total No of mutants	1356		965		146		186		59	
N-source	**Phx Treatment**									
	Total mutants		**No of *niaD***		**No of *cnx***		**No of *nrt***		**No of *nirA***	
	No	%	No	%	No	%	No	%	No	%
Arg	200	15.6	98	49	82	41	13	6.5	7	3.5
His	300	23.4	183	61	96	32	15	5	6	2
Lys	50	3.9	42	84	6	12	1	2	1	2
Pro	200	15.6	124	62	64	32	6	3	6	3
U.A	25	2	15	60	8	32	1	4	1	4
Glu	200	15.6	128	64	56	28	8	4	8	4
Asp	200	15.6	132	66	60	30	4	2	4	2
Trp	50	3.9	30	60	17	34	2	4	1	2
Tyr	50	3.9	31	62	16	32	2	4	1	2
Cys	-	-	-	-	-	-	-	-	-	-
Total No of mutants	1283		783		413		52		35	
N-source	**UV Treatment**									
	Total mutants		**No of *niaD***		**No of *cnx***		**No of *nrt***		**No of *nirA***	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Arg	250	19.5	199	79.6	36	14.4	19	7.6	-	-
His	300	23.4	256	85	27	9	15	5	-	-
Lys	100	7.8	88	88	8	8	4	4	-	-
Pro	350	27.3	325	93	18	5	7	2	-	-
U.A	25	2	21	84	3	12	1	4	-	-
Glu	300	23.4	264	88	23	8	12	4	-	-
Asp	300	23.4	270	90	21	7	7	2	-	-
Trp	100	7.8	76	76	20	20	4	4	-	-
Tyr	50	3.4	42	84	6	12	2	4	-	-
Cys	-	-	-	-	-	-	-	-	-	-
Total No of mutants	1775		1543		159		73		-	

Mutant strains were selected at two temperature regimes (25 ºC and 37ºC) on glucose supplemented minimal medium containing potassium chlorate (600 mM) and a sole N-source (10 mM). [a] Arg denotes Arginine; His: Histidine; Lys:Lysine; Pro: Proline; U.A: Uric acid; Glu: Glutamic acid; Asp: Aspartic acid; Trp: Tryptophane, Cys: Cysteine and Tyr: Tyrosine. Total number of screened colonies from mutagenesis plates was 3600; 1500; 1900 and 1900 for spontaneous; DEO; Phx and UV treatment respectively. Total number of confirmed resistant mutants was 1725; 1356; 1283 and 1775 for spontaneous, DEO, Phx and UV treatments, respectively.

Effect of applied Treatments on Proportions of Bromate Resistant Mutants

Results presented in Table 2 indicate that 976 mutant strains from a total of 1530 (i.e. 63.8%) screened colonies were isolated on the bases of bromate resistance (250 mM), after four mutagenic treatments (i.e. spontaneous; DEO; Phx and UV treatment). However, the obtained mutants' yield after spontaneous, DEO, Phx and UV treatments were 7.5%, 76.8%, 85.6% and 88%, respectively. The selected mutants were found to represent two phenotypic classes. The first, showed high resistance to bromate but complete sensitivity to chlorate (RS-type) and these were designated *brn* mutant strains (i.e. bromated resistant nitrate utilizing). The second type of mutants showed high resistance to both selective agents (i.e. bromate and chlorate; RR-type) and designated *cbrn* strains (chlorate and bromated resistant nitrate utilizing). The *brn* mutant strains were found to constitute 93.3% of the total confirmed mutants, whereas *cbrn* strains have constituted just 6.76%. However, the highest percentages of RS mutants (i.e. 58.6% and 48%; expressed as percentages from the total confirmed RS strains) were obtained with UA after spontaneous and Phx treatments, whereas Trp has generated 29% and 42% of Rs strains after UV and DEO treatments, respectively (Table 2). Results of multivariate ANOVA indicate that the selection temperature, the used *N*-source with the treatments and the interaction between both factors have significantly influenced the ratios of mutants generated after spontaneous, DEO, Phx and UV treatments. Consequently, results presented in Table 3 indicate that the calculated *F*-values for strains with the RS and the RR phenotypes were significantly higher than the tabulated *F*-values in all tested treatments. Results indicate that three (UA, Pro, and Trp) out of ten tested *N*-sources have served effectively as sole sources of nitrogen with all treatments. Uric acid (UA) was serving at 25º C rather than 37º C as sole *N*-source with all treatments except with Phx treatment, where the selection of mutants was successful at both temperatures (25º C and 37º C). The generated mutants' yields with UA were 60%, 39%, 50% and 28% after spontaneous, DEO, Phx and UV treatments, respectively (Table 2). The amino acid Pro was serving as sole *N*-source for mutants selection at 25º C with spontaneous and DEO treatments, whereas, with Phx and UV treatments the selection was performed at both temperatures (25º C and 37º C) using the same amino acid (Pro). However, when Trp was used with the four tested mutagenic treatments the selection of mutants was successful at both selection temperatures. Furthermore, the amino acids Arg, Glu and His were not effectively acting as sole *N*-sources for the isolation of mutants at any selection temperature after spontaneous treatment. In contrast, all mentioned amino acids were effectively serving as sole *N*-sources at 37º C with the UV treatment. Moreover, mutant strains with the RR phenotype (i.e. *cbrn* strains) were only selected at 25º C in all treatments tested (except Phx; mutants were obtained at both selection temperatures) when either Pro or UA was used. However, the proportions of obtained mutants (i.e. with the RR phenotypes) from the total confirmed RR mutant strains were in the range of 0.0% to 100 %; 47% to 53% and 46% to 54% after spontaneous, DEO and UV treatments, respectively. The mutants' yields obtained with UA and Pro after Phx treatment were 22.2% and 77.8%, respectively (Table 2). Results of multivariate ANCOVA (Covariance) indicate that the chemical (DEO and Phx) and physical (UV) treatments as compared to spontaneous treatment have significantly influenced the ratios of generated bromate mutants (i.e. RS and RR types), where the calculated *F*-values were higher than the tabulated values (Table 4). However, the selection temperature had significantly influenced the effect of DEO treatment on mutants' ratios, where the calculated *F*-values i.e. 54.56 and 15.24 for RS and RR mutant types, respectively were higher than the tabulated *F*-value (4.17) at *df1* and *df 29* (Table 4). Moreover, the effect of Phx treatment was greatly influenced by the *N*-source used. The obtained *F*-values for RS and RR strains were 12.11 and 4.84, respectively, whereas, the tabulated *F*-value was 2.69. The proportion of mutant strains with the Rs phenotype only was significantly influenced by selection temperature, where the obtained *F*-value was 27.26 as compared to 4.17 for the tabulated value (Table 4). The effect of UV treatment was

highly influenced by the *N*-source used, where the calculated *F*-value i.e. 8.08 and 8.79 for RS and RR phenotypes, respectively were higher than the tabulated *F*-value (2.49) at *df5* and *df35* (Table 4). However, the selection temperature showed no significant influence on the treatment action, where the calculated *F*-value was less than the tabulated value (Table 4).

Table 2. Numbers and percentages of confirmed bromate (250 mM) resistant *Aspergillus niger* mutant classes generated after spontaneous and induced mutagenic treatments.

Spontaneous Treatment						
N-source [a]	**Confirmed mutant classes**		**brn mutants (strains with RS phenotypes)**		**Cbrn mutants (strains with RR phenotypes)**	
	No	**% from total**	**No**	**% from total Rs**	**No**	**% from total RR**
UA	18	60	17	58.6	1	100
Pro	6	20	6	20.7	-	-
Trp	6	20	6	20.7	-	-
Total screened mutants	400					
Total confirmed mutants	30		29		1	
% of mutant yield	7.5		96.7		3.3	
DEO treatment						
N-source [a]	**Confirmed mutant classes**		**brn mutants (strains with RS phenotypes)**		**Cbrn mutants (strains with RR phenotypes)**	
	No	**% from total**	**No**	**% from total Rs**	**No**	**% from total RR**
UA	114	39	98	38	16	47
Pro	126	43	108	42	18	53
Trp	40	13.7	40	15.5	-	-
Arg	8	2.8	8	3	-	-
Glu	4	1.4	4	1.6	-	-
Total screened mutants	380					
Total confirmed mutants	292		258		34	
% of mutant yield	76.8		88.4		11.64	
Phx treatment						
N-source [a]	**Confirmed mutant classes**		**brn mutants (strains with RS phenotypes)**		**Cbrn mutants (strains with RR phenotypes)**	
	No	**% from total**	**No**	**% from total Rs**	**No**	**% from total RR**
UA	108	50	94	48	14	77.8
Pro	42	19.6	38	19.4	4	22.2
Trp	34	15.9	34	17	-	-
Arg	-	-	-	-	-	-
Glu	6	2.8	6	3	-	-
His	24	11	24	12	-	-
Total screened mutants	250					
Total confirmed mutants	214		196		18	
% of mutant yield	85.6		91.6		8.4	
UV treatment						
N-source [a]	**Confirmed mutant classes**		**brn mutants (strains with RS phenotypes)**		**Cbrn mutants (strains with RR phenotypes)**	
	No	**% from total**	**No**	**% from total Rs**	**No**	**% from total RR**
UA	124	28	118	27.6	6	46
Pro	118	27	111	26	7	54
Trp	124	28	124	29	-	-
Arg	18	4	18	4.2	-	-
Glu	50	11.4	50	12	-	-
His	6	1.4	6	1.4	-	-
Total screened mutants	500					
Total confirmed mutants	440		427		13	
% of mutant yield	88		97		3	

Mutant strains were selected at two temperature regimes (25 ºC and 37ºC) on glucose supplemented minimal medium containing potassium bromate (250 mM) and a sole N-source (10 mM). [a] U.A: denotes Uric acid; Pro: Proline; Trp: Tryptophane; Arg Arginine; His: Histidine; Glu: Glutamic acid. [b] RS denotes bromate resistant but chlorate sensitive strain. [c] RR denotes bromate resistant and chlorate resistant strain.

Table 3. Multivariate ANOVA test for the influence of temperature and *N*-source on treatments` efficiency in obtaining bromate mutants.

Source	Dependent Variable	F-value	F-critical	df
Spont/N-source	**RS**	**423.0**	**5.14**	**2.0**
	RR	**121.0**		
Temperature	**RS**	**1513.0**	**5.99**	**1.0**
	RR	**121.0**		
N-source*Temperature	**RS**	**379.0**	**5.14**	**2.0**
	RR	**121.0**		
Error	**RS**			**6.0**
	RR			
DEO/N-source	RS	466.0	3.48	4.0
	RR	**1135.0**		
Temperature	**RS**	**5550.0**	**4.96**	**1.0**
	RR	**3025.0**		
N-source*Temperature	**RS**	**356**	**3.48**	**4.0**
	RR	**1135**		
Error	**RS**			**10.0**
	RR			
Phx/N-source	RS	709.0	3.48	4.0
	RR	**762.0**		
Temperature	**RS**	**3713.0**	**4.96**	**1.0**
	RR	**864.0**		
N-source*Temperature	**RS**	**795.0**	**3.48**	**4.0**
	RR	**357.0**		
Error	**RS**			**10**
	RR			
UV/N-source	RS	6870.0	3.11	5.0
	RR	**781.0**		
Temperature	**RS**	**4284.0**	**4.75**	**1.0**
	RR	**48.0**		
N-source*Temperature	**RS**	**13013.0**	**3.11**	**5.0**
	RR	**154.0**		
Error	**RS**			**12.0**
	RR			

Table 4. Multivariate ANCOVA for the influence of treatments as compared to spontaneous treatment in generating bromate mutant classes.

Source	Dependent Variable	F-value	F-critical	df
UV treatment	**RS**	**23.9**	**4.15**	**1.0**
	RR	**6.75**		
N-source	**RS**	**8.08**	**2.49**	**5.0**
	RR	**8.79**		
Temperature	**RS**	**0.83**	**4.15**	**1.0**
	RR	**2.79**		
N-source*Temperature	**RS**	**7.86**	**2.49**	**5.0**
	RR	**4.60**		
Error	**RS**			**35.0**
	RR			
Phx treatment	**RS**	**18.07**	**4.17**	**1.0**
	RR	**8.31**		
N-source	**RS**	**12.0**	**2.69**	**4.0**
	RR	**4.84**		
Temperature	**RS**	**27.26**	**4.17**	**1.0**
	RR	**3.15**		
N-source*Temperature	**RS**	**1.74**	**2.69**	**4.0**
	RR	**1.21**		
Error	**RS**			**29.0**
	RR			
DEO treatment	**RS**	**5.37**	**4.17**	**1.0**
	RR	**6.77**		
N-source	**RS**	**12.80**	**2.69**	**4.0**
	RR	**6.03**		
Temperature	**RS**	**54.56**	**4.17**	**1.0**
	RR	**15.24**		
N-source*Temperature	**RS**	**4.89**	**2.69**	**4.0**
	RR	**6.03**		
Error	**RS**			**29.0**
	RR			

DISCUSSION

The highest yield of chlorate resistant mutants was obtained after UV treatment (93.4%) this was followed by a 90% yield obtained with the DEO treatment. Concerning chlorate toxicity it had been previously proposed that chlorate itself is not toxic, but it renders toxic when it is converted (*In vivo*) to chlorite by the action of the molybdoenzyme nitrate reductase. i..e. chlorate serves as an analogue for nitrate during reduction in bacteria, fungi, algae and higher plants (10, 19). However, this might not be the mechanism of action for chlorate, because not all mutants lacking nitrate reductase are chlorate resistant, i.e. both chlorate sensitive and resistant mutants lacking nitrate reductase. A possible explanation for chlorate toxicity is that it mimics nitrate and lead to a shutdown of nitrogen metabolism, as chlorate can not act as nitrogen source (11). In addition, an important feature should be met to demonstrate that chlorate and nitrate share a common transport system is that they should each competitively inhibit the uptake of the other ion (19). Concerning the Specificity and effectiveness of employed mutagenic treatments in inducing forward mutations in *Aspergillus niger* at several gene loci, the *niaD* mutants were obtained with the highest percentage (73.97% of the total generated mutants) with all mutagenic treatments whatever, the used *N*-source or the selection temperature. However, *cnx* mutants have ranked the second, in terms of total mutants yield (17.8%) obtained with all treatments (Table 3). Furthermore, Phx treatment as compared with other treatments has generated the highest *cnx* ratio (32%). The high relative frequency of this mutant type may suggest that the *niaD* gene contains one or more mutational hot spots that became easily altered with reduced capacity for DNA repairing. These findings disagreed with that obtained previously by Cove (9, 10) which indicated that *cnx* genes were more susceptible to mutations than *niaD* gene. However, our results agreed with the findings of Kanan et al. (18). Moreover, it seems likely that *nrt* (generated with a 6.4% yield of the total mutants) and *nirA* (obtained with a yield of 1.9%) genes were relatively less prone to mutations whatever the applied treatment, the *N*-source or the selection temperature used and this agreed with the findings of Cove (9) and also with that of Kanan et al. (18). The success of certain *N*-source in generating high ratio of specific mutants type under specific treatments` conditions but not the other, could be related to the type and position of mutation in the altered gene(s) involved in the utilization of different *N*-sources. This kind of alteration (either in the transcriptional factors or in *N*-source utilization genes) could make one nitrogen source unable to be metabolized under certain treatments` conditions but not the others. These suggestions confirm not unexpectedly the previous findings (9, 12, 18, 20, 21) which stated that the effective transcription of genes involved in *N*-sources utilization, the type of *N*-source used, the selection temperature and the interactions between them had significantly influenced the ratio of selected mutants. The spontaneously generated mutations comprise the ultimate source of natural genetic variation that is seen in populations. The frequency at which spontaneous mutations occur is low, since these are the resultants of low level inherent metabolic errors, or they may actually caused by unknown agents present in the environment (30). The DEO mutagen was preferentially affecting the treated conidiospores more than UV and Phx treatments, and this confirm the previous findings of Yunes, et. al. (37) stated that DEO was more carcinogenic and mutagenic than the other analogues. The DEO mutagen reacts preferentially with the N_7 of guanosine and the N_3 of adenine, then induces both mono-adducts and inter-strand cross-links by specific genotoxic pathway in absence of cell death, (34, 37). However, the UV-induced thymine dimers cause mutations in two ways. (I) dimers perturb the structure of DNA double helices and interfere with accurate DNA replication. (II) Errors occur during the cellular processes that repair defects in DNA (30, 35). Furthermore, previous results revealed that potassium bromate was mutagenic to bacteria, and this activity was mediated by the formation of oxidative damage to DNA, where the reduction of bromate under aerobic and anaerobic conditions was attributed to chemical rather than biological reduction (28). However, as chlorate, bromate was found to act as electron acceptor for bacteria, thus it is likely to be used in

the same enzymatic reduction (15). This may lead to the suggestion that bromate toxicity arises as a result of mimicking nitrite (being its toxic analogue), so it might alter specific genes responsible for the active uptake of nitrite, which suggests that *brn* strains (with the RS phenotypes) may specify a nitrite specific system independent from the nitrate transport system specified by *nrt* gene (with the SR phenotypes). Furthermore, the occurance of *cbrn* strains with RR phenotypes may specify specific components for a complex bispecific transporter that is responsible for the transport of both nitrate and nitrite. The mutation at the *cbrn* locus may be influenced by the nature of *nrt* and *brn* genes, which suggests overlapping sequence, where the *cbrn* alteration may arise as a result of double loss mutation in both genes. Furthermore, if the recombinant progeny (i.e. non-parental recombinants) are recovered from out crosses between the wild-type and *nrt* or *brn* mutants, this suggests that chlorate and bromate resistant may occur due to a complex locus that co-segregates as a single gene or two tightly linked genes. This locus is independent from the chlorate specific one which is represented by *nrt* mutants showing complete sensitivity to bromate but high resistance to chlorate (SR-type). In addition, bromate resistance in *brn* strains that show high resistance to bromate but not to chlorate i.e. RS phenotypes may result from a distinct locus that is independent from the bispecific one (RR-type) or the chlorate specific locus. These suggestions would agree with that of Kanan (17) who stated that results of recombination analysis and biochemical studies on the *A. nidulans brn* and *cbrn* mutant strains suggest that these strains may specify a nitrite specific and nitrate/nitrite bi-specific transporters, respectively.

CONCLUSIONS

As a consequence of the double selection for nitrate assimilation defective mutants on chlorate and bromate, new phenotypes (i.e. with RS and RR phenotypes) were observed. This selection system which is based on bromate resistance is considered valuable in terms of applying it in addition to the classical chlorate system as a selective agent for mutants` selection. The isolation of mutant strains (*brn* mutants) with the RS phenotype, i.e. showing the antagonistic phenotypes of the SR chlorate mutants is also valuable for the characterization of nitrite specific transporters. The most relevant and innovative findings of this research work is the isolation of mutants with the RR phenotypes (i.e. *cbrn* mutant strains) which are new form of nitrate assimilation defective mutants that could be useful for studying the bispecific nitrate/nitrite transport system. However, 93.3% of the total isolated bromate resistant mutants were of the *brn* type (i.e. RS phenotypes), whereas the *cbrn* mutant strains (RR phenotypes) have constituted just 6.8%. The *brn* strains were obtained at a percentage of 58.6% and 48% of the total RS strains when UA was used as sole *N*-source with the spontaneous and Phx treatments. In addition, Trp has generated 29% and 42% of RS strains after UV and DEO treatments, respectively. The chemical treatments (i.e. DEO and Phx) were more efficient in generating *cbrn* strains where, the obtained percentage was in the range of 8.4% to 11.6% of the total bromate strains. Uric acid (UA) and Proline (Pro) were the only *N*-sources that have served effectively for the generation of *cbrn* mutant strains in all treatments tested. Moreover, the *cbrn* strains were only selected at 25º C with all treatments except Phx treatment which generates RR mutants (in the range of 22% to 77.8%) at both selection temperatures. The obtained results indicate that a mutants` yield of approximately 69% of the four loci (*niaD*, *cnx*, *nrt* and *nirA* genes) was obtained on the bases chlorate (600 mM) toxicity, using four mutagenic treatments (spontaneous, DEO, Phx and UV). However, all chlorate resistant mutants were completely sensitive to bromate (250 mM). The highest mutants` yield (93.4%) was obtained with UV treatment, whereas the least (47.9%) was achieved with the spontaneous treatment. The tested selection temperatures did not significantly influenced the ratios of generated mutants. The amino acids Arg, His, Pro, Glu, Asp and Tyr were the best serving *N*-sources for mutants selection, where approximately equal ratios of mutants were obtained at both selection temperatures. However, Lys and Trp did not serve as sole *N*-sources for mutants selection at

25º C with all treatments except the DEO treatment. In addition, Cys was not the *N*-source of choice for mutants selection at any of the tested temperatures. The *niaD* mutants have ranked the first among all chlorate resistant mutants in terms of achieved mutant yield (73.97% of the total mutants), by all tested treatments. However, the UV treatment has generated the highest *niaD* ratio (86.9%), whereas, the least was obtained with Phx treatment. The obtained mutants` yield in *cnx* genes was 17.8% of the total mutants generated from all treatments (except DEO treatment). However, a 32% of the total mutants generated after Phx treatment was of *cnx* type which was the highest among tested treatments. The obtained *nrt* mutants` yield was ranked the third among isolated mutant types, where only 6.4% of the total confirmed mutants were achieved by all treatments (except DEO; generates 13.8%). The *nirA* mutant` yield was the least obtained (1.9%) with all treatments. However, DEO treatment was the best to use for the isolation of these types of mutants. In contrast, no single *nirA* mutant was obtained with any of the ten tested *N*-sources after UV treatment.

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