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Production and structural modeling of a novel asparaginase in *Yarrowia lipolytica*

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Abstract

Asparaginase catalyzes the conversion of asparagine into aspartic acid and ammonia. The enzyme has various industrial applications and it is considered as an anticancer drug for treatment of certain leukemias. In the current study, production of asparaginase was investigated by *Yarrowia lipolytica* as well as optimized its production and determined its molecular characteristics by in silico analysis. *Y. lipolytica* DSM3286 produced 17.14 U/ml of asparaginase in flask culture. Optimization of asparaginase production was done by response surface methodology and the enzyme production increases up to 102.85 U/ml. The enzyme production reached 210 U/ml in a bioreactor which is 12-fold more than flask culture containing non-optimized medium. Asparaginase gene of *Y. lipolytica* was identified and isolated on the basis of comparison with asparaginase gene sequences of other microorganisms. The gene has 981 nucleotides and its protein has 326 amino acids. According to in silico analysis, the secondary structure of the enzyme is composed of 9 α-helixes and 11 β-sheets. *Y. lipolytica* produces type II asparaginase with high affinity for asparagine which is a suitable eukaryotic asparaginase for treatment of hematopoietic cancers. Hence, *Y. lipolytica* could be recommended as a new eukaryotic microbial source for the production of this important therapeutic enzyme.

Keywords: *Yarrowia lipolytica*, Asparaginase, Identification, Production, Anticancer drug

1. Introduction

Asparaginase (E.C. 3.5.1.1) catalyzes the hydrolysis of asparagine to aspartic acids and ammonia. This enzyme has been used as a therapeutic drug for certain types of hematopoietic diseases such as acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL) [1]. The enzyme prevents acrylamide formation and increases the taste and nutritional value of foods [2]. Furthermore, it is used as an enzyme model in drug delivery systems and biosensor to detect the level of asparagine [3, 4]. Asparaginase has been widely found in plants, animal and microorganisms. Microbial asparaginase has attracted much attention in recent years because of its biotechnological applications and simplicity of large-scale production [5, 6]. Various genera of microorganisms can produce L-asparaginase such as *Aspergillus*, *Candida*, *Pseudomonas*, *Corynebacterium*, *Enterobacter*, *Erwinia*, *Staphylococcus*, and *Thermus* [7]. *Escherichia* and *Erwinia* are main microbial asparaginase producers for medical and therapeutic purposes. However, they are prokaryotic sources for asparaginase production and their enzymes have a wide spectrum of side effects in long-term therapy including hypersensitivity and immune inactivation due to the fact that is a foreign protein [8]. Finding new eukaryotic sources for the production of asparaginase can be considered to overcome these problems. Yeasts are good eukaryotic candidates for asparaginase production due to post-translational protein modification and suitable glycosylation. The yeast *Yarrowia lipolytica* is nonpathogenic and classified by as a generally regarded as safe (GRAS) microorganism by the US Food and Drug Administration (FDA) for human manufacturing processes. This yeast has many advantages to other yeasts like high secretion efficiency, low over-glycosylation, good product yield as well as using the cheap and cost-effective medium to growth and metabolite production [9]. In the current study, the production of asparaginase by *Y. lipolytica* was investigated to introduce new eukaryotic sources for the production of asparaginase. Response surface methodology (RSM) as a statistical technique was used to optimize asparaginase

production in flask and bioreactor cultures. Furthermore, L-Asparaginase gene of *Y. lipolytica* was isolated and its molecular characteristics were determined using bioinformatics study.

2. Materials and Methods

2.1. Yeast strain and culture conditions

Y. lipolytica DSM3286 was prepared from the German collection of microorganisms and cell cultures (DSMZ), Germany. Yeast strain was cultured in YPD medium, containing 10 yeast extract, 20 peptone, and 20 dextrose in grams per liter, as preculture and incubated at 29 ºC for 24 h [10]. Asparaginase was produced by *Y. lipolytica* in Czapek Dox medium containing 2 glucose, 10 asparagine, 0.52 KCl, 1.52 K_2HPO_4, 0.3 $NaNO_3$, 0.05 $ZnSO_4$ and 0.05 $FeSO_4$ in grams per liter [11]. 20 ml of Czapek Dox medium in 100-mL flask was inoculated with 1% inoculum, and incubated at 29 ºC and 200 rpm. Sampling was performed every 24 h for measurement of enzyme activity and yeast cell growth [12].

Qualitative assay for asparaginase activity

The qualitative assay of asparaginase was carried out using a rapid plate method in minimal asparagine agar (MSA) medium containing 10 L-asparagine, 0.5 $MgSO_4$, 0.5 KCl, 0.2 NaCl, 0.12 phenol red and 15 agar in grams per liter. A yeast colony was inoculated into MSA medium and incubated at 29 ºC [11].

2.2. Quantitative assay for asparaginase activity

A spectrophotometric method was used for the quantitative assay of asparaginase activity [13]. A reaction mixture containing 0.5 ml of L-Asparagine (0.04 M), 0.5 ml of Tris buffer (0.1 M and pH 7.2), 0.5 ml of enzyme preparation and 0.5 ml of distilled water was incubated at 37 ºC for 30 min. The reaction was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid. 0.1 ml of

the above mixture and 0.2 ml of Nessler's reagent were added to 3.7 ml of distilled water. The absorbance of the sample was measured at 450 nm by spectrophotometer (Shimadzu spectrophotometer UV-1800, Japan) after incubating the mixture at room temperature for 15 min. One unit of asparaginase is defined as the amount of enzyme which produces one μmol of ammonia from L-asparagine per minute at 37 °C and pH 7.

2.3. Design of experiments for the optimization of asparaginase production

The four important variables affecting the production medium of asparaginase, glucose, L-asparagine, K_2HPO_4, and pH were selected to optimize the production medium (Table 1). The central composite design (CCD) of response surface methodology (RSM) was used for optimization of asparaginase production and determination of the interactive effects of four variables by the software Design-Expert Version 7.0.0 (Stat-Ease, Inc., Minneapolis, USA). The software recommended 30 experiments with different composition of variables.

2.4. Production of asparaginase in bioreactor

The asparaginase production was done in a 5-L bioreactor (Winpact FS-01-A, Taiwan) containing 2 L of optimized medium. The bioreactor was equipped with two RDT6 Rushton turbines and a built-in digital controller for pH, temperature, agitation, and dissolved oxygen (DO). A pH sensor (Mettler-Toledo InPro3030/325, Urdorf, Switzerland) and a DO sensor (Mettler-Toledo InPro6800/12/320, Urdorf, Switzerland) were controlled by online monitoring set point for pH and DO concentration, respectively. The medium was inoculated with 5 % (v/v) inoculum. Fermentation was carried out at 29 ºC, pH 7.0, and a fixed stirring speed of 500 rpm and an airflow rate of 1 vvm. Enzymatic activity and cell growth were measured every 2 hours during 24 h [14].

2.5. Identification and sequencing of asparaginase gene

The genome of *Y. lipolytica* DSM3286 was extracted using ZR Fungal/Bacterial MiniPrep™ Kit (Zymo Research, USA). The forward primer (ATGGTGTACGTAGCACTCCATC) and reverse primer (TCACCTCTCTCTATATATCTGTATAGAGCTAAC) were designed according to YALI0F30723g locus in chromosome F of *Y. lipolytica* strain CLIB122 which is similar to L-asparaginase of *Schizosaccharomyces pombe* [15, 16]. The 2X Master Mix Phusion U (Thermo Fisher Scientific, USA) was used to carry out PCR according to the recommended conditions [17]. The sequencing of the PCR product was done by Eurofins Genomics, Germany.

2.6. In silico analysis and structural modeling

After sequencing of the desired gene, the sequence was compared with stored sequences in NCBI (https://blast.ncbi.nlm.nih.gov) using blastn and blastp. The phylogenetic tree was drawn by MEGA7 software (7.0.026 version). Multiple sequence alignment and conserved areas were predicted based on amino acid sequences using NCBI (https://www.ncbi.nlm.nih.gov).

The secondary structure of the protein was obtained using PSS finder provided by Soft Berry (http://www.softberry.com). Signal peptide sequence was determined using Phobius site (http://phobius.sbc.su.se/). The functional structure of the protein and the amino acids of the active site were determined by RaptorX site (http://raptorx.uchicago.edu/).

3. Results

3.1. Asparaginase production

Y. lipolytica DSM3286 showed a pink halo around its colony in MSA medium after 24 h. The formation of pink halo around colony indicates asparaginase activity [11]. Then, the yeast strain was cultured in Czapek Dox for the production of asparaginase. Figure 1 shows asparaginase

production and cell growth by the yeast strain during 96 h. As shown in the figure, the enzyme activity of *Yarrowia lipolytica* DSM3286 was 17.14 U/ml after 24 h.

3.2. Optimization of asparaginase production

We analyzed the results of 30 experiments using the software (Table 2). They were fitted with the following model which expressed in actual variables in equation:

Asparaginase activity (U/ml) = +44.75 + 6.70 × glucose + 0.32 × asparagine – 5.31 × K_2HPO_4 – 2.75 × pH + 0.91 × glucose × asparagine + 2.02 × glucose × K_2HPO_4 – 4.72 × asparagine × K_2HPO_4 + 9.80 × asparagine × pH+ 2.34 × K_2HPO_4 × pH + 6.87 × glucose × asparagine × K_2HPO_4 – 14.88 × asparagine × K_2HPO_4 × pH

The amount of asparaginase production can be predicted by the above model. The model for the production of asparaginase was significant according to the analysis of variance (Table 3). The P value of the model (<0.0007) is less than 0.05 which indicates model terms are significant. The coefficient of determination (R square) value ensures a reasonable adjustment of the model to the experimental data. Model is significant when it is close to 1. Here, the R square was 0.65 which is good and satisfying.

Three-dimensional plots show interactions between levels of variables in asparaginase production. The optimum level of each variable is recognized on the basis of hump in the plots (Fig. 2).

Glucose and asparagine had the most significant effect on asparaginase production among the four variables according to the Fig. 2 and various analysis performed by the software. After optimization of the medium, asparaginase production reached up to 102.85 U/ml. The optimized medium is composed of 8 glucose, 15 asparagine, 4.5 K_2HPO_4 in grams per liter and pH 7.

3.3. Production of asparaginase in bioreactor

The 2 L initial work volume of optimized medium was used to the production of asparaginase in a 5-L bioreactor. Maximum production of asparaginase was obtained 210 U/ml by *Y. lipolytica* DSM3286 after 20 h in precisely controlled conditions of the bioreactor (Fig. 3).

3.4. Isolation and in silico analysis of asparaginase gene

We found a similarity to asparaginase of *Sc. pombe* and YALI0F30723g locus in chromosome F of *Y. lipolytica* strain CLIB122 according to bioinformatics analysis. Hence, forward and reverse primers were designed for YALI0F30723g locus. Figure 4 shows the agarose gel electrophoresis image of asparaginase gene of *Y. lipolytica* DSM3286 after PCR of the yeast genome by specific primers. The asparaginase gene length of *Y. lipolytica* DSM3286 was 981 bp. The sequence of the gene was deposited in the GenBank database with accession number MG 988315.

The sequence of the gene was compared with available sequences in NCBI using BLAST. The nucleotide and amino acid sequences of asparaginase gene show similarity to asparaginase sequences in other species of microorganisms like *Sc. pombe* and *Aspergillus niger*. The neighbor-joining method was used to draw a phylogenetic tree based on nucleotide sequences [18, 19]. The phylogenetic tree of similar sequences to the asparaginase gene of *Y. lipolytica* DSM3286 is shown in Fig. 5.

The asparaginase of *Y. lipolytica* DSM3286 has 326 amino acids. Multiple sequence alignment of amino acids sequences were applied to check the conserved areas of asparaginase in different microorganisms (Fig. 6). The signal peptide sequence has fifteen amino acids which begin with methionine and ends with histidine at position 15. The mature protein is made up of nine alpha-helixes and eleven beta-sheets according to the secondary structure. Figure 7 shows the third structure of the asparaginase of *Y. lipolytica* DSM3286.

4. Discussion

One of the attractive biotechnology research areas is finding new microbial metabolites and compounds with pharmaceutical and therapeutic applications. Asparaginase as a therapeutic enzyme has been used for hematopoietic diseases such as acute lymphoblastic leukemia and non-Hodgkin lymphoma [1]. There are three families of asparaginase that divide based on function, structure and amino acid sequence. These enzymes include bacterial-type L-asparaginases, plant-type L-asparaginases, and rhizobial-type L-asparaginases. Bacterial-type L-asparaginase has been subdivided types I and II [20]. Type I L-asparaginases have low affinity for L-asparagine. Type II L-asparaginase has a high affinity for L-asparagine and low specific activity against L-glutamine. Hence, type II L-asparaginase is more attractive for treatment of cancers which are destroyed by remove of available asparagine [21]. Prokaryotes are main microbial asparaginase producers and their enzymes have many side effects for patients [8]. Many researchers reported asparaginase production in molds and yeasts [13]. Different species of *Aspergillus, Penicillium, Mucor Fusarium* and *Scopulariopsis* can produce asparaginase [21]. *Saccharomyces cerevisiae* strains are capable to produce asparaginase [22]. The yeast *Y. lipolytica* has suitable glycosylation and post-translational protein modification like a human [23]. This yeast was studied as an appropriate candidate for the production of asparaginase. *Y. lipolytica* DSM3286 produced maximum amount of asparaginase in Czapek Dox medium after 24 h (Fig. 1).

Optimization of asparaginase production in various fungal and bacterial species have been reported. The production of the enzyme could be elevated by optimization of the levels of different factors using RSM [14]. RSM was used for optimization the production of asparaginase in *Pectobacterium carotovorum* MTCC 1428 which 8.3-fold increase in production of the enzyme compared to the non-optimized medium by optimizing four factors

consist of glucose, asparagine, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ [24]. Asparaginase production in *Bacillus cereus* MAB5 reached 51.54 U/ml via optimizing four parameters of medium containing soybean meal, asparagine, sodium chloride and wood chips [25]. Asparaginase production in *Y. lipolytica* DSM3286 was increased up to 6-fold after optimization of four variables of the medium. Glucose and asparagine had the most significant effect on asparaginase production (Fig. 2).

Aspergillus niger AKV-MKBU for asparaginase production in a bench scale 5-L bioreactor using groundnut oil cake (GOC) as a low-cost substrate. The highest enzyme activity was observed 15.78 U/ml after six-day [26]. Karanam and Medicherla (2010) used palm kernel cake as the substrate for the production of L-asparaginase in solid state fermentation by *Yarrowia lipolytica* NCIM 3472. They used of Doehlert experimental design for the optimization of enzyme production. The enzyme activity reached to 39.8623 U/gds at optimum conditions [27]. *Y. lipolytica* DSM3286 produce 210 U/ml of asparaginase in a 5-L bioreactor containing an optimized medium. This amount was 12-fold more than in flask culture with the non-optimized medium after 18 h (Fig. 3). Because well-controlled conditions like precise agitation, aeration, pH, and temperature are provided for microorganisms, therefore maximum asparaginase produced in bioreactor shorter time than flask culture.

Many investigations have been carried out to determine the molecular structure of L-asparaginase [4]. The gene of asparaginase in *Y. lipolytica* DSM3286 has 981 bp which is almost the same length as other microorganisms. For example, the length of asparaginase in *Erwinia chrysanth* and *Streptomyces* is 1044 and 800, respectively [28, 29]. *Y. lipolytica* DSM3286 is composed of 326 predicted amino acids. Alignment of the predicted amino acids sequences of *Y. lipolytica* DSM3286 asparaginase with similar sequences in other microorganisms is shown in Fig. 6. Many of amino acids in *Y. lipolytica* DSM3286 asparaginase is conserved in compare with other microorganisms.

Asparaginase has a signal peptide with 15 amino acids in *Y. lipolytica* DSM3286. Therefore, it is a secretory protein. Same sequences had been found in other secretory proteins and enzymes in *Y. lipolytica* [30].

Asparaginase of *Y. lipolytica* DSM3286 is similar to asparaginase of *Schizosaccharomyces pombe* as well as YALI0F30723g locus in chromosome F of *Y. lipolytica* strain CLIB122 [15, 16]. In silico analysis showed that asparaginase of *Y. lipolytica* DSM3286 is type II asparaginase with high affinity for asparagine and low specific activity against glutamine. Fortunately, the enzyme is a suitable eukaryotic asparaginase for treatment of hematopoietic cancers like acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL).

5. Conclusions

For the first time, L-asparaginase gene of *Y. lipolytica* was isolated and its molecular characteristics were determined using bioinformatics study. *Y. lipolytica* produces type II L-asparaginase according to bioinformatics analysis which is a good eukaryotic candidate for the production of asparaginase because it is more effective against tumor cells. Hence, *Y. lipolytica* could be introduced as a new source for production of a eukaryotic type of asparaginase. However, it needs further studies to determine enzyme properties such as cloning and purification the enzyme and evaluation of its effect in cancerous cell culture.

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Figure captions

Fig 1. Asparaginase production and cell growth of *Y. lipolytica* DSM3286 in Czapek Dox medium during 96 h. All the results presented are the mean values ± SD for three independent replicates.

Fig. 2. Three-dimensional response surface plots for asparaginase production showing the interaction effects of four selected variables. **a)** interaction between K_2HPO_4 and glucose; **b)** interaction between K_2HPO_4 and asparagine; **c)** interaction between pH and asparagine; and **d)** interaction between glucose and asparagine.

Fig. 3. Time courses of asparaginase activity, temperature pH, and DO in the culture of *Y. lipolytica* DSM3286 in the optimized medium in the 5-L bioreactor.

Fig. 4. Image of agarose gel electrophoresis of asparaginase gene of *Y. lipolytica* DSM3286. (M): 1 kb DNA ladder; (S): asparaginase gene of *Y. lipolytica* DSM3286.

Fig. 5. The phylogenetic tree for similar DNA sequences to the asparaginase gene of *Y. lipolytica* DSM3286. MG_988315 asparaginase of *Yarrowia lipolytica* DSM3286; XM_0188890892.1 asparaginase of *Fusarium verticillioides* 7600; XM_001389847.2 asparaginase of *Aspergillus niger* CBS513.88; NM_001020452.2 asparaginase of *Schizosaccharomyces pombe*; XM_013164838.1 asparaginase of *Schizosaccharomyces octosporus* yFS286 and XM_506063.1 YALI0F30723p locus of *Yarrowia lipolytica* CLIB122.

Fig. 6. Alignment of the predicted amino acids sequences of *Y. lipolytica* DSM3286 asparaginase with similar sequences. Query 10001: MG_988315 asparaginase of *Yarrowia lipolytica* DSM3286; Query 10002: XP_018747297.1 asparaginase of *Fusarium verticillioides* 7600; Query 10003: XP_001389884.1 asparaginase of *Aspergillus niger* CBS 513.88; Query 10004: NP_595021.1 asparaginase of *Schizosaccharomyces pombe*; Query 10005: XP_013020292.1 asparaginase of *Schizosaccharomyces octosporus* yFS286 and Query 10006: XP_506063.1 YALI0F30723p locus of *Yarrowia lipolytica* CLIB122. Conserved residues are highlighted in red, blue shows no gaps, misalignment shows into the bracket form, gray shows containing gaps (less than 50% of the sequences contain gaps are shown in gray uppercase and greater than 50% are shown in gray lowercase).

Fig. 7. The predicted third structure of the asparaginase in *Y. lipolytica* DSM3286.

Table 1. Actual and coded variables and their levels for central composite design (CCD) of response surface methodology (RSM) in order to optimize asparaginase production

Variables	Levels of variables					
	Actual	Coded	Actual	Coded	Actual	Coded
A: Glucose (g/l)	4	-1	6	0	8	+1
B: L-Asparagine (g/l)	5	-1	10	0	15	+1
C: K_2HPO_4 (g/l)	1.5	-1	3	0	4.5	+1
D: pH	5	-1	6	0	7	+1

Table 2. The designed experiments using RSM and their observed results for asparaginase production

Run order	Variables				Observed results (U/L)
	Glucose	L-Asparagine	K_2HPO_4	pH	
1	-1	+1	+1	1	30.32
2	0	+1	0	0	38.03
3	0	0	-1	0	39.72
4	0	-1	0	0	36.18
5	+1	+1	+1	-1	22.78
6	0	0	0	0	41.63
7	+1	-1	+1	-1	45.53
8	-1	0	0	0	38.62
9	0	0	0	0	41.52
10	-1	+1	-1	-1	43.13
11	-1	-1	+1	-1	41.72
12	0	0	0	-1	23.48
13	-1	-1	-1	-1	19.28
14	0	0	+1	0	24.32
15	-1	-1	-1	+1	43.41
16	+1	+1	+1	+1	102.85
17	-1	-1	+1	+1	80.38
18	0	0	0	+1	41.70
19	0	0	0	0	43.39
20	+1	0	0	0	18.93
21	-1	+1	-1	+1	72.03
22	+1	-1	-1	+1	60.12
23	0	0	0	0	46.38
24	+1	+1	-1	-1	20.87
25	+1	-1	+1	+1	18.91
26	0	0	0	0	79.28
27	0	0	0	0	59.98
28	+1	+1	-1	+1	63.12
29	+1	-1	-1	-1	37.96
30	-1	+1	+1	-1	20.36

Table 3. Analysis of variance for the response surface model for the production of asparaginase by *Y. lipolytica* DSM3286

Factors	df	Sum of squares	Mean square	F value	P value
Model	11	7811.53	710.11	6.02	<0.0007
A	1	808.15	808.15	6.32	0.0238
B	1	1.83	1.83	6.538E-003	0.9366
C	1	507.32	507.32	1.30	0.2713
D	1	136.35	163.35	2.12	0.1659
AB	1	13.34	13.34	12.25	0.0032
AC	1	65.57	65.57	7.37	0.0160
BC	1	356.74	356.74	15.49	0.0013
BD	1	1537.62	87.75	2.74	0.1187
CD	1	87.75	87.75	9.41	0.0078
ABC	1	754.19	754.19	2.50	0.1348
BCD	1	3542.33	3542.33	0.39	0.0158

A: Glucose, B: L-Asparagine, C: K_2HPO_4, C: pH, df: Degrees of freedom

Highlights

- Isolation and identification of asparaginase gene in *Y. lipolytica* for the first time.
- *Y. lipolytica* produces 210 U/ml of asparaginase after optimization of fermentation conditions.
- *Y. lipolytica* produces type II L-asparaginase according to in silico analysis.
- Introduce *Y. lipolytica* as a new microbial source for the production of eukaryotic asparaginase.

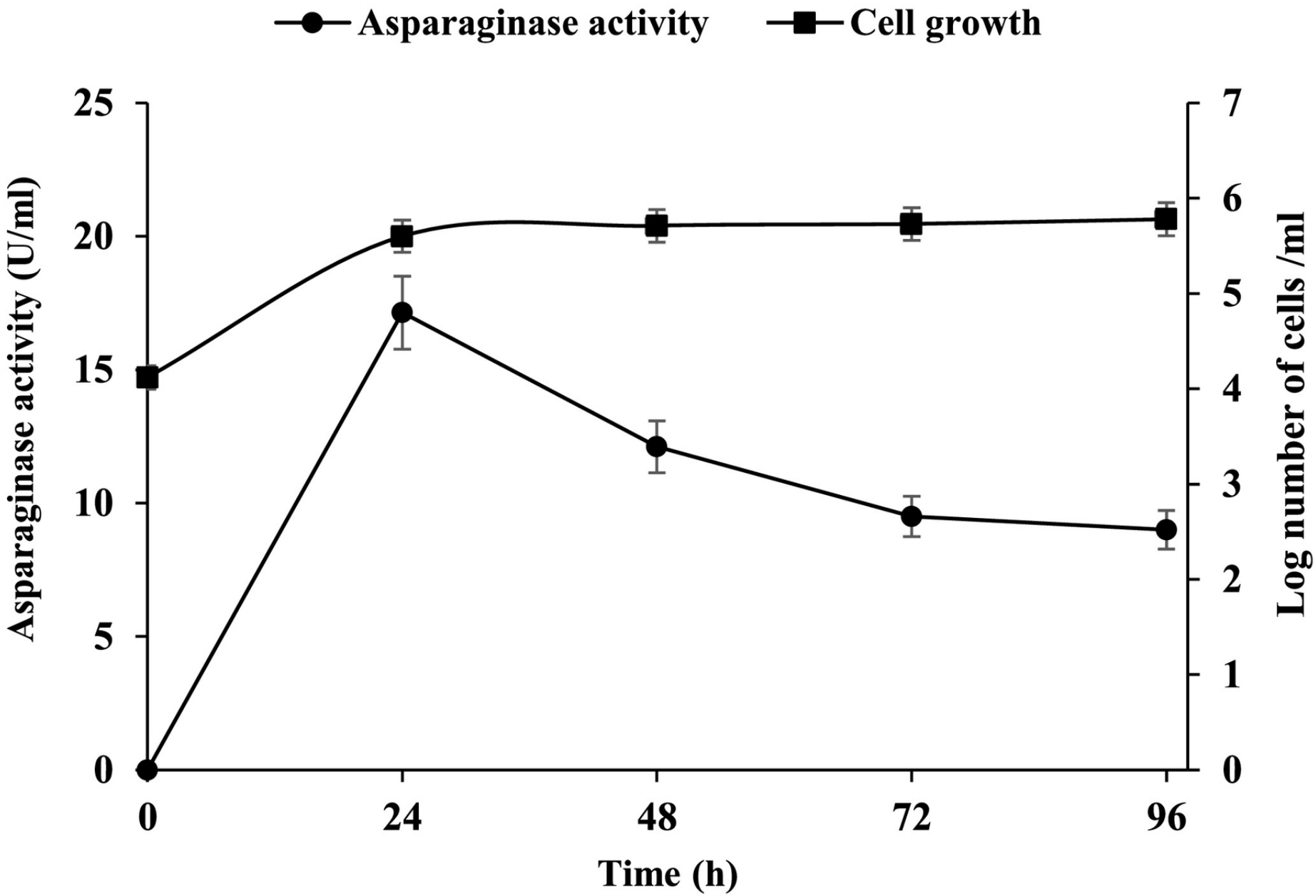
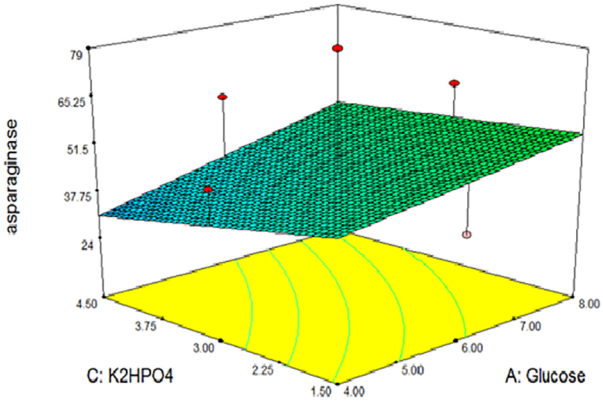
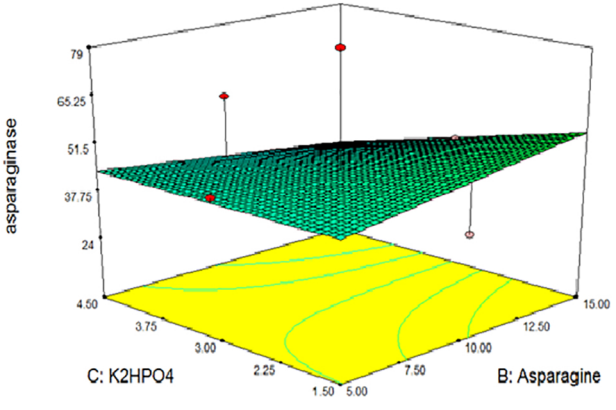

Asparaginase activity
Cell growth
Asparaginase activity (U/ml)
Log number of cells /ml
25
20
15
10
5
0
7
6
5
4
3
2
1
0
0
24
48
72
96
Time (h)

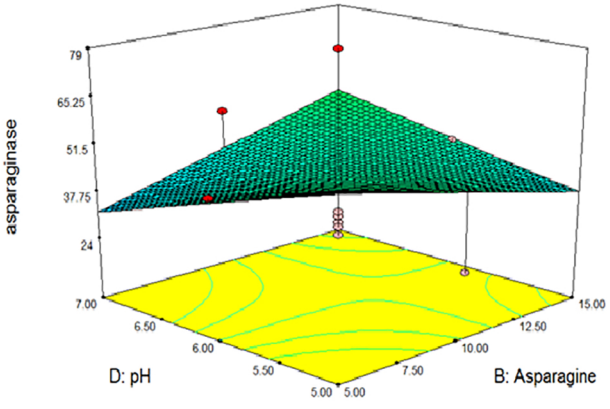
Figure 1



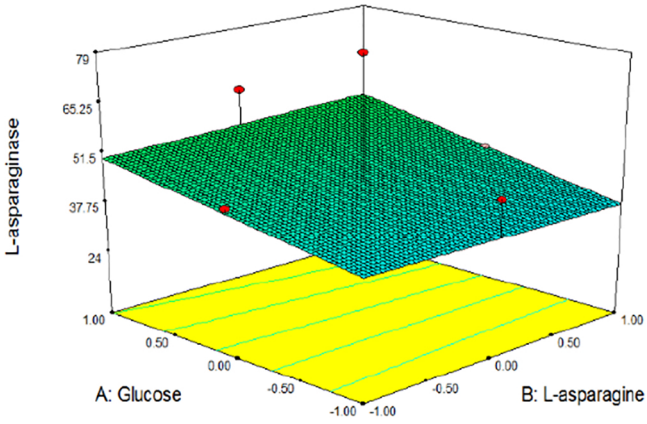
(a)



(b)



(c)



(d)

Figure 2

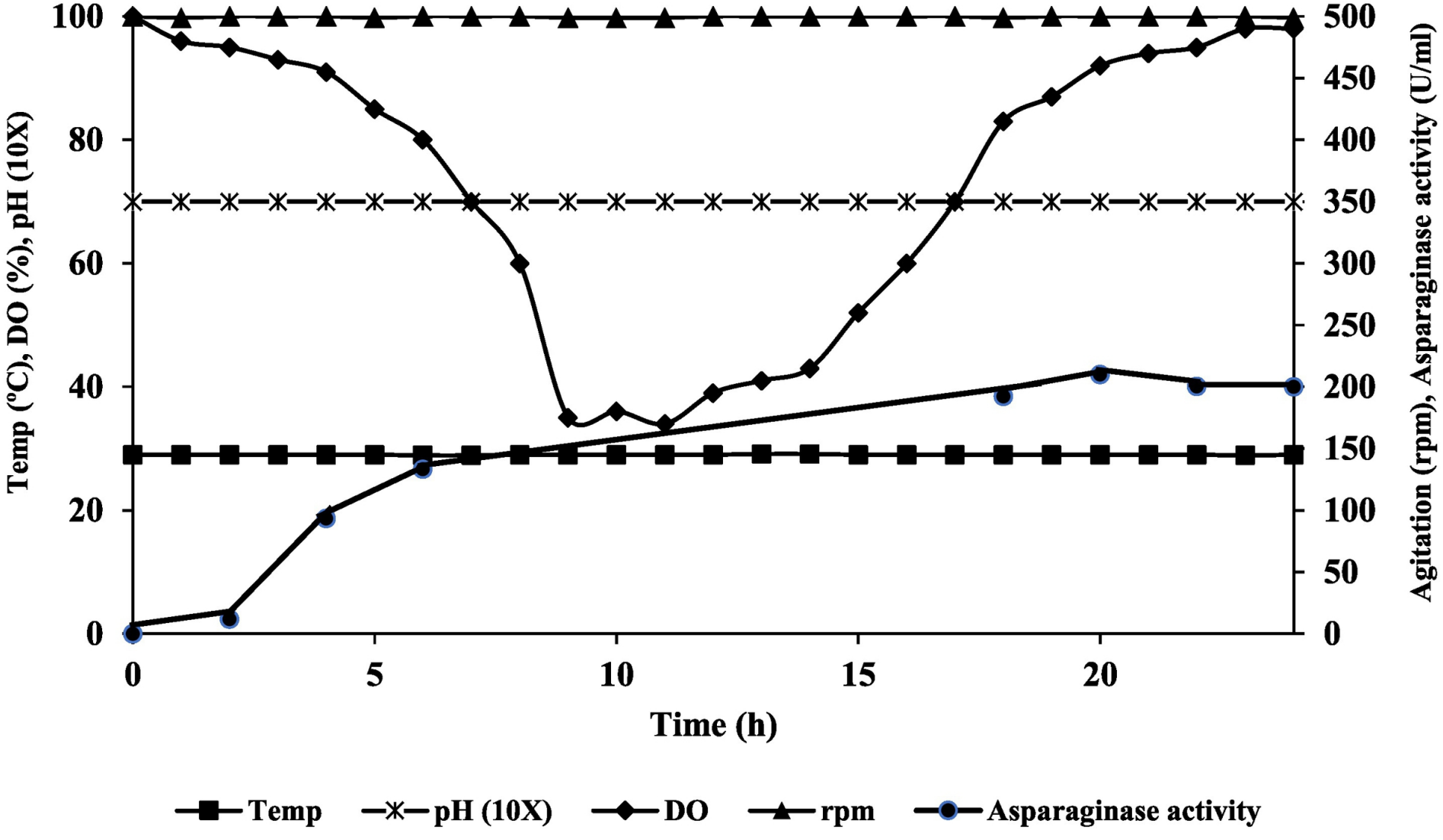

Temp (°C), DO (%), pH (10X)
Agitation (rpm), Asparaginase activity (U/ml)
Time (h)
0
20
40
60
80
100
0
50
100
150
200
250
300
350
400
450
500
0
5
10
15
20
Temp
pH (10X)
DO
rpm
Asparaginase activity

Figure 3

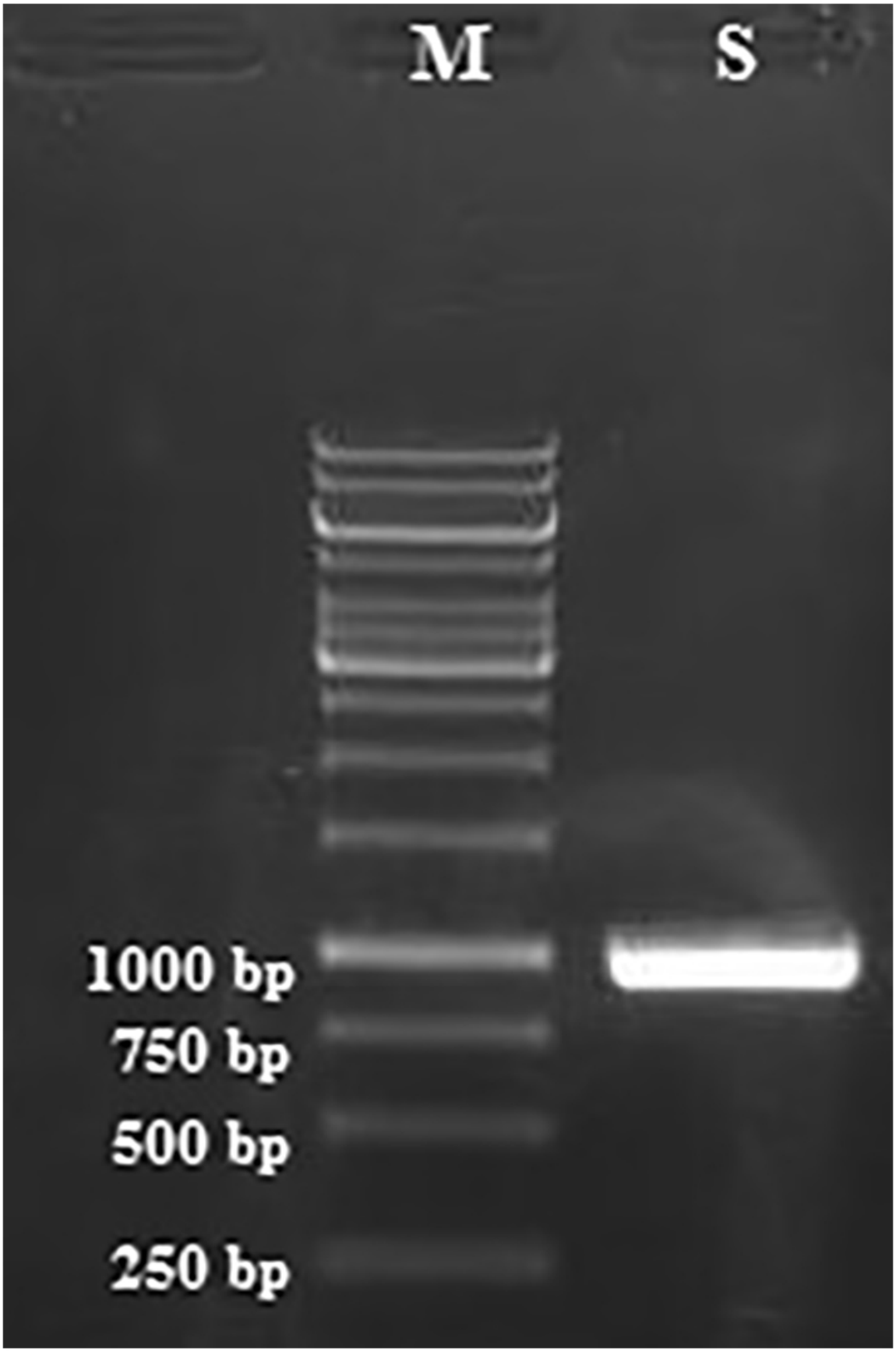


Figure 4

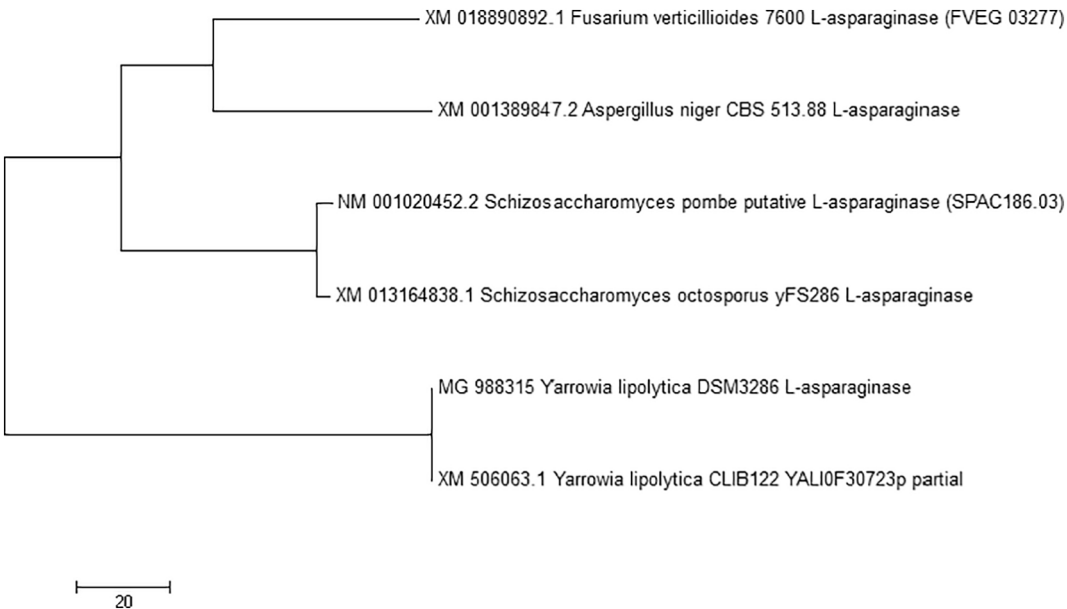

XM 018890892.1 Fusarium verticillioides 7600 L-asparaginase (FVEG 03277)
XM 001389847.2 Aspergillus niger CBS 513.88 L-asparaginase
NM 001020452.2 Schizosaccharomyces pombe putative L-asparaginase (SPAC186.03)
XM 013164838.1 Schizosaccharomyces octosporus yFS286 L-asparaginase
MG 988315 Yarrowia lipolytica DSM3286 L-asparaginase
XM 506063.1 Yarrowia lipolytica CLIB122 YALI0F30723p partial
20

Figure 5

```
Query_10001   1   M--------VYVALHLGAGTHSHE   ------NYVRYKRLAKKACELAISEV--------------KAGKSVGEAAVSA   49
Query_10002   1   --MSPSLQSLLALTTLVGSALASP[8]TRAVS-DFECYNASLPNITIYATGGTIAGSAASSDQTTGYKAGALGIKTLIDA   82
Query_10003   1   MpLKPILLSALASLASASPLLYSR[8]TNANGLNFTQMNTTLPNVTIFATGGTIAGSDSSSTATTGYTSGAVGVLSLIDA   85
Query_10004   1   M-WRSIISFLFFSVALCQPFLFQK   RSSNISDFISFNASLPNVTIFAMGGTIAGYASSSTETVDYAAGSVGIATLVDA   76
Query_10005   1   M-WSCIIAFFFFSVSLCHPTLIYK   RESNASDLISFNASLPNVTIFAMGGTIAGSAASNAETADYKAGAVGIQTLVEA   76
Query_10006   1   M--------VYVALHLGAGTHSHE   ------NYVRYKRLAKKACELAISEV--------------KAGKSVGEAAVSA   49

Query_10001   50  CRVLEDSPLVNAGYGSQLDIDGDVACDAGYIDTDFVRPA----CVSGITRSHPISTAKALSQSYkapSLPLERHRPVHIH  125
Query_10002   83  VPQLCNVSNVRGVQIANVDSGDINSTILTTLAHRIQDDL-DSEHMQGVVVTHGTDTLEESSFFL---DLTVKSEKPVVLV  158
Query_10003   86  VPSMLDVANVAGVQVANVGSEDITSDILISMSKKLNRVVcEDPTMAGAVITHGTDTLEETAFFL---DATVNCGKPIVIV  162
Query_10004   77  VPAIKNFSNIRGVQVTNVGSEELTPANVLNLTQLILAEV-AKPDVHGIVVTHGTDSLEETAMFL---DMTVNTTKPIVVV  152
Query_10005   77  VPDIKNVSNVRGIQVTSVGSENLTPANVLNLTQLVINEV-AKDDVHGIVVTHGTDSLEETAMFL---DLTVNTTKPIVVV  152
Query_10006   50  CRVLEDSPLVNAGYGSQLDIDGDVACDAGYIDTDFVRPA----CVSGITRSHPISTAKALSQSYkapSLPLERHRPVHIH  125

Query_10001   126 GQENVESYLGIEGDVDLKspEATRVWEKWKRLEQNAQVPSSVPEDTVGVIVGDKEHMAIATSSG-----GPILKSKGRVG  200
Query_10002   159 GSMRPATALSADGPLNLL--SAVRLASSKSAMGRGAMIVLNDKIGSARYTIKTNANSLDTFKAEDQGYLGSFENVEPIFW  236
Query_10003   163 GAMRPSTAISADGPFNLL--EAVTVAASTSARDRGAMVVMNDRIASAYYVTKTNANTMDTFKAMEMGYLGEMISNTPFFF  240
Query_10004   153 GAMRPSTAISADGPMNLL--NAVVVAASNRSIGRGTMILLNDRIGSAFYTTKTNGNMLDTFKSYEAGFLGMILDQRPHFF  230
Query_10005   153 GAMRPSTAISADGPMNLL--NAVAVAASDRSVGRGTMILLNDRIGSAFYTTKTNGNTLDTFKSYEAGFLGMIVDQRPHFY  230
Query_10006   126 GQENVESYLGIEGDVDLKspEATRVWEKWKRLEQNAQVPSSVPEDTVGVIVGDKEHMAIATSSG-----GPILKSKGRVG  200

Query_10001   201 PVATYGCGFSLQKGSRT--------------IGVCTTGCGEDIITSRLSGRVLEALAEAQEPDTGISALFDKLQGSFQSS  266
Query_10002   237 YPATRPLGHHYFNISTSstkTALPQVDVLYGHQEADPKLFQEAIENGAEGIVLAGLGAGGWPDKAVEEIKKVLNETEVPV  316
Query_10003   241 YPPVKPTGKVAFDITNV---TEIPRVDILFSYEDMHNDTLYNAISSGAQGIVIAGAGAGGVTTSFNEAIEDVINRLEIPV  317
Query_10004   231 YSPATPTGKVHFDVSNT---TELPAVEILYGYQGLNPNLAKAAVDLGAKGLVLAGMGAASWTDPGNEVIDGLISNQSIPV  307
Query_10005   231 YSPATPTAKVQFDIRNT---TVLPQVDILYGYQGLNPTLAKAAVNSGAKGLVLAGMGAASWTDPGNDVIDGLIRNQSIPV  307
Query_10006   201 PVATYGCGFSLQKGSRT--------------IGVCTTGCGEDIITSRLSGRVLEALAEAQEPDTGISALFDKLQGSFQSS  266

Query_10001   267 PVTLGCL[5]EGHVSIYS-LHKAPSMISAVKSGHDKTN--IQWLQKDPNTVVSSIQIYRER---  326
Query_10002   317 VVSRRTA   WGYV------DERPFGIGAGYLNPSKARIQLQLALEKKLSIEEIKDIFEYAS--  369
Query_10003   318 VQSMRTV   NGEVPLSDvSSDTATHIASGYLNPQKSRILLGLLLSQGKNITEIADVFALGTda  378
Query_10004   308 VYSHRTM   DGFSDYYY------NGIPSYFQNPQKARYMLMLSINAGYSIQNITDIFSLEY--  360
Query_10005   308 VYSHRTM   DGFSDYSY------NGIPAYFHNPQKARYMLMLAINAGYSIQNITDMFHLEY--  360
Query_10006   267 PVTLGCL[5]EGHVSIYS-LHKAPSMISAVKSGHDKTN--IQWLQKDPNTVVSSIQIYRER---  326
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Figure 6

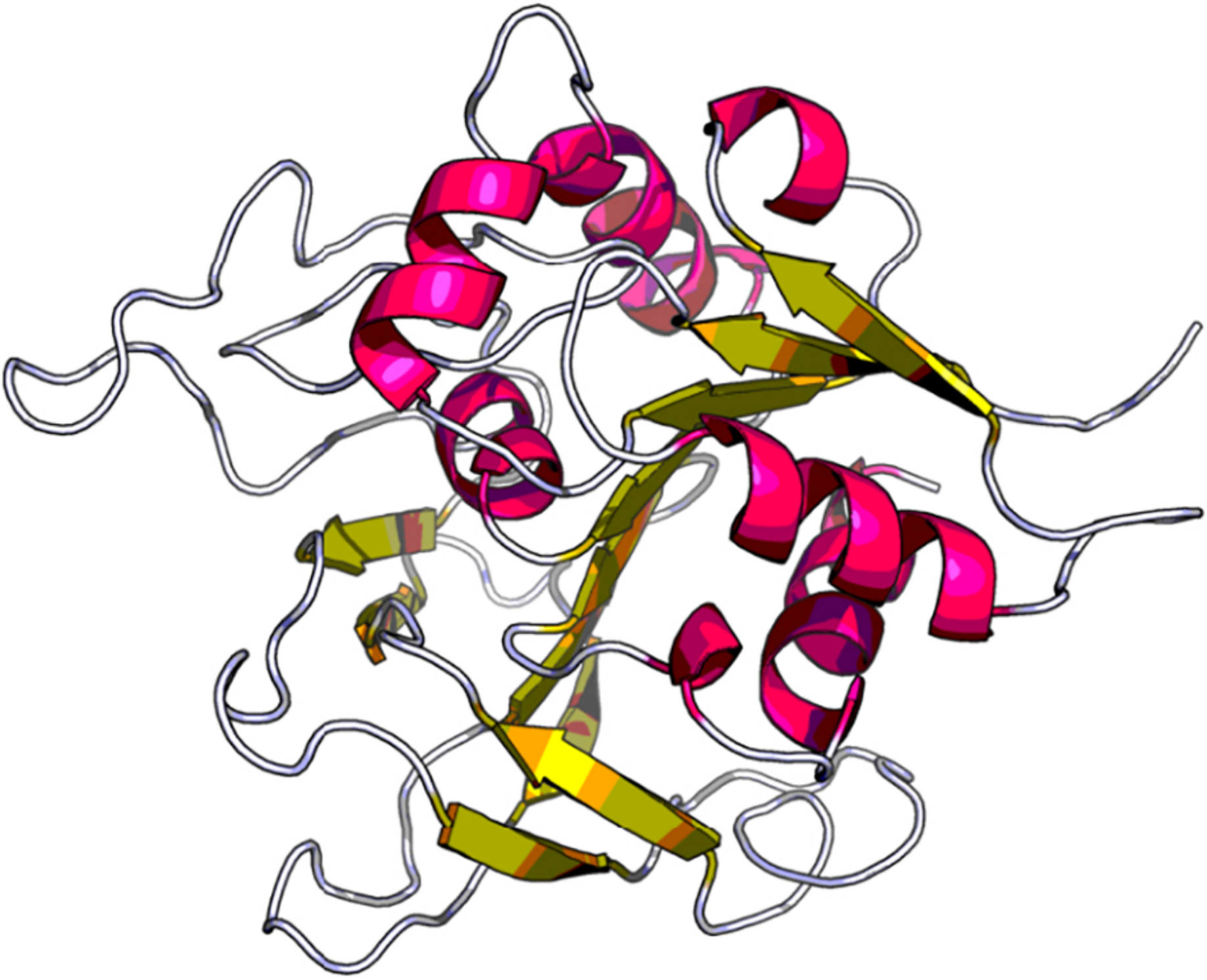

Figure 7