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ORIGINAL ARTICLE

Cloning and expression of a full-length glutamate decarboxylase gene from a high-yielding γ-aminobutyric acid yeast strain MJ2

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Abstract A yeast strain MJ2 that was found to produce a higher amount of γ-aminobutyric acid (GABA) was isolated from the surface of kiwi. Phylogenetic analysis based on the ITS sequence and morphological, biochemical studies indicated that it may belong to *Saccharomyces cerevisiae*. Under optimum conditions in Czapek's broth medium with 0.5 % monosodium glutamate, it produced GABA at a concentration of 5.823 g/L after 48 h. A full-length glutamate decarboxylase gene (*Scgad*) was cloned by PCR amplification. The open reading frame (ORF) of the *Scgad* gene was composed of 1,755 nucleotides and encoded a protein (585 amino acids) with a predicted molecular weight of 65.897 kDa. The deduced amino acids sequence of *Scgad* shows 100 %, 65 % and 62 % similarity with *S. cerevisiae*, *Candida glabrata* and *Kluyveromyces lactis GAD* in the polypeptide level, respectively. The *Scgad* gene was expressed in *Escherichia coli* BL21 (DE3) cells, and the expression was confirmed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis. The results suggested that the *S. cerevisiae* GAD (*Sc*GAD) was successfully encoded in *E. coli* BL21 (DE3) cells. Furthermore, the enzyme activity of *Sc*GAD encoded in *E. coli* BL21 (DE3) had been significantly enhanced using artificial neural network linked with genetic algorithm (ANN-GA) method.

Keywords *Saccharomyces cerevisiae* · γ-aminobutyric acid (GABA) · Glutamate decarboxylase (GAD) · Expression · ANN-GA

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Introduction

Yeasts are characterized by a wide dispersion of natural habitats, common on plant leaves and flowers, soil and salt water (Vallesa et al. 2007; Nielsen et al. 2008). The most well-known and commercially significant yeasts are the related species and strains of *Saccharomyces cerevisiae* (Roger 2004). These organisms have long been utilized to ferment the sugars of rice, wheat, barley, and corn to produce alcoholic beverages and in the baking industry to expand or raise dough. *S. cerevisiae* is commonly used as baker's yeast and for some types of fermentation (Bruno et al. 2009). Yeast is often taken as a vitamin supplement because it is highly enriched in proteins an B vitamins such as niacin, folic acid, riboflavin, and biotin.

Glutamate decarboxylase (GAD: EC 4.1.1.15) catalyzes the irreversible α-decarboxylation of L-glutamate to γ-aminobutyric acid (GABA). GAD is the rate-limiting enzyme for GABA biocatalysis (Ueno 2000). It is widely distributed amongst eukaryotes and prokaryotes (Nomura et al. 2000). GABA is a well-characterized inhibitory neurotransmitter for animals, with hypotensive and analgesic properties (Krnjevic and Schwartz. 1966; Takahashi et al. 1995; Adeghate and Ponery. 2002; Zenab et al. 2006). Therefore, GABA has potential as a functional bioactive component in foods and pharmaceuticals (Park and Oh 2007). Some reports indicate that GABA is, in microorganisms, functionally involved in the germination of the *Bacillus megaterium* spore (Foester and Foester 1973). It has also been reported that GABA production confers resistance to an acidic pH in *Escherichia coli* and *Lactococcus lactis* (Sanders et al. 1998; Castanie-Cornet et al. 1999; Warnecke and Gill 2005).

Recently, much interest has been generated around the utilization and mass production of GABA as a bioactive component for food. GABA green tea, gabaron tea, and red mold rice have been reported to exert antihypertensive

effects in human subjects (Kohama et al. 1987; Su et al. 2003; Wang et al. 2003; Wang and Lin 2007). A mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbreuckii*, isolated from commercially available yoghurt, produced a large amount of GABA (Cross et al. 2004). Currently, various lactic acid bacteria (LAB), *Lactobacillus plantarum* (Park and Oh 2004), *Lactobacillus brevis* (Ueno et al. 1997), *Lactobacillus paracasei* (Komatsuzaki et al. 2005), have also been found to produce a large amount of GABA. However, the yeast strains of high-yielding GABA had few reports and the functions and properties of GABA and GAD in yeast are still unknown (Takahashi et al. 2004).

In the present study, a screened yeast strain of high-yielding GABA was used as the *gad* gene sources. A full-length *gad* of *S. cerevisiae* was cloned by PCR. The gene was sequenced and analyzed by homology and function. Besides, the similarity of *gad* between *S. cerevisiae* and other yeasts was explored. Recombinant expression vectors that contained *S. cerevisiae gad* were constructed and transformed into *Escherichia coli* BL21 (DE3) prokaryotic expression host cells. The strains of efficient expression were selected and the expression products of *S. cerevisiae gad* were analyzed by SDS-PAGE. And the enzyme activity of *Sc*GAD was determinated by artificial neural network linked with genetic algorithm (ANN-GA) method (Sivapathasekaran et al. 2010)

Materials and methods

Yeast strains and plasmids

The yeast strains producing GABA were isolated from the fruit surface of pears, apples, grapes and kiwi, etc. The isolated strains were incubated in Czapek's broth liquid medium as following described in culture media containing 0.5 % monosodium glutamic acid (MSG) at 28 °C for 48 h. The cells were centrifuged, and the supernatant was analyzed for formation of GABA by using paper chromatography. Microorganisms that produced high levels of GABA were selected and identified by studying their morphological characters, biochemical properties and by TIS sequence determination.

Escherichia coli DH5α and BL21 (DE3) codon plus (Novagen, USA) were used as the cloning and expression host cells, respectively. Plasmid pGEMT-Easy vector (Promega, USA) was used for DNA cloning and sequencing, and pET-30a (Novagen, USA) was used for the expression of GAD protein.

Culture media. (1) The LB medium consists of 0.5 % yeast extract, 10 % peptone, 10 % NaCl and 2.0 % agar, ddH_2O 1,000 mL, pH 7.2; (2) YEPD medium consists of 1.0 % yeast extract, 2.0 % peptone, 2.0 % glucose, and 2.0 % agar, ddH_2O 1,000 mL, pH 6.0; (3) Czapek's medium consists of 3 % glucose, 0.1 % yeast extract, 0.1 % K_2HPO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.05 % KCl, 0.001 % $FeSO_4$ and 2.0 % agar, ddH_2O 1,000 mL, pH 6.0. Liquid media were free of agar and all the media were sterilized at 121 °C for 20 min before use.

Cloning and DNA sequencing

The *S. cerevisiae* MJ2 was inoculated on YEPD liquid medium and incubated at 28 °C and 150 rpm for 48 h in a reciprocal shaker. The cells of *S. cerevisiae* MJ2 were collected by centrifuging at 6,000×*g*. Genomic DNA was isolated from the *S. cerevisiae* MJ2 using TIANamp Yeast DNA Kit (Tiangen Biotech. Co. Ltd., Beijing, China)and was used as the template DNA for PCR cloning. *gad* 1 (forward): 5′-ggg *CCT AGG* ATG TTA CAC AGG CAC GGT TCT AAG CAG AAG-3′ (*Avr*II restriction site underlined and protecting base in small letter) and *gad* 2 (reverse): 5′-aaggaaaaaa *GCG GCC GC* TCA ACA TGT TCC TCT ATA GTT TCT C-3′ (*Not*I restriction site underlined and protecting base in small letter) were designed from *gad* both ends sequence of *S. cerevisiae* in GenBank (Gene systematic name:YMR250W) using the soft of Vector NTI 9. Amplification by PCR was carried out at 94 °C for 1 min, 48 °C for 1 min 30 s, 72 °C for 1 min for 35 cycles using *Taq* DNA polymerase (Takara, Japan). The PCR product was purified using the Wizard DNA Purification Kit (Promega, Madison, WI), ligated into pGEMT-Easy vector by T4 DNA ligase, and then transformed into *E. coli* DH5α. The cloned DNA was sequenced by dideoxynucleotide termination procedures (Sanger et al. 1977) by Shenggong Co. Ltd., China.

Construction of pET-*gad* vector

The entire ORF region of *Scgad* was amplified by PCR using both forward primer (5′-acgc *GTC GAC* ATG TTA CAC AGG CAC GGT TCT AAG CAG AAG-3′) (*Sal*I restriction site underlined and protecting base in small letter) and reverse primer (5′-ccg *CTC GAG* TCA ACA TGT TCC TCT ATA GTT TCT C-3′ (*Xho*I restriction site underlined and protecting base in small letter, the same below). The resulting PCR product (*Scgad*) was purified using the Wizard DNA Purification Kit (Promega, Madison, WI) and digested with *Sal*I/*Xho*I. An approximately 1.8 kb DNA was then inserted into the pET-30a expression vector to generate the recombinant expression vector construct pET-*gad*.

Expression of *Scgad* gene in *E. coli*

To examine *Scgad* gene expression, a DNA fragment containing *Scgad* was amplified by PCR using two primers (primer 1, 5′-acgc *GTC GAC* ATG TTA CAC AGG CAC GGT TCT AAG CAG AAG-3′; primer 2, 5′-ccg *CTC GAG*

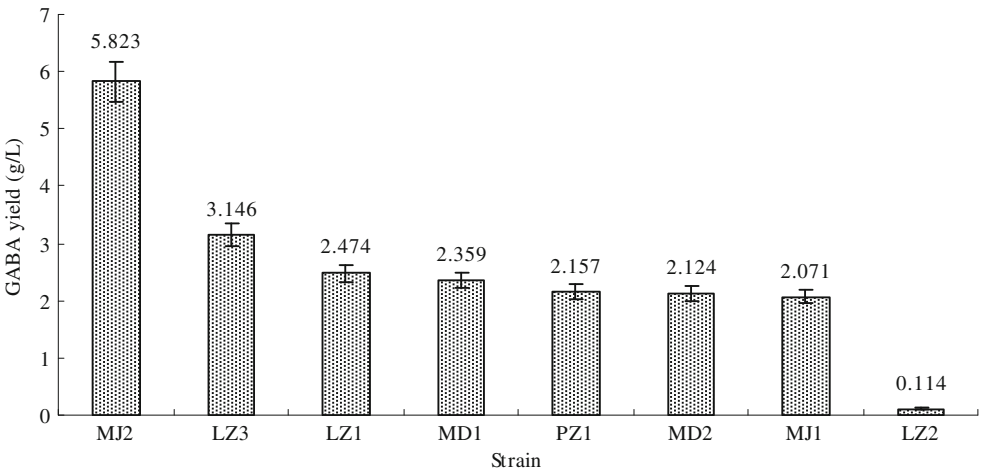

Fig. 1 The GABA yield of some selected yeast strains. Note: Results are mean values of triplicate determinations ± standard deviation. Yeasts were cultivated in Czapek's broth with 0.5 % MSG at 28 °C for 48 h

TCA ACA TGT TCC TCT ATA GTT TCT C-3′). Amplification by PCR was carried out at 94 °C for 1 min, 48 °C for 1 min 30 s, 72 °C for 1 min for 35 cycles using Taq DNA polymerase (Takara, Japan). The resulting expression plasmid was designated as pET-*gad*. The pET-*gad* was transformed into *E. coli* BL21 (DE3).

Escherichia coli BL21 (DE3) cells harboring the *Scgad* gene were grown in LB medium containing ampicillin (100 μg/mL) at 37 °C to an OD_{600} of approximately 0.6 and the gene expression was induced with 1 mM IPTG at 37 °C for 7 h. The cells were centrifuged and the pellet was washed with resuspension buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM DTT, 5 mM $MgCl_2$, 10 % glycerol, 1 mM PLP). The cells were then disrupted by lysozyme digestion followed by sonication as previously described (Park and Oh 2007; 2004), and the supernatant fraction was recovered by centrifugation at 14,000×*g* for 30 min at 4 °C. The supernatant was used for the analysis of GAD (*Sc*GAD) activity and SDS-PAGE (Sodium dodecyl sulphate−polyacrylamide gel electrophoresis) (Sambrook and Russell 2001).

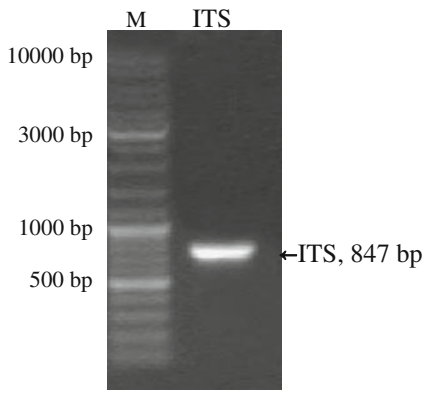

Fig. 2 The PCR amplified ITS of *S. cerevisiae* MJ2 M, DNA molecular weight marker; ITS, PCR product of ITS (847 bp)

Characterization of *Sc*GAD

To further obtain more detailed characteristics of *Sc*GAD in *E. coli* BL21 (DE3) cells, four characteristic parameters for *Sc*GAD activity including pH, PLP, temperature dependence (Temp.D) and temperature on stability (ToS) were investigated by single-factor-at-a-time experiments. In advance, the boundaries of the experimental domain of the four variables were determined, that is, these were various pH ranging from 3 to 7, PLP ranging from 0 to 100 μM, Temp.D, at a range from 20 °C to 60 °C, and ToS at different time, ranging from 20 to 80 °C. Subsequently, the best set of pH, PLP and Temp.D on *Sc*GAD activity were studied using artificial neural network linked with genetic algorithm (ANN-GA) method, and all data obtained from activity assay were plotted using MATLAB version 7.8.1 (Mathworks Inc. MA, USA).

Artificial intelligence-based optimization scheme

ANN is a biologically inspired computational tool that maps the complex non-linear relationship in modeling and simulation systems (Desai et al. 2008). GA, on the other hand, is

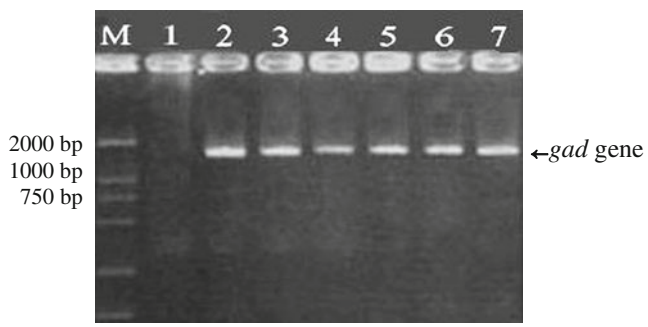

Fig. 3 The agarose gel electrophoresis analysis of *gad* PCR amplification from *S. cerevisiae* MJ2 Lane 1, control; Lanes 2~7, *gad* PCR product; M, DL2000 Marker

Fig. 4 The 585 amino acid residues deduced from *Scgad* ORF from *S. cerevisiae* MJ2

1	MLHRHGSKQK	NFENIAGKVV	HDLAGLQLLS	NDVQKSAVQS
41	GHQGSNNMRD	TSSQGMANKY	SVPKKGLPAD	LSYQLIHNEL
81	TLDGNPHLNL	ASFVNTFTTD	QARKLIDENL	TKNLADNDEY
121	PQLIELTQRC	ISMLAQLWHA	NPDEEPIGCA	TTGSSEAIML
161	GGLAMKKRWE	HRMKNAGKDA	SKPNIIMSSA	CQVALEKFTR
201	YFEVECRLVP	VSHRSHHMLD	PESLWDYVDE	NTIGCFVILG
241	TTYTGHLENV	EKVADVLSQI	EAKHPDWSNT	DIPIHADGAS
281	GGFIIPFGFE	KEHMKAYGME	RWGFNHPRVV	SMNTSGHKFG
321	LTTPGLGWVL	WRDESLLADE	LRFKLKYLGG	VEETFGLNFS
361	RPGFQVVHQY	FNFVSLGHSG	YRTQFQNSLF	VARAFSFELL
401	NSSKLPGCFE	IVSSIHESIE	NDSAPKSVKD	YWEHPQAYKP
441	GVPLVAFKLS	KKFHEEYPEV	PQAILSSLLR	GRGWIIPNYP
481	LPKATDGSDE	KEVLRVVFRS	EMKLDLAQLL	IVDIESILTK
521	LIHSYEKVCH	HIELASEQTP	ERKSSFIYEM	LLALASPQDD
561	IPTPDEIEKK	NKLKETTTRN	YRGTC	

a commonly used globalized optimization technique to solve diverse optimization problems in biochemical engineering, and is based on the evolutionary methods of natural selection of the best individuals in a population (Che et al. 2011). Hybrid ANN-GA methodology has been an effective tool with a wide range of applications in various fields (Chen et al. 2004; Lu et al. 2011). Just because of this, the artificial intelligence-based optimization tool, in this paper, was employed to obtain the maximum of *Sc*GAD activity.

Analysis of GABA and *Sc*GAD activity

GABA-generating capacities of *S. cerevisiae* MJ2 and other yeast strains were determined by paper chromatography and HPLC (Waters, Milford, MA) as described by Kim et al. (2007). The authentic GABA and glutamate were used as controls. *Sc*GAD in *E. coli* BL21 (DE3) cells was extracted as described by Zhang *et al*. (2007) and the specific activity was assayed by a radiometric method as described before (Sha et al. 2005). In brief, GAD reaction system was consisted of 8 μL L-[^{14}C] glutamic acid, 42 μL of 5 mM MSG in 50 mM potassium phosphate and proper amount of sample or enzyme standard solution to give a final volume of 100 μL. The reaction was conducted in disposable culture tubes, which were capped with rubber stoppers and incubated at 37 °C for 2 h with shaking. After incubation, 0.1 mL of 0.5 N H_2SO_4 was injected to terminate the reaction. A certain amount of ^{14}C labelled CO_2, produced during the enzyme reaction, was collected using hyamine base and measured in β counter. One enzyme unit was defined as 1 μM of product formed per min at 37 °C. The enzymatic activity of *Sc*GAD was detected in this assay.

Results and discussion

Isolation and identification of *S. cerevisiae* MJ2

The yeasts producing GABA were isolated from the surface of fruits. During the screening stage, all yeast strains (56 strains) producing GABA from MSG were selected (The GABA yields of some selected yeast strains were showed in Fig. 1). Among the screened microbes, one yeast strain from the surface of kiwi showed a particularly high GABA production by paper chromatography analysis. The production of GABA was confirmed by HPLC (Fig. 1). The isolated strain was identified as *S. cerevisiae* by examining its morphological characteristics and biochemical characteristics (data not shown). To further confirm the identification of the MJ2 strain, ITS was amplified and 847 bp (Fig. 2) of the DNA sequence was determined by Shenggong Co. Ltd., China. The GenBank database was used to search for genes

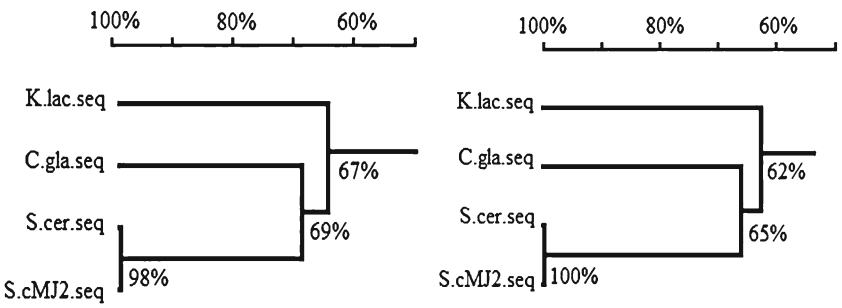

Fig. 5 The homology analysis of *Scgad* gene from *S. cerevisiae*. Left: the homology of *Scgad* sequences, Right: the homology of amino acid sequence deduced from *Scgad* ORF

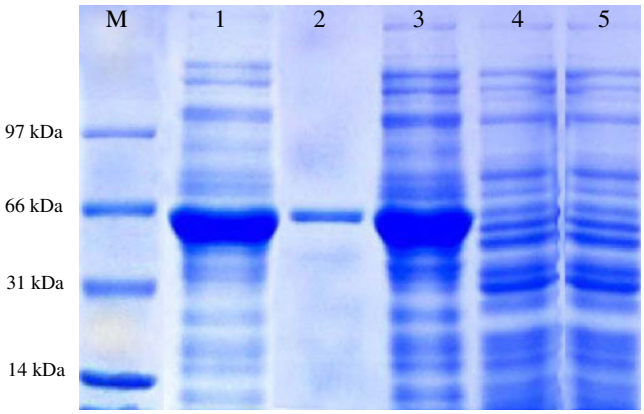

Fig. 6 SDS-PAGE analysis of *Scgad* gene expression protein in *E. coli,* M: Molecular weight markers; lanes 1 and 3:*Scgad* expression protein; lane 2:purified *Scgad* protein; lane 4:The sample before induced by IPTG ;lane 5:The sample of negative control

similar to the ITS sequence, revealing that the highest nucleotide sequence similarity was 100 % with *Saccharomyces cerevisiae* (GenBank accession no. AB280541.1, AB279758.1 and AB279757.1). Therefore, the strain MJ2 was considered to be a strain of *S. cerevisiae*. When the GABA-generating capacity of *S. cerevisiae* MJ2 strain was compared with that of other ordinary strains with identical substrate concentrations of 0.5 % (w/v) monosodium glutamate (MSG), the *S. cerevisiae* MJ2 exhibited a GABA productivity of 5.823 g/L, which was remarkably higher than that of other ordinary strains (Fig. 1). *S. cerevisiae* MJ2 could be a potentially useful strain for the industrial production of GABA and the development of health foods rich in GABA.

Cloning, sequencing and characterization of the *Scgad* gene

To clone *Scgad* from *S. cerevisiae* MJ2 strain, genomic DNA was isolated and used as a template DNA for PCR cloning. PCR for full-length *gad* was carried out using primers designed from sequence of *gad* gene of *S. cerevisiae* in GenBank (Gene systematic name:YMR250W; accession no. Q04792). Through the PCR reaction, a full-length *Scgad* gene (about 2,000 bp) was cloned (Fig. 3).

The PCR product was purified, cloned into pGEM-T, and sequenced. The nucleotide sequence of the full-length *Scgad*

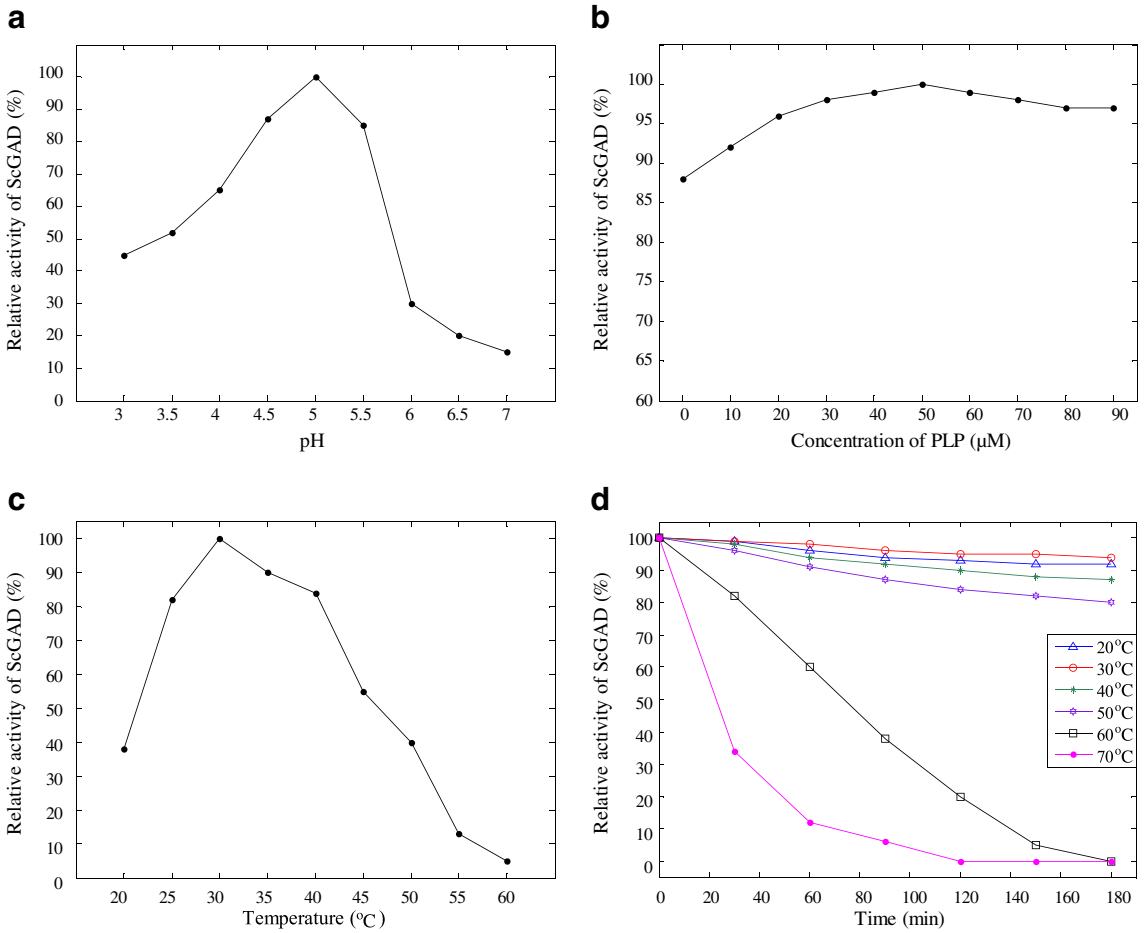

Fig. 7 **a** Effect of pH on activity of ScGAD. **b** Effect of PLP on activity of ScGAD. **c** Effect of temperature dependence on activity of ScGAD. **d** Effect of temperature on stability of ScGAD. ScGAD activity was measured in the potassium phosphate buffer consisting of 100 μL of 50 mM sodium phosphate, 100 mM L-glutamate

gene consisted of 1758 bases (ORF 1755 bp), which encoded a protein of 585 amino acid residues (Fig. 4) with a calculated molecular weight of 65.897 kDa. A computer-based homology search program by NCBI revealed that the *Scgad* gene had 98 %, 69 % and 67 % sequence homology to the *gad* sequences deposited in GenBank for *Saccharomyces cerevisiae gad* (NC_001145.2), *Candida glabrata gad* (NC_006031.1), and *Kluyveromyces lactis gad* (NC_006039.1), respectively. The amino acid sequence deduced from *Scgad* ORF showed 100 %, 65 %, and 62 % identity to the *S. cerevisiae* GAD, *C. glabrata* GAD, and *K. lactis* GAD sequences, respectively (Fig. 5). The *Scgad* gene sequence was landed in NCBI Genebank under the accession code of GU074586.

Expression of *Scgad* gene

Saccharomyces cerevisiae gad was expressed in the pET-30a, as described in materials and methods. The cells harboring pET-*gad* were induced by the addition of 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) to the LB culture media. The extract from *E. coli* cells transformed with the expression vector lacking any inserted sequence (negative control) was also assayed by SDS-PAGE analysis with equivalent amounts of protein being loaded. SDS-PAGE analysis showed that the molecular weight of the GAD proteins analyzed was estimated to be about 65.0 kDa (Fig. 6). This value is consistent with the size (65.897 kDa) calculated from the amino acid sequence. This result indicated that the *S. cerevisiae* MJ2 *gad* gene had indeed encoded *Sc*GAD and was successfully expressed.

Characterization of *Sc*GAD

The optimum pH of *Sc*GAD was between 4.5 and 5.5, and the optimal pH was preliminary determined at pH 5.0 (Fig. 7a). The enzyme was stable within the pH range of 3.5-5.5. The remaining activity was about 45 % and 15 % at pH 3.0 and 7.0, respectively.

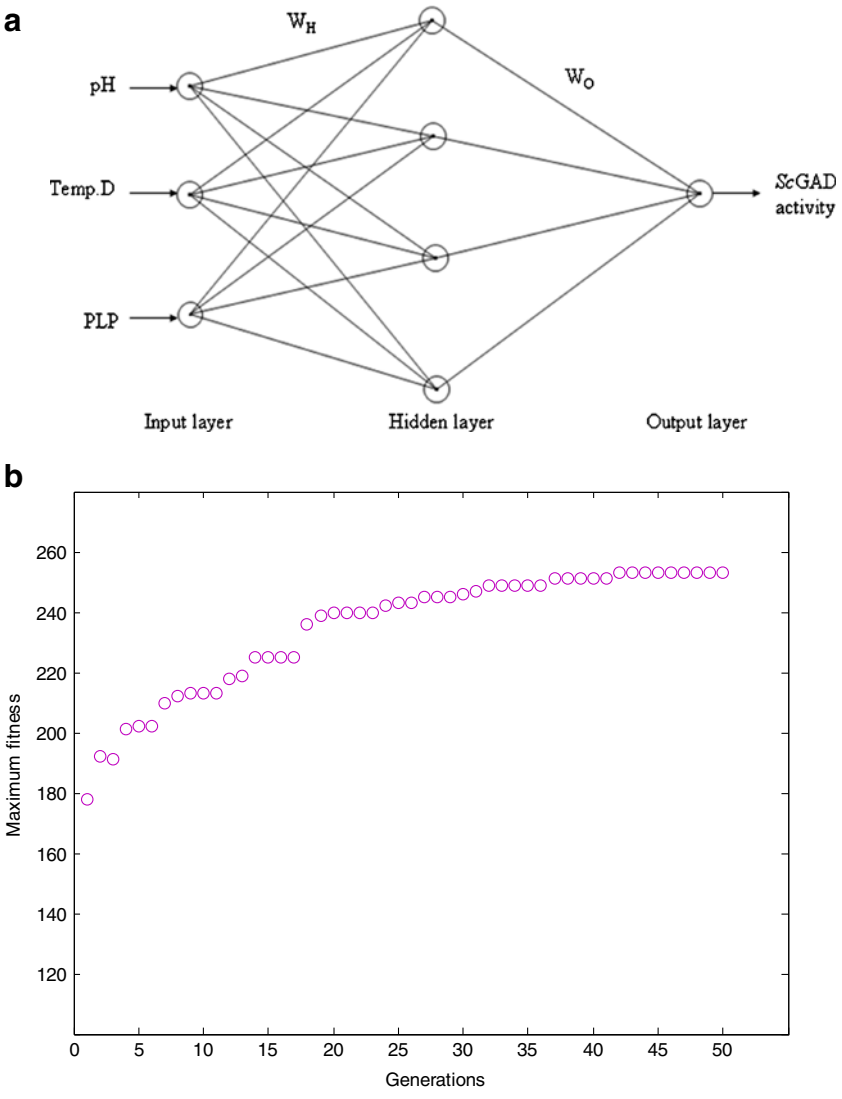

Fig. 8 **a** Neural Network architecture used for ScGAD activity optimization. WH = weight connections between input and hidden nodes and WO = weight on connects between hidden and output nodes. **b** Representative plots generated from the optimization by GA using MATLAB 7.8.1. Best fitness values with successive generations showed gradual convergence to the optimum value. Flow chart of a typical GA based optimization model

As a pyridoxal-dependent decarboxylase, GAD requires PLP for its activity. Figure 7b showed the effect of the additional concentration of PLP on the activity of *Sc*GAD. The result showed that *Sc*GAD activity increased indistinctively by addition of PLP. The possible reason might be that the GAD of *S. cerevisiae* MJ2 integrated very strongly with the coenzyme PLP and they existed mostly as holo GAD.

The optimal Temp.D for GAD activity was determined by the standard assay ranging from 20 °C to 60 °C, and the ToS of the *Sc*GAD was determined by measuring the remaining activity after incubating the enzyme for various times separately at 20 °C, 30 °C, 40 °C, 50 °C, 60 °C and 70 °C. The optimum Temp.D of *Sc*GAD was 30 °C (Fig. 7c). The *Sc*GAD was stable after 180 min of incubation at the temperatures between 20 °C and 50 °C, while the enzyme had only about 20 % and 0 of initial activity after 120 min of incubation at 60 °C and 70 °C, respectively (Fig. 7d).

To obtain a more detailed relationship between the *Sc*GAD activity and three critical variables, namely pH, PLP, Temp.D. ANN-GA model as an appropriate technique was employed to compute the optimal conditions for enhancing the *Sc*GAD activity. Briefly, in the present study three neurons were used in the input layer, four in the hidden layer and one in the output layer of the network to model the dependence of the *Sc*GAD activity on the three critical variables, i.e., a single hidden layer comprising of four neurons was used (Fig. 8a). Once the neural network was successfully created, GA was used to optimize and determine the maximum possible *Sc*GAD activity and the point in the input space where this maximum was to be obtained. Figure 8b shows the variation of maximum fitness with generation for a maximum iteration of 50. It can be seen from this figure that the GA has reached a maximum value after the end of 50 iteration steps. The results revealed that the highest GAD activity of extracts of cells expressing PET/*Sc*GAD was 252.8 nmol CO_2/min/mg protein at pH 5.2, Temp.D 28 °C, and 50 μM of PLP, which was over 19-fold higher than the GAD activity of untransformed cells (12.36 nmol CO_2/min/mg protein) or uninduced cells (13.23 nmol CO_2/min/mg protein). All these data proved that the *Sc*GAD encoded in *E. coli* BL21 (DE3) had efficient enzyme activity for GABA production.

Conclusion

In this study, a new GABA producing microorganism, *S. cerevisiae*, was isolated from the surface of kiwi. Also, a full-length *gad* gene from *S. cerevisiae* MJ2 was cloned, and the amino acid sequence of *gad* was compared with the primary structure of GAD proteins from other sources. The protein was successfully expressed in *E. coli* BL21 (DE3) cells and the enzyme activity had been significantly enhanced. The results suggested that this strain and recombinant *gad* could be used for the industrial production of GABA and the development of health foods rich in GABA. In addition, it could be a potentially useful strain for the industrial production of GABA.

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