EJB Electronic Journal of Biotechnology ISSN: 0717-3458 Vol.5 No.2, Issue of August 15, 2002

Received December 21, 2001 / Accepted June 4, 2002

RESEARCH ARTICLE

Novel technique for microbial production of 3,4-Dihydroxy Phenyl L-alanine by a mutant strain of *Aspergillus oryzae*

Sikander Ali*
Biotechnology Research Laboratories
Department of Botany
Faculty of Sciences
Government College
Lahore, Pakistan
Tel: 92 42 9211634
Fax: 92 42 7243198
E-mail: alisbiotech@yahoo.com

Ikram-ul-Haq
Biotechnology Research Laboratories
Department of Botany
Faculty of Sciences
Government College
Lahore, Pakistan
Tel: 92 42 9211634
Fax: 92 42 7243198
E-mail: ikrhaq@yahoo.com

Qadeer, M.A.
Centre of Excellence in Molecular Biology
Thokar Niaz Baig
C/B Road, Lahore, Pakistan
Tel: 92 42 7588977
Fax: 92 42 723198
E-mail: qadeerma@yahoo.com

Financial support: Principal, Government College Lahore, Pakistan.

Keywords: amino acid, *Aspergillus oryzae*, fermentation, L-DOPA, L-tyrosine, Parkinson's disease.

Abbreviations:

Substrate consumption parameters:
μ (h^{-1}) = specific growth rate
$Y_{x/s}$ = g cells/g substrate utilized
Q_s = g substrate consumed/l/h
q_s = g substrate consumed/g cells/h
Q_x = g cells formed/l/h

Production formation rates:
Q_p = g citric acid produced/litre/h
$Y_{p/s}$ = g citric acid produced/g substrate consumed
$Y_{p/x}$ = g citric acid produced/g cells formed
q_p = g citric acid produced/g cells/h.

L-DOPA is a useful drug for Parkinson's disease. This investigation deals with the biosynthesis of L-DOPA by parental (GCB-6) and mutant (UV-7) strains of *Aspergillus oryzae*. There was a marked difference between the mycelial morphology and pellet type of the parental and UV-irradiated mutant cultures. The mutant strain of *Aspergillus oryzae* UV-6 showed pellet-like mycelial morphology and improved tyrosinase activity. Mould mycelium was used for biochemical conversion of L-tyrosine to L-DOPA since tyrosinase is an intracellular enzyme. The mutant was found to give 3.72 folds higher production of L-DOPA than the parental strain. The comparison of kinetic parameters was also done which showed greater ability of the mutant to yield L-DOPA (*i.e.* $Y_{p/x}$ 32.73 mg/mg with parent and 95.71 mg/mg with mutant). When cultures grown for various incubation periods, were monitored for Q_p, Q_s and q_p, there was significant enhancement ($p<0.0025$-0.005) in these variables by the mutant strain of *Aspergillus oryzae* UV-7 over GCB-6 on all the rates.

L-DOPA (3,4-dihydroxy phenyl L-alanine) is a useful drug

*Corresponding author

in the treatment of Parkinson's disease and Myocardium following neurogenic injury (Raju et al. 1993). It occurs naturally in beans of *Vicia faba* and seeds of *Mucana pruriens*. Its production has been described by a number of workers (Sih et al. 1969; Haq et al. 1998; Fling and Paul, 2001). L-DOPA is produced from L-tyrosine by one-step oxidation reaction by submerged fermentation (Haneda et al. 1973). The optimisation of cultural conditions is necessary for the successful fermentation process. The key enzyme responsible for biosynthesis of L-DOPA is 'tyrosinase' (Rosazza et al. 1995). Tyrosinases are widely distributed and highly purified enzymes, derived from microbial (*Aspergillus, Rhizopus* and *Neurospora* spp) and plant sources (*Agaricus* and *Vicia* spp). However, in microorganisms tyrosinase activity is generally very weak and L-tyrosine and L-DOPA are rapidly decomposed to other metabolites. Thus, stoichiometric formation of L-DOPA is difficult to achieve (Kumagai et al. 1969; James and Fling, 2001). The mycelial activity of *Aspergillus oryzae* or *Aspergillus flavus* catalysing L-tyrosine to L-DOPA was observed in acidic range below pH 5.0 (Singh, 1999; Haq et al. 2000). In present study, the authors have described the increase in biomass of *Aspergillus oryzae* and consequently the production of L-DOPA, using shake flasks. Tyrosinase is an intracellular enzyme. So, mould mycelium was used for biochemical conversion of L-tyrosine to L-DOPA.

Materials and Methods

Organism

Aspergillus oryzae strain GCB-6 was used in the present study. The wild culture of *Aspergillus oryzae* was taken from Biotechnology Research Labs, Department of Botany, Government College, Lahore. It was maintained on potato dextrose agar medium and stored at 4ºC in a cold cabinet.

Improvement of strain after UV-irradiation

The *Aspergillus oryzae* strain GCB-6 was improved after UV-irradiation. The dose of ultraviolet irradiation was given at a rate of 1.2×10^6 J/m^2/S.

(a) Conidia from 3-5 days old cultures were harvested in phosphate buffer containing (g/l); K_2HPO_4 3.5, KH_2PO_4 1.5 at pH 7.2. The conidial suspension was exposed to UV-irradiation for different time intervals (15-120 min.), aseptically. The irradiated conidial suspension was poured on agar-malt extract with tyrosine dishes (Roy and Das, 1978).

(b) The mutant strain of *Aspergillus oryzae* UV-3, a hyper producer of L-DOPA obtained after UV-irradiation was used for mycelial mutation. Hundred ml of vogel medium containing (g/l); trisodium citrate 2.5, NH_4NO_3 2.0, KH_2PO_4 5.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4.7H_2O$ 0.2, Peptone 2.0, Yeast extract 1.0 at pH 5.5 with 15-20 glass beads (2.0mm, diameter) in 1-L cotton wool plugged conical flask was sterilized at 15 lbs/inch^2 (121ºC) for 15 minutes. Small amounts of conidia from the slant (3-5 days old) were aseptically transferred with the help of an inoculating needle to the flask. The flask was incubated at 30ºC in an incubator shaker at 200 rpm for 24 hours. The inoculum was kept homogeneous and optical density was maintained at 1.0 with the help of a photoelectric colorimeter, using a 530 nm filter. Five ml of the vegetative inoculum was taken in a petriplate and then UV-treatment was given from 15-25 minutes, intervals, following the method of Pontecarvo et al.1969. The mutant cultures were incubated at 30ºC for 3-4 days for sporulation.

Cultivation of mycelium

The submerged culture method (Raju et al. 1993) was employed for cultivation of the mycelium. A conidial inoculum was prepared in 10 ml of Monoxal O.T. (Dioctyl ester of sodium sulpho succinic acid). Twenty-five ml of cultivation medium containing (% w/v); glucose 2.0, polypeptone 1.0, NH_4Cl 0.3, KH_2PO_4 0.3, $MgSO_4.7H_2O$ 0.02, yeast extract 1.0 at pH 5.0 was used in 250 ml shake flasks. The medium was autoclaved at 15-lb/inch^2 pressure (121ºC) for 15-minutes and seeded with 1.0 ml conidial suspension. The flasks were then incubated at a rotary incubator shaker (200 rpm) in 30ºC for 48 hours. The mycelium was harvested by filtering through a funnel and washed free of adhering medium with ice-cold water. The mycelium was dried in filter paper folds and it was stored at 5ºC in a refrigerator.

Reaction procedure

The reaction for L-DOPA production from L-tyrosine was carried out in a suspension of intact mycelia. The mycelia were suspended in reaction mixture (Haneda et al. 1973). Fifteen ml of acetate buffer (pH 3.5, 50mM) containing (mg/ml); L-tyrosine 2.5, L-ascorbic acid 5.0 and intact mycelia 75.0 were taken in 250 ml Erlenmeyer flasks. The reaction was carried out aerobically at 50ºC for 60 minutes in a hot plate with magnetic stirrer. The sample was withdrawn, centrifuged (6,000/g) and the supernatant was kept under dark for further investigation.

Assay methods

L-DOPA and L-tyrosine were determined colorimetrically according to the method of Arnow, 1937.

(a) L-DOPA: One ml of the supernatant was taken and in it 1.0 ml of 0.5N HCl along with 1.0 ml of nitrite molybdate reagent was added. A yellow color appeared. Then 1.0 ml of 1.0N NaOH was added, which gave red coloration and the total volume was made up to 5.0 ml. The color intensity was read by a photoelectric colorimeter (Model: AE-II, ERMA, Japan) using a green wratten filter of 530 nm and the amount of L-DOPA was determined from Arnow's standard curve of L-DOPA.

(b) L-tyrosine:One ml of supernatant from the reaction mixture was taken and in it 1.0 ml of mercuric sulfate reagent was added. It was placed in boiling water bath for 10 minutes. It was cooled and 1.0 ml of nitrite reagent was added. Total volume was made up to 5.0 with distilled water. It was compared in a colorimeter and the amount of L-tyrosine was determined from Arnow's standard curve of L-tyrosine.

Kinetic parametric studies and data statistics

The kinetic parameters were studied according to the procedures of Pirt, 1975. The data was statistically analyzed using Duncan's multiple range and one-way ANOVA (Snedecor and Cochran, 1980).

Results and Discussion

The data of Table 1 shows the screening of UV-irradiated mutant strains of *Aspergillus oryzae* for the production of L-DOPA in shake flasks. All the fermentations were carried out at 30ºC temperature with 160 rpm, agitation rate. The production of L-DOPA by mutant cultures ranged from 0.08-1.28 mg/ml. Of all the mutant cultures examined, the strain UV-7 gave the highest yield of L-DOPA. It might be due to the fact that UV-irradiation has altered the actual structure of DNA by photolysis *i.e.*, formation of pyrimidine dimers. The structural change in DNA is related with the activity of the enzyme tyrosinase. Thymidine-thymidine dimers promote mycelial growth in the form of round pellets and subsequently enzyme activity, which resulted in the greater excretion of L-DOPA from the mycelial cells. Only a few research reports have quoted similar type of findings (Kumagai et al. 1969; Singh, 1999). In the present study, the mutant strain of *Aspergillus oryzae* UV-7 was found to give 3.72 folds higher production of L-DOPA as compared to the parent culture, which is satisfactory.

A comparative study of L-DOPA production and the kinetic parameters of the wild type culture of *Aspergillus oryzae* and its best UV-irradiated mutant strain is presented in Table 2. The specific growth rate (μ), cell mass and product yield coefficients ($Y_{x/s}$, $Y_{p/x}$, $Y_{p/s}$, Q_p and q_p) and substrate consumption rates (Q_s, q_s) of UV-7 showed higher values as compared to the wild-culture as well as other mutant strains of *Aspergillus oryzae*. On the basis of standard deviation among the three replicates of flasks, the values with different letters differ significantly at $p<0.05$. The uptake of tyrosine was less effective when *Aspergillus oryzae* was grown under submerged culture conditions. Although complete uptake of substrate from the media was observed at the lowest concentrations employed (0.5 and 1.0 mg/mg), this decreased to only 50% at 1.5 mg/ml and to about 20% at the highest concentration employed (3.0 mg/ml). This is different to that observed by Sarin et al.1980 and Singh, 1999 who reported product formation at higher concentrations of substrate. However, biomass formation was not effective in their case (*i.e.*, $Y_{x/s}<0.0042$ mg/mg).

Biosynthesis of L-DOPA by parental (GCB-6) and mutant (UV-7) strains of *Aspergillus oryzae* using shake flasks is shown in Table 3. The cultures were incubated for different time intervals (24-96 hours). At 24 hours of incubation, the production of L-DOPA was low (0.21 mg/ml in case of parental strain and 0.16 mg/ml in case of mutant strain of *Aspergillus oryzae*). The maximum production of L-DOPA by the parental GCB-6 was 0.36 mg/ml, whereas 1.34 mg/ml L-DOPA was obtained by mutant UV-7, 48 hours after incubation. The over all values of the growth yield coefficient $Y_{x/s}$ (mg/mg) of mutant culture are higher than the mutant strain. There was a great reduction in the formation of L-DOPA when the incubation period was increased beyond 48 hours. At 96 hours of incubation period, the production of L-DOPA became significantly low, with both the parental (0.18 mg/ml) as well as mutant strain (0.92 mg/ml). The decrease in L-DOPA production with the increased incubation period might be due to the overgrowth of fungi and also the age factor. Enzyme activity is directly related with the age and development of fungi. Findings have been reported by Fling and Paul, 2001 but focusing on properties of L-DOPA as well as amino acids. Our finding (1.34 mg/ml L-DOPA) is the most encouraging value ever recorded and economically significant due to the greater production and decreased in incubation period. The consumption of L-tyrosine was 0.86 mg/ml and 0.75mg/ml by parental (GCB-6) and mutant (UV-7), respectively.

The comparative time course and kinetic parameters *i.e.*, volumetric rates (Q_p, Q_s and Q_x in mg/ml/h) and specific rate constants (q_p and q_s in mg/mg/h) on the production of L-DOPA by parental (GCB-6) and mutant (UV-7) is also shown (Table 4 and Table 5). Maximum growth in terms of specific growth and production rate was significantly different ($p<0.005$ and $p<0.0120$ respectively) during growth of GCB-6 and UV-7, 24-96 hours after incubation. When cultures grown for different incubation periods, were monitored for Q_p, Q_s and q_p, there was significant enhancement ($p<0.0025$-0.005) in these variables by mutant strain of *Aspergillus oryzae* UV-7 over GCB-6 on all the rates. The mutant UV-7 exhibited improved $Y_{p/s}$ and $Y_{p/x}$ for L-DOPA over the GCB-6. Lowest values of operational kinetic parameters were obtained, 84 hours after the incubation followed by 96 hours incubation. The $Y_{p/s}$ (product/substrate utilized g/g) in case of UV-7 was 4.2 folds better than GCB-6.

Concluding Remarks

Mutation can raise the status of microorganisms to hyper produce the actual product required. The mutant strain of *Aspergillus oryzae* UV-7 showed pellet-like mycelial morphology and improved tyrosinase activity due to the formation of thymidine-thymidine base pairs in its DNA structure. The product *i.e.*, L-DOPA is a low cost and high yield product. By optimising the effect of phosphate sources on the mould growth, this mutant could be exploited for L-DOPA production.

References

ARNOW, L.E. Colorimetric determination of the components of L-3, 4-dihydroxy phenyl alanine-tyrosine mixtures. *Journal of Biochemistry*, January 1937, vol. 88, no. 1, p. 531-533.

FLING, G. and PAUL, J. Radiation induced production of L-3,4 dihydroxy phenylalanine by *Aspergillus oryzae*. *Journal of Analytical Chemistry*, March 2001, vol. 67, p. 185-187.

HANEDA, K.; WATANABE, S. and TAKEDA, P. Production of L-DOPA from L- Tyrosine by microorganisms. *Journal of Fermentation Technology*, March 1973, vol. 51, no. 6, p. 398-406.

HAQ, I.; ALI, S.; MANZOOR, S. and QADEER, M. A. Optimization of cultural conditions for the production of L-DOPA from L-tyrosine by *Aspergillus oryzae. Pakistan Journal of Botany*, April 2000, vol. 32, no. 2, p. 255-258.

HAQ, I.; IQBAL, F. and QADEER, M.A. Production of L-DOPA from L-tyrosine by *Aspergillus oryzae. Biologia*, November 1998, vol. 44, no. 1 and 2, p. 31-39.

JAMES, P. and FLING, G. Tyrosinase activity of different fungal strains. *Biochemistry and Biophysics*, June 2001, vol. 112, p. 98-103.

KUMAGAI, L.S.; FLING, M. and SARIN, A.N. Mushroom tyrosinases. *Journal of Biological Chemistry*, July 1969, vol. 68, p. 178-185.

PIRT, S. John. *Principles of Microbe and Cell Cultivation*. Blackwells Scientific, London, pp. March 1975. 274 p. ISBN 0470690380.

PONTECARVO, J.; ROPER, J.A.; HEMMOUS, L.M. and BUFTAN, W. Basic techniques of UV-irradiation. *Advanced Genetics*, September 1969, vol. 5, p. 141-183.PONTECARVO, J.; ROPER, J.A.; HEMMOUS, L.M. and BUFTAN, W. Basic techniques of UV-irradiation. *Advanced Genetics*, September 1969, vol. 5, p. 141-183.

RAJU, B.G.S.; RAO, G.H. and AYYANNA, C. Bioconversion of L-tyrosine to L-DOPA using *Aspergillus oryzae*. CBS Publishers, Visakhapatnam, India, November 1993, p. 106-110.

ROSAZZA, K.C.; PAUL, G.M. and WOLF, S. The microbial synthesis of L-DOPA. *Biotechnology Letters*, January 1995, vol. 16, no. 3, p. 210-215.

ROY, D. and DAS, R.M. Mutation of microorganisms to complete change in physiological sequences. *Amniotic and Technological Chemistry*, April 1978, vol. 159, no. 12, p. 172-183.

SIH, C.J.; FOSS, P.; ROSAZZA, J. and LEMBAGAR, M. Microbial synthesis of L-3, 4-dihydroxy phenylalanine. *Journal of Amniotic and Chemistry Society*, March 1969, vol. 91, p. 62-64.

SINGH, H. Mechanism of oxidation of L-tyrosine by fungal tyrosinases. *Journal of Chemistry*, March 1999, vol. 172, p. 83.

SNEDECOR, George Waddel and COCHRAN, William G. *Statistical methods*, 7th edition, Iowa State University, USA. January 1980, 524 p. ISBN 0813815614.

APPENDIX

Tables

Table 1. Screening of UV-irradiated mutant strains of *Aspergillus oryzae* for the production of L-DOPA in shake flasks.

Mutant strains of *Aspergillus oryzae*	L-tyrosine used (mg/ml)	Dry cell mass (mg/ml)	L-DOPA produced (mg/ml)	Mycelial morphology
Parent (GCB-6)	0.92	0.008	0.32	Fine round pellets
UV-1	0.95	0.004	0.16	Gelatinous mass
UV-2	1.21	0.005	0.25	"
UV-3	1.06	0.008	0.74	Small pellets
UV-4	1.35	0.011	0.30	Gelatinous mass
UV-5	0.87	0.010	0.08	"
UV-6	0.94	0.008	0.82	Small pellets
UV-7	0.86	0.007	1.28	Intermediate pellets
UV-8	1.42	0.009	0.10	Gelatinous mass
UV-9	1.68	0.014	0.26	"
UV-10	0.98	0.012	0.18	"

Incubation temperature 30ºC; Initial pH 5.5; Agitation intensity 160 rpm.

Table 2. Comparative study of L-DOPA production and kinetic parameters of parental strain of *Aspergillus oryzae* and its best UV-irradiated mutant strains.

Kinetic parameters	L-DOPA			
	Parent	UV-3	UV-6	UV-7
μ (h^{-1})	0.0067±0.02	0.0154±0.02	0.0170±0.02	0.0267±0.02
$Y_{x/s}$ (mg/mg)	0.0087±0.01	0.0087±0.02	0.0086±0.01	0.0081±0.03
Q_s (mg/ml/h)	0.0192±0.02	0.0220±0.01	0.0196±0.02	0.0179±0.03
q_s (mg/mg/h)	0.0062±0.02	0.0071±0.01	0.0062±0.01	0.0058±0.03
$Y_{p/x}$ (mg/mg)	40.000±0.01	92.500±0.01	102.50±0.01	182.86±0.02
$Y_{p/s}$ (mg/mg)	0.3478±0.02	0.6981±0.02	0.8723±0.02	1.4883±0.02
Q_p (mg/ml/h)	0.0066±0.01	0.0150±0.02	0.0165±0.02	0.0266±0.02
q_p (mg/mg/h)	0.0021±0.01	0.0050±0.01	0.0055±0.02	0.0086±0.02

μ = specific growth rate (h^{-1}); $Y_{x/s}$ = cell mass yield (mg/mg); Q_s = slope of substrate utilized (mg/ml/h) / time of fermentation (h); q_s = specific substrate uptake rate (mg/mg/h) = (μ x $Y_{x/s}$); $Y_{p/x}$ = product (mg/ml) / cell mass (mg/ml); $Y_{p/s}$ = product (mg/ml) / substrate utilized (mg/ml); Q_p = slope of product (mg/ml/h) / time of fermentation (h); q_p = specific citric acid production rate (mg/mg/h) = (μ x $Y_{p/s}$); ± indicates the standard deviation among the three parallel replicates. Within the rows, the values differ significantly at $p<0.05$.

Table 3. Biosynthesis of L-DOPA by parental (GCB-6) and mutant (UV-7) strains of *Aspergillus oryzae* using shake flasks.

Incubation period (Hours)	L-DOPA produced (mg/ml)		L-tyrosine used (mg/ml)		% L-DOPA*		Growth yield coefficient, $Y_{x/s}$ (mg/mg)	
	Parent	Mutant	Parent	Mutant	Parent	Mutant	Parent	Mutant
24	0.21	0.16	0.48	0.26	43.75	61.54	0.013±0.002	0.015±0.002
36	0.34	0.68	0.62	0.48	54.84	80.42	0.013±0.001	0.019±0.002
48	0.36	1.34	0.86	0.74	41.86	87.55	0.014±0.001	0.019±0.002
60	0.32	1.26	1.10	0.80	29.09	70.52	0.011±0.001	0.017±0.002
72	0.29	1.12	1.39	0.86	20.86	62.50	0.009±0.001	0.015±0.001
84	0.24	1.06	1.78	0.96	13.48	50.02	0.008±0.001	0.013±0.001
96	0.18	0.92	1.96	1.38	9.18	37.24	0.008±0.001	0.011±0.002

L-tyrosine added 2.5 mg/ml, Incubation temperature 30ºC, Agitation intensity 160rpm, *on the basis of L-tyrosine used. $Y_{x/s}$ = Dry cell mass (mg/ml) / Substrate utilized (mg/ml), ± indicates the standard deviation among the three parallel replicates, the values differ significantly at $p<0.008$.

Table 4. Comparative study of L-DOPA production and kinetic parameters (volumetric rates) by using parental (GCB-6) and mutant (UV-7) strains of *Aspergillus oryzae*.

Incubation period (Hours)	Volumetric rates (mg/ml/h)					
	Q_p		Q_s		Q_x	
	Parent	Mutant	Parent	Mutant	Parent	Mutant
24	0.008±0.002	0.007±0.001	0.020±0.002	0.011±0.002	0.0025±0.001	0.0017±0.0001
36	0.009±0.001	0.019±0.001	0.017±0.002	0.013±0.002	0.0022±0.001	0.0025±0.0001
48	0.007±0.001	0.028±0.001	0.018±0.002	0.015±0.001	0.002±0.002	0.0029±0.0002
60	0.005±0.001	0.021±0.002	0.018±0.002	0.013±0.001	0.0017±0.001	0.0023±0.0002
72	0.004±0.003	0.012±0.002	0.019±0.002	0.012±0.001	0.0017±0.0002	0.0018±0.0002
84	0.003±0.001	0.011±0.001	0.021±0.002	0.011±0.002	0.0017±0.0001	0.0015±0.0003
96	0.002±0.001	0.014±0.001	0.020±0.003	0.014±0.001	0.0001±0.0002	0.0017±0.0001

Q_p = Slope of product (mg/ml) / time of fermentation (h); Q_s = Slope of sustrate utilized (mg/ml) / time of fermentation (h); Q_x = Slope of cell mass formation (mg/ml) / time of fermentation (h); ± indicates the standard deviation of three parallel sets of replicates, within the column, values differ significantly at $p<0.005$.

Table 5. Comparative study of L-DOPA production and kinetic parameters (specific rate constants) by both parental (GCB-6) and mutant (UV-7) strains of *Aspergillus niger*.

Incubation period (Hours)	Specific rate constants (mg/mg/h)			
	q_p		q_s	
	Parent	Mutant	Parent	Mutant
24	0.00184±0.0001	0.00107±0.0005	0.00420±0.0001	0.00173±0.0002
36	0.00321±0.0002	0.01283±0.003	0.00585±0.0002	0.00905±0.0001
48	0.00270±0.0001	0.03740±0.002	0.00645±0.0002	0.02065±0.005
60	0.00170±0.0001	0.02646±0.002	0.00586±0.0004	0.01680±0.005
72	0.00117±0.00005	0.01742±0.0005	0.00560±0.0002	0.01334±0.002
84	0.00068±0.00002	0.01338±0.002	0.00509±0.0005	0.01212±0.003
96	0.00335±0.0001	0.00881±0.0005	0.00364±0.0003	0.01322±0.0001

q_p = specific citric acid production rate (mg/mg/h) = (μ x $Y_{p/s}$); q_s = specific substrate uptake rate (mg/mg/h) = (μ x $Y_{x/s}$). The values differ significantly at $p<0.0025$ while ± indicates the standard deviation among the replicates.