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MULTISTEP MUTAGENESIS FOR THE OVER-EXPRESSION OF CELLULASE IN *HUMICOLA INSOLENS*

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Abstract

The present study describes the improvement of a thermophilic fungal strain *Humicola insolens* for cellulase production. *H. insolens* TAS-13 has the potential to produce 1.00 U/ml/min CMC-ase, 0.43 U/ml/min FP-ase and 0.30 U/ml/min β-glucosidase, which was improved through UV and chemical mutagenesis like N-methyl-N-nitro-N-nitrosoguanidine (MNNG), nitrous acid (HNO_2), ethyl methyl sulphonate (EMS) or Ethidium bromide (EtBr). Two alternative mutation steps were carried out and mutants were screened for cellulase production. After first step mutation, 26 mutants were isolated and among them 5 best mutants (TAS_{UV-4}, TAS_{NG-7}, TAS_{HN-4}, TAS_{EB-2} and TAS_{EMS-1}) were further screened on the basis of hypercellulolytic ability. These mutants gave 17.36, 41.17, 5.25, 23.07 & 29.07% CMC-ase, 18.87, 30.64, 6.52, 10.41 & 17.30% FP-ase and 19, 61.54, 62.02, 14.28 & 23.07% β-glucosidase, respectively more than their wild strain *H. insolens* TAS-13. When these mutant were further mutated alternatively with the same mutagens under the same conditions, a total of 33 mutants were picked up as second generation mutants. Among these, a mutant strain TAS-$13_{UV-4}{}^{NG-5}$ proved to be the best mutant, which produced 43.19% CMC-ase, 60.15% FP-ase and 59.78% β-glucosidase more than the first generation mutant TAS-13_{UV-4}. Furthermore, this mutant was highly stable upto many generations possessing cellulolytic ability.

Introduction

Thermophilic fungi have a powerful ability to degrade polysaccharide constituents of biomass like cellulose and are the potential source of cellulolytic enzymes with scientific and commercial interest. These fungi can make the process more economical due to their thermostable enzymes, high rate of cellulolysis and ability to sacrify under non-aseptic conditions (Maheshwari *et al*., 2000; Sohail *et al.,* 2009). However, there is a tendency of variation in nature and productivity of cellulases among different strains (Oberson *et al*., 1992). In spite of extensive research for more active enzyme preparations from a large variety of microorganisms, the enzymatic sacrification of cellulose so far has not been reached to the conversion level of cellulose to glucose by the wildly produced enzymes. Hence, strain improvement by mutations is a successful method for better efficiency, but it is largely a trial and error process involving laborious steps of procedures in performance (Baker, 1991; Iftikhar *et al.,* 2010).

The techniques for the induction of mutation and strain selection have long been accepted as routine practice to improve the yield of cellulases from different fungi by the use of chemicals such as ethyl methyl sulphonate (EMS), N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and (HNO_2) nitrous acid (Kaudewitz, 1959; Bautz-Freese & Freese, 1961; Guerola *et al*., 1971). When fungi are grown with mutagens at sub-lethal concentrations, rate of enzyme production increased (Rudravaram *et al*., 2003). Exposure of thermophilic fungal conidia to ultraviolet (UV) and gamma (γ) irradiations is also

being used to obtain mutants yielding higher cellulase production (Jagger, 1961; Witkin, 1969). The idea of treating conidia and vegetative hyphae with various mutagens to search for improved mutant among the surviving progeny has now been recognized as the best mean to secure strains of improved potency (Chand *et al*., 2005).

Industrial application success for improved strains depends on their genetics and physiological characterization with a system that allows quick diagnosis. These diagnostic procedures for mutation are based upon a number of techniques in which resistant type of mutants can also be used for this purpose. This technique allows quick selection of improved and mutated strains that become able to grow under new conditions in the presence of 2-deoxy-D-glucose/benomyl/amphotericin concentrations, which cannot be done by wild strains. Various spontaneous resistant mutants were isolated by different workers like Azin and Noroozi (2001), Molina *et al*., (2001), Chand *et al*., (2005) and Haq *et al*., (2008). This work was initiated to develop a mutant strain of higher secretary rate of cellulase by keeping in mind the potential of thermothilic strain *Humicola insolens* TAS-13. A multistep mutation strategy based on the alternative UV and chemical treatments was followed. The mutants developed as a result were compared with the wild as well as neighboring sister mutant strains.

Materials and Methods

Organism: The culture of *Humicola insolens* TAS-13 used in this work was isolated previously qualitatively by standard plate screening method (Choi *et al*., 1978). In the present data, this strain was improved through UV and various chemical mutagens in terms of cellulase active unit production using submerged fermentation in 250 ml Erlenmeyer conical flasks. For maintenance, the strain was grown on potato dextrose agar medium (4.0% PDA) at 45°C and after maximum sporulation, stored at 4±1°C in cold room.

Random mutagenesis and mutant selection: In the present investigations, two steps of mutagenesis were followed for the development of a highly mutated and stable strain. In the first step, the wild strain was mutated by exposing to various mutagens like ultraviolet irradiations (UV), N-methyl-N-nitro-N-nitrosoguanidine (MNNG), nitrous acid (HNO_2), Ethidium bromide (EtBr) and Ethylmethyl sulphonate (EMS). The mutated strains possessing higher rate of cellulolysis were screened out. During the second step, all the best mutants of first step mutation were again exposed to the same mutagens and double mutated strains were obtained. These strains were screened for the cellulolytic potential and among these, best one was isolated and further work was continued on that mutant.

The fungal conidia were allowed to undergo UV based mutagenesis and 95% death rate was achieved after Azin & Noroozi (2001). A mutation method as described by Adelberg (1965) was followed for MNNG exposure. Nitrous acid (HNO_2) treatment was given after the method of Sinha & Chakrabarty (1977), Carlton & Brown (1981) and Azin & Noroozi (2001). Ethidium bromide treatment was based on the description of Dhawan *et al*., (2003). Ethyl methyl sulphonate (EMS) was used as a mutagen after Shonukan & Nwafor (1989).

Minimal inhibitory concentration (MIC) was obtained by growing the parental strain on Eggins and Pugh mineral salts agar medium enriched with Sigmacell-101 (0.5%) and 2-deoxy-D-glucose (0.0-0.5%) at 45±1ºC as reported by Azin & Noroozi (2001) and Molina *et al*., (2001). After exposure to mutagenic agents, about 100 µl of each suspension

containing treated mycelia was aseptically transferred to the individual Petri plates containing Eggins and Pugh mineral salts cellulose agar medium supplemented with (g/L); Sigmacell-101 (5.0), Rose Bengal (0.01), Triton X-100 (5.0), 2-deoxy-D-glucose (above the MIC of parental strain) and agar (20). The plates were incubated at 45±1ºC and were examined regularly after repeated 12 h time interval to study the growth pattern. The colonies were selected qualitatively, showing the clear zone formation after cellulose hydrolysis and were allowed to grow on PDA slants for culture maintenance. These were then tested quantitatively for enzyme production by shake flasks fermentation.

Fermentation technique: Conidia from 4-6 days old slant cultures were used as inoculum for fermentation. Twenty five millilitre of the Eggins & Pugh (1962) fermentation medium was taken in a 250 ml cotton plugged conical flask. After sterilization and cooling at room temperature, it was inoculated with 1.0 ml conidial inoculum and incubated in a rotary shaking incubator with 200 rpm at 45±1°C for 72 h. After the completion of incubation period, the fermented broth was centrifuged at 10,000×g for 10 minutes and the mycelia free supernatant was used for the analysis of cellulases. CM-cellulase activity was determined by the method of Wood & Bhat (1988). Filter paper-cellulase activity was measured by the method of Mandels & Sternberg (1976) and β-glucosidase activity was estimated according to the method used by Rajoka & Malik (1997). The reducing sugars, released in case of CMC-ase and FP-ase was measured by standard dinitrosalicylic acid method (Miller, 1959).

Statistical analysis: Treatment effects were compared by the method of Snedecor & Cochrane (1980). Post Hoc Multiple Comparison tests were applied under one-way ANOVA. Significance has been presented in the form of probability ($p<0.05$) values.

Results

Wild strain *H. insolens* TAS-13 was grown on Eggins & Pugh mineral salts agar medium with Sigmacell-101 (1.0%) in the presence of 2-deoxy-D-glucose (0.0-0.5%) at 45±1ºC for 84 h. It was found that 0.3% 2-deoxy-D-glucose was minimal concentration at which wild strain was unable to grow. The conidial suspension of wild strain H. insolens TAS-13 was exposed to UV irradiation (1.2×102 J/m2/s) for 15-90 minutes with 6.0 cm distance from the source. After 75 minutes exposure, more than 95% kill rate was observed and maximum frequency of two 2-deoxy-D-glucose resistant mutants was achieved (Fig. 1A). After repeated exposures, seven 2-deoxy-D-glucose resistant mutants were selected, which were tested for cellulase production by submerged fermentation (Fig. 2). This quantitative test for cellulase production of entire seven mutants revealed that mutant TAS-13UV-4 gave significantly high titre of cellulase i.e., 1.21, 0.53 & 0.37 U/ml/min of CMC-ase, FP-ase and β-glucosidase, respectively than its wild strain. All the mutants differed significantly at 0.047, 0.018 and 0.035 LSD, respectively.

After the repeated treatments of *H. insolens* TAS-13 mycelia with MNNG for 40 minutes (Fig. 1B), eight 2-deoxy-D-glucose resistant mutants were selected and tested quantitatively in shake flasks for their potential of CMC-ase, FP-ase and β-glucosidase bio-synthesis (Fig. 2). The data shows that *H. insolens* TAS-13_{NG-7} was the best mutant, which produced 1.70, 0.62 & 0.78 U/ml/min of CMC-ase, FP-ase and β-glucosidase, respectively. It differed significantly at $p\leq0.05$, having 0.039, 0.057 & 0.051 LSD with wild strain and other MNNG mutants.

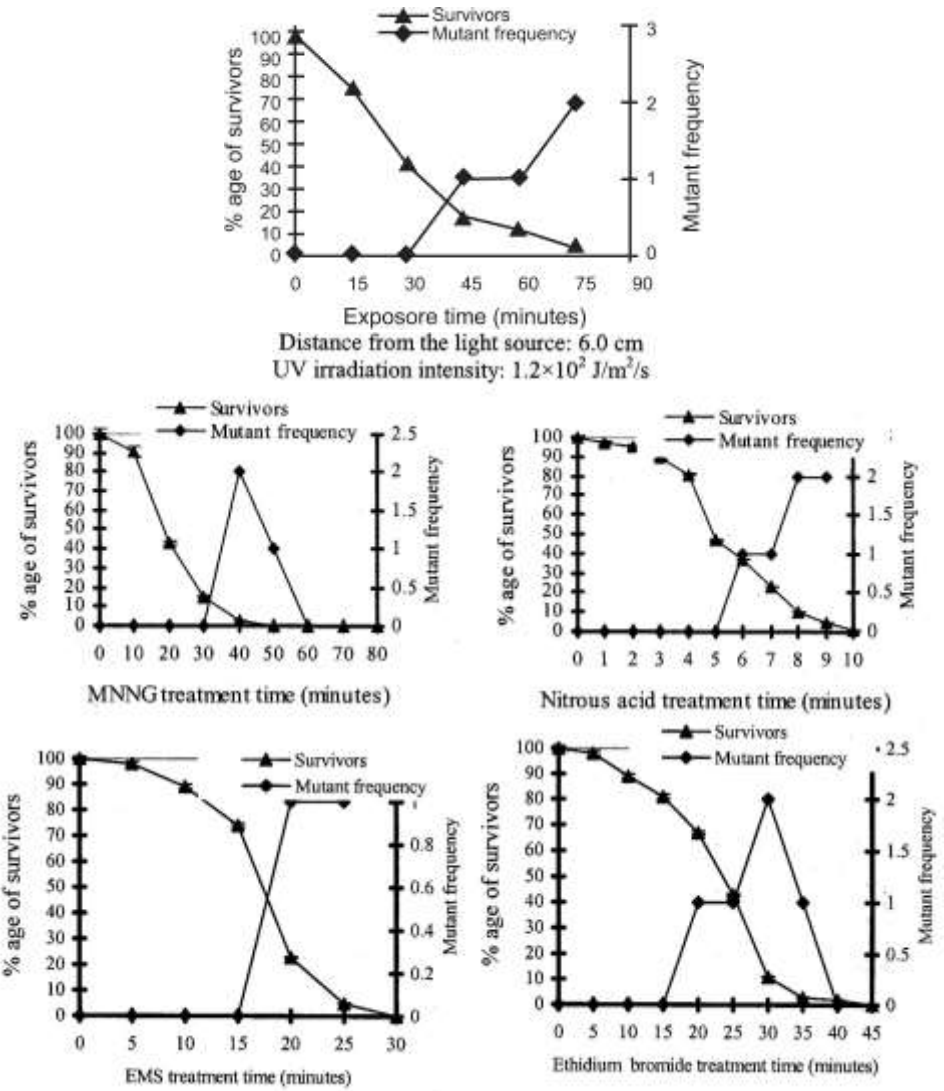

Fig. 1. Survival/kill curve of *H. insolens* TAS-13 in response of mutagens.

When *H. insolens* TAS-13 mycelial suspension was treated repeatedly for 09 minutes with HNO_2 (Fig. 1C), four 2-deoxy-D-glucose resistant mutants were selected, which were then tested for the production of CMC-ase, FP-ase and β-glucosidase (Fig. 2). These four mutants differed among themselves and with wild strain significantly at $p \leq 0.05$. The least significant difference among these strains was 0.047 for CMC-ase, 0.039 for FP-ase and 0.061 for β-glucosidase. Strain TAS-13_{HN-4} was found to be the best cellulolytic mutant with better CMC-ase (1.16 U/ml/min), FP-ase (0.45 U/ml/min) and β-glucosidase (0.38 U/ml/min) production as compared to other mutants selected after HNO_2 treatment.

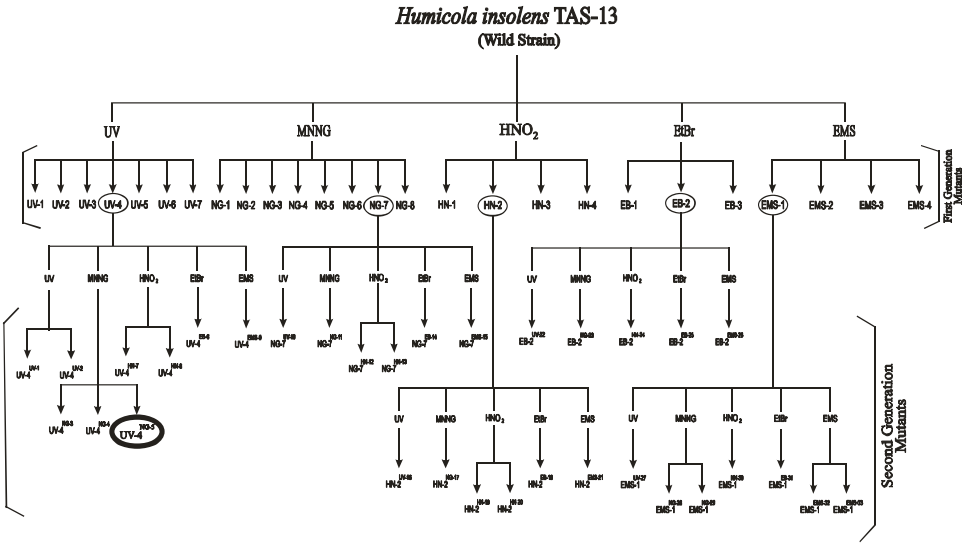

Fig. 2. Genealogy of the 2-deoxy-D-glucose resistant cellulase mutants of *Humicola insolens* TAS-1
(Light encircled were best among first generation mutants, while dark encircled was the best among all mutants)

The mycelial suspension of wild strain was treated with 0.5M EMS for 20 minutes repeatedly (Fig. 1D) and only four mutants were selected (Fig. 2). These mutants differed at $p \leq 0.05$ level of significance with 0.050, 0.045 and 0.070 LSD for CMC-ase, FP-ase and β-glucosidase, respectively. TAS-13_{EMS-1} was found to be the best among sister mutants, which produced 1.41, 0.52 & 0.39 U/ml/min CMC-ase, FP-ase and β-glucosidase, respectively.

In order to get the best mutant with EtBr, the mycelial suspension of wild strain was subjected to the sub-lethal doze for 30 minutes again and again (Fig. 1E). Three best mutants were selected and among them, strain TAS-13_{EB-2} was found to be the best producer of cellulase enzymes (Fig. 2). It significantly differed from wild and other EtBr mutants at $P \leq 0.05$ with 0.066, 0.041 and 0.077 LSD for CMC-ase, FP-ase and β-glucosidase, respectively. The data also showed that this mutant strain gave maximum titre of 1.30, 0.48 & 0.35 U/ml/min of CMC-ase, FP-ase and β-glucosidase, respectively.

All the best mutants selected in step-I, were alternatively treated with UV irradiations, MNNG, HNO_2, EtBr or EMS. Thirty-three different mutants were selected and screened for cellulase production (Fig. 2). The data showed that TAS-$13_{UV-4}{}^{NG-5}$ produced maximum cellulolytic activity i.e., CMC-ase 2.13 U/ml/min, FP-ase 1.33 U/ml/min and β-glucosidase 0.92 U/ml/min. The least significant difference among 33 mutants and wild strain were 0.017 for CMC-ase, 0.015 for FP-ase and 0.013 for β-glucosidase at $p \leq 0.05$ level of significance.

All the best mutants of step-I (TAS-13_{UV-4}, TAS-13_{NG-7}, TAS-13_{HN-2}, TAS-13_{EB-2} and TAS-13_{EMS-1}) and Step-II (TAS-$13_{UV-4}{}^{NG-5}$) selected on the basis of best cellulase production were compared among themselves and with wild strain (TAS-13). Comparison was made with randomized complete block design by applying one-way ANOVA and Duncan's multiple range test on each variable e.g., CMC-ase, FP-ase and β-glucosidase at $p \leq 0.05$. Comparison test revealed that least significant difference was 0.0095 for CMC-ase and β-glucosidase, while 0.0086 for FP-ase. *H. insolens* TAS-13_{UV-}

$_4^{NG-5}$ was found to be the best mutant strain as compared to all others and gave 115% CMC-ase, 303% FP-ase and 196% β-glucosidase more than the wild strain.

Discussion

Several factors motivate the development of microorganisms for consolidated bio-processing (CBP) in the set up of an industry. The microorganisms should possess all the properties that are required for cost-effective implementation (Lynd *et al*., 2002). Extensive work is being carried out in many laboratories to select an organism capable of producing concentrated cellulase titre for large scale conversion of lignocellulosic biomass. However, the thermostability of enzyme is more important on the industrial scale during different treatment steps of temperature fluctuation. The production of such thermostable enzymes can be achieved from the genera growing under high degrees of temperature range.

Members of thermophilic genus have been extensively used particularly due to their ability to secrete extracellular cellulase enzymes. These strains have also been mutagenized and genetically modified to develop a mutant strain capable of producing high levels of cellulases. Because, thermotolerant strains have a unique character to pass over the environmental stress and are highly susceptible to various physical as well as chemical mutagenic agents. Hence, it has become a routine practice in the field of biotechnology to develop a mutant through random mutagenesis (Azin & Noroozi, 2001). Efforts were made to mutate the wild strain *H. insolens* TAS-13 for enhanced cellulase production through UV, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), Nitrous acid (HNO_2), Ethyl methyl sulphonate (EMS) or Ethidium bromide (EtBr).

H. insolens TAS-13 showed different patterns of lethality and survival rate, when exposed to UV, MNNG, HNO_2, EMS and EtBr for different time intervals. It has been observed that the development of a hyper mutant is time and dose dependent. Survival rate was 77% at 15 minutes exposure to UV irradiations (Fig. 1A). Among all the mutagens tested, MNNG was a more toxic mutagen, which killed 97% progeny within only 4 minutes of treatment time and gave maximum frequency of mutant development (Fig. 1B). Nitrous acid survival curve indicated that 9 minutes exposure time is optimal for the induction of 95% death rate (Fig. 1C). When the strain was treated with EMS, 95% killing rate was observed after 25 minutes of exposure (Fig. 1D). However, EtBr was unique among all the mutagens tested, by giving recovery of 11% survivals with a prolonged exposure time of 30 minutes (Fig. 1E). Hence, it is concluded that EtBr is less lethal to wild strain (Guerola *et al*., 1971).

It has been observed that wild strains and their mutants showed different physiological characters under different conditions. A similar condition was provided to separate wild and mutant progeny in the presence of a toxic glucose analogue i.e., 2-deoxy-D-glucose (DOG). On 0.3% dose of DOG, mutants were frequently grown in contrast to their wild type strain. This is because, mutants have adopted a unique glucose-deregulated mechanism, which is either absent or developed poorly in wild strains. Similar results have also been reported by Van-Uden & da-Costa (1980), Kawamori *et al*., (1985, 1986), Zaldivar *et al*., (1987) and Azin & Noroozi (2001).

H. insolens TAS-13 was given alternative treatments of mutagens for the sake of mutant development (Fig. 1) with hyper-cellulolytic activity and resistance to catabolic repression in the presence of 0.3% DOG. Among 26 first generation mutants, TAS-13_{UV-4}, TAS-13_{NG-7}, TAS-13_{HN-4}, TAS-13_{EB-2} and TAS-13_{EMS-1} gave better cellulase yield than

their other sister mutants. *H. insolens* TAS-13_{UV-4} gave 17.36, 18.87 & 19%, TAS-13_{NG-7} 41.17, 30.64 & 61.54%, TAS-13_{HN-4} 15.25, 6.52 & 62.02%, TAS-13_{EB-2} 23.07, 10.41 & 14.28% and TAS-13_{EMS-1} 29.07, 17.30 & 23.07% more CMC-ase, FP-ase and β-glucosidase, respectively than their wild strain *H. insolens* TAS-13.

When these mutants were further exposed to UV, MNNG, HNO_2, EMS and EtBr mutagens, a total of 33 mutants were screened out. Among these second generation mutants, *H. insolens* TAS-$13_{UV-4}{}^{NG-5}$ was the best, which produced 43.19%, 60.15% & 59.78% and 53.05%, 67.67% & 67.39% more CMC-ase FP-ase and β-glucosidase than did its first generation mutant TAS-13_{UV-4} and wild strain TAS-13, respectively (Table 1). The stability with respect to cellulase production was evaluated frequently for one year. Results showed that this mutant was highly stable upto many generations. Further more, when this mutant was exposed to day light, then it had shown resistance against photo-reactivation. It might be due to ultraviolet radiations, which are known to induce both the base substitution and base deletion mutations in organisms and second mutagen (MNNG) which might have caused permanent change in DNA sequence or stabilized the pre-induced UV mutation (Schwartz & Beckwith, 1969).

Zaldivar *et al*., (2001) and Chand *et al*., (2005) obtained mutants, which produced cellulase enzymes with 5-fold increase in β-glucosidase and one fold increase in both CMC-ase and FP-ase, through such mutagens treatments. Moreover, Berger *et al*., (1966) has also reported weak induction of frameshift mutations by UV irradiation. These radiations alter the genome by making pyrimidine dimmers, which are photo-chemically repairable.

Table 1. Screening of mutants for the production of cellulase in shake flask [Each value is a mean average of three parallel replicates, ± denotes standard deviation among the replicates and numbers followed by different letters differ significantly at $p \leq 0.05$. Carbon source: 1.0% Sigmacel-101, Incubation temperature: 45°C, Inoculum: 2.50×10^7 conidia/25ml of medium, pH: 5.0, Time of incubation: 84 h].

Strains	Enzyme activities (U/ml/min)		
	CMC-ase	FP-ase	β-glucosidase
Wild	1.00 ± 0.010^{g}	0.43 ± 0.015^{g}	0.31 ± 0.040^{g}
TAS-13_{UV-4}	1.21 ± 0.010^{e}	0.53 ± 0.020^{c}	0.32 ± 0.010^{f}
TAS-13_{NG-7}	1.70 ± 0.030^{b}	0.62 ± 0.009^{b}	0.78 ± 0.040^{c}
TAS-13_{HN-2}	1.18 ± 0.030^{f}	0.46 ± 0.030^{f}	0.79 ± 0.030^{b}
TAS-13_{EB-2}	1.3 ± 0.020^{d}	0.48 ± 0.010^{e}	0.35 ± 0.020^{e}
TAS-13_{EMS-1}	1.41 ± 0.020^{c}	0.52 ± 0.010^{d}	0.39 ± 0.050^{d}
TAS-$13_{UV-4}{}^{NG-5}$	2.13 ± 0.001^{a}	1.34 ± 0.005^{a}	0.92 ± 0.015^{a}
LSD	**0.0095**	**0.0086**	**0.0095**

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