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Recombinant *Candida utilis* for the production of biotin

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Abstract Biotin is an important nutritional supplement but is difficult to manufacture effectively. Here we present a trial of biotin production using the food yeast *Candida utilis*. In this system, we cloned the *C. utilis* biotin synthase (BIO2) gene, the gene of the rate-limiting enzyme for biotin biosynthesis, and assembled it under the control of a strong promoter. A series of plasmids were constructed to direct the integration of the BIO2 gene, either high-copy integration with 18S rDNA fragment or low-copy integration with *URA3* or *HIS3* fragment. The BIO2 gene can be successfully integrated into the *C. utilis* chromosome and can drive biotin production using these plasmids. The biotin yield in this system can reach 100-fold above the endogenous level in a small-scale culture. Although the biotin production is not stable if the selection pressure is removed, this system has the potential to produce biotin-rich feed or food additives directly without the requirement of further purification.

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Introduction

Biotin (hexahydro-2-oxo-1*H*-thieno [3,4-d]imidazo-4-pentanoic acid), also known as vitamin H, is one of the critical nutrients for growth of animals, humans, plants, and microorganisms; as a result, it serves as an important medicinal and food additive. Biotin plays an essential role as a coenzyme in enzyme-catalyzed carboxylation and decarboxylation reactions, fatty acid biosynthesis, gluconeogenesis, and amino acid metabolism. Most animals and many microorganisms are unable to synthesize biotin effectively and therefore must rely on biotin from other sources to survive.

The biotin biosynthetic pathway in bacteria, especially that in *Escherichia coli* and *Bacillus sphaericus* (Gloeckler et al. 1990), has been investigated thoroughly. In *E. coli*, the first step is converting pimelic acid to pimelyl-CoA (Pm-CoA) through the gene products of *bioC* and *bioH*. Then, Pm-CoA is converted into biotin in four further enzymatic steps, from converting Pm-CoA to 7-keto-8-aminopelargonic acid (KAPA) by BioF (KAPA synthetase), to 7,8-diaminopelargonic acid (DAPA) by BioA (DAPA aminotransferase), to dethiobiotin (DTB) by BioD (dethiobiotin synthase), and finally to biotin by BioB (biotin synthase). The reaction catalyzed by BioB is thought to be chemically difficult. It involves the insertion of a sulfur atom between the unactivated methyl and methylene carbon atoms adjacent to the imidazole ring of DTB to form the thiolane ring of the vitamin biotin. The genes *bioABFCD* are encoded in *E. coli* on a bidirectional operon located at 17 min on the *E. coli* chromosome (Bachmann 1983). Transcription of the *bioA.BFCD* operon is divergent and corepressed by biotin and the *birA* gene product, a bifunctional protein that serves as a repressor and converts biotin to biotinyl adenylate, the true corepressor (Barker and Campbell 1981). Highly conserved homologs to the *E. coli* proteins BioF, A, D, B have been found in *B. sphaericus* (Gloeckler et al. 1990), *Bacillus subtilis* (Bower et al. 1996), in yeasts such as *Saccharomyces cerevisiae* (Zhang et al. 1994), and in plants such as *Arabidopsis thaliana* (Weaver et al. 1996).

Currently, most of the biotin commercially available is synthesized by a chemical process of more than ten different steps having several disadvantages, including high energy input and the production of considerable amounts of chemical waste (Fleckenstein et al. 2001). Therefore, one of the major concerns of the biotin industry is to achieve a cost-effective fermentative process to produce biotin. During the past decades, many attempts have been made to develop a fermentation process for preparing biotin using microorganisms. It has been possible, by cloning the biotin operon on multicopy plasmids, to increase biotin synthesis to 1 μg/ml in microorganisms transformed with these genes (Streit and Phillip 1996). The deregulation of *bio* operon expression via selection of *birA* mutants was also investigated to increase the production yield of biotin (Ifuku et al. 1999). Even the cell-free system was employed to further improve the efficiency of the conversion of DTB to biotin (Ohshiro et al. 1994; Ifuku et al. 1992). However, due to the low efficiency of carbon recovery from the nutrients into biotin and the accumulation of the direct intermediate, DTB, these fermentation processes have not yet been industrialized.

Since yeast has long been used for the production of fermentation products such as alcoholic products, we considered using yeast as the host for biotin production. The food yeast *Candida utilis*, which has been used for the production of food additive and animal feeds, may well be the best candidate. In the present study, we have cloned, sequenced, and characterized the biotin synthase (BIO2) gene from the food yeast *C. utilis*. The BIO2 gene was then assembled into the plasmid capable of integrating into the chromosome of the yeast and catalyzing the biotin formation from DTB. Without further processes to isolate and purify biotin from the fermentation broth, the dried yeast powder with high biotin level may thus have the potential to be used directly as a food additive, cosmetic additive, or animal feed.

Materials and methods

Bacterial strains and plasmids

The strains used in the study include *C. utilis* (also known as *Torula utilis*, *Pichia jadinii*) strain Y25 obtained from Taiwan Sugar Research Institute for cloning and assay, *E. coli* strain R901 (Sm^R *Δbio*) for complementation study, CJ236 (*ung-dut-thi-1 relA* pCJ105 [Cam^R]) for site-directed mutagenesis, and JM101 (*supE thi Δ[lac-proAB] F' [traD36 proAB lacIQ lacZDM15]*) for mutagenesis and cloning.

Plasmid pQE30, purchased from Qiagen (USA), was used for cloning, expression, and vector construction. pAS2-1 (GenBank accession number U30497), obtained from Clontech (USA), was used for gene expression and for construction of *E. coli*-yeast shuttle vector. pGEM-7Zf (+) (GenBank accession number X65310) from Promega (USA) was used for subcloning. Plasmid pUC19 and phagemid M13mp18 (Yanisch-Perron et al. 1985; GenBank accession number X02513) from Clontech were used for gene cloning, site-directed mutagenesis, and sequencing. Lambda EMBL3 vector (GenBank accession numbers U02426, 02427) was used for cloning. The packaging system from Stratagene (USA) was used for the construction of the genomic library.

Molecular cloning and sequencing of the BIO2 gene of *C. utilis*

The chromosomal DNA of *C. utilis* was isolated (Phillippsen et al. 1991), partially digested with *Sau*3A, ligated with the EMBL3 vector, and packaged with the in vitro packaging extract to obtain the lambda EMBL3 genomic library (Shiuan and Campbell 1988). The library was then screened with a 315-bp-long DNA probe for the BIO2 gene. The probe was designed by first aligning the biotin synthase of *S. cerevisiae* and *E. coli*, in which two conserved regions, EDCKYC and YNHNID, were identified and used to synthesize the degenerate primers, 5′-TGTNCNGARGAYTGYAANTATTG-3′ and 5′-GTRTCNANRTTRTGGTTGTA-3′, where Y represents T and C, R represents A and G, and N represents any of the four bases. Then, the degenerate primers were used for polymerase chain reaction (PCR) with *C. utilis* chromosomal DNA as the template to obtain the 315-bp fragment. The 315-bp DNA was sequenced and confirmed to be part of the BIO2 gene by sequence comparison before being end-labeled with [γ-^{32}P]ATP and being used to screen the library (delete). From approximately 1,000 lifted plaques, 20 positive clones were isolated and purified and the lambda DNA prepared. The 14-kb DNA insert was further digested with *Bam*HI/*Hin*dIII and subcloned to verify the location of the BIO2 gene. A 5,278-nucleotide (nt) insert covering the 315-bp probe was identified and further sequenced using the dideoxynucleotide chain-termination method (Sanger et al. 1977) and was deposited in the GenBank database under accession number AF212161.

Complementation of *C. utilis* BIO2 gene in *E. coli* biomutant

The entire 5,278-nt DNA sequence was analyzed first by looking for the open reading frame (ORF) through the NCBI ORF finder and making a BLAST comparison with the nonredundant database. Two overlapping ORFs, nt 3,915–5,102 and nt 4,044–5,102, were found to share high similarities with known biotin synthase genes. Both ORF fragments were generated by PCR and subcloned into the *Bam*HI site of the expression plasmid pQE30 to obtain pQE30A and pQE30B, respectively. To determine the correct start position of BIO2, plasmid pQE30A and pQE30B were used to transform the biotin-operon-deficient strain R901 and incubated on a minimal agar plate supplemented with the biotin precursor DTB (50 μg/ml) and tetrazolium chloride indicator (0.004%). Colonies that can express sufficient amount of biotin would complement

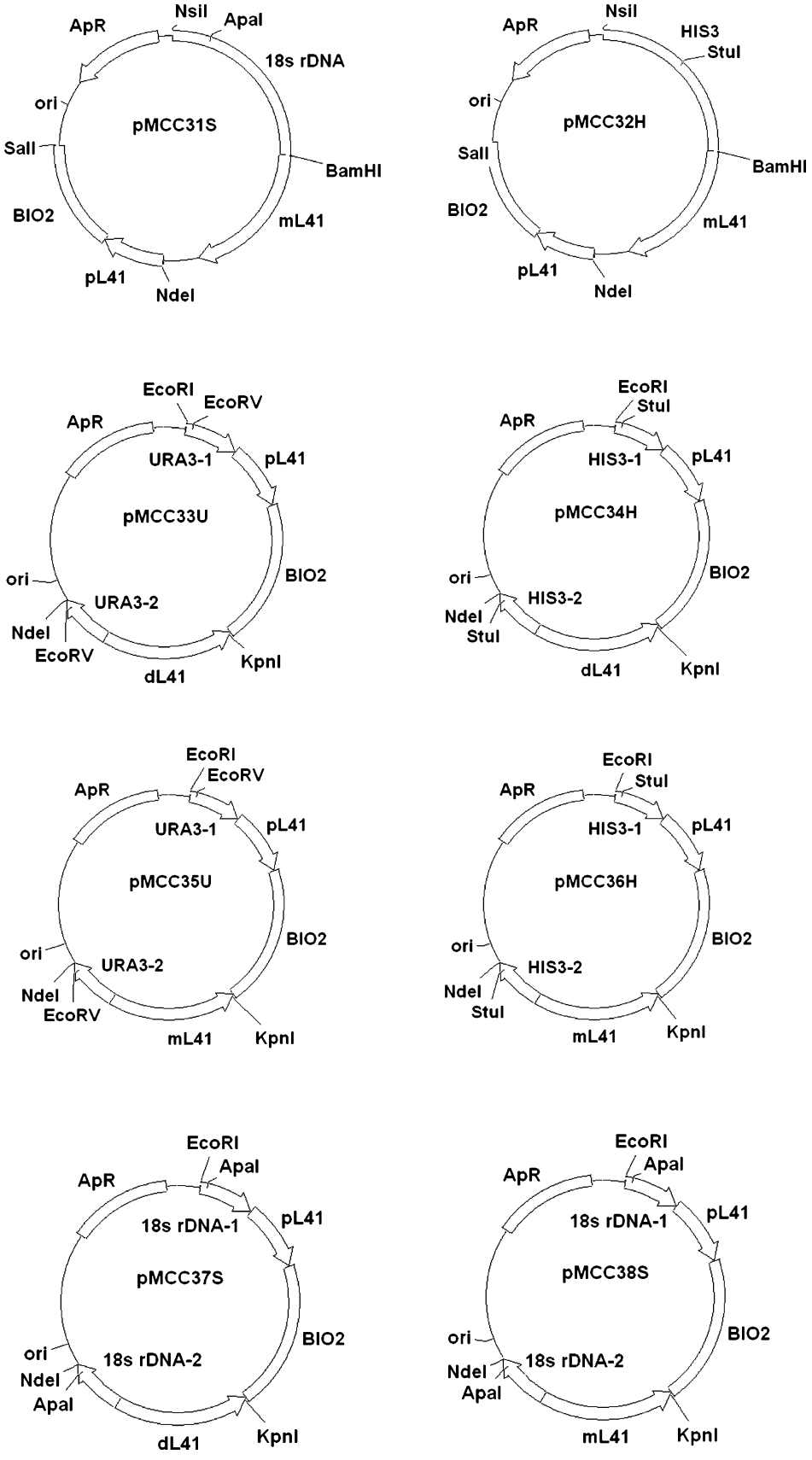

Fig. 1 The integrative plasmids constructed in the present study. Each plasmid carries the integration site, the regulatory sequences, the selection marker, and the BIO2 gene

Table 1 The sequences of PCR primers used in plasmid construction

Gene (accession no.)	Fragment size (kb)	Primer sequence	Plasmid constructed
URA3-1 (E11619)	0.5	For:5'-GGGCGCCAGCTCGATAAAACGGTAT-3' *Kas*I	pWS31
		Rev:5'-GGAATTCGAGATGAGCTTGAAATTTTT-3' *Eco*RI	
URA3-2 (E11619)	0.5	For:5'-AACTGCAGATTGGATTCATCGCACAGAGA-3' *Pst*I	pWS32
		Rev:5'-CCCAAGCTTGACTAGTCTGTTGTGTAGCAT-3' *Hin*dIII *Spe*I	
pGAP (E11621)	0.97	For:5'-GGAATTCGCTTACAGCGAGCACTCA-3' *Eco*RI	pWS33
		Rev:5'-GCTCTAGAGTTGTTTGTAAGTGTGTT-3' *Xba*I	
mL41 (E11620)	1.23	For:5'-ACGCGTCGACGGTGAGCGTACTTGCA-3' *Sal*I	pWS34
		Rev:5'-AAACTGCAGGGATACCGAACCAAGT-3' *Pst*I	
tGAP (E11622)	0.82	For:5'-ACGCGTCGACATTGTATGACTTTTATTTATG-3' *Sal*I	pWS35
		Rev:5'-ACGCGTCGACCGTGTAATACCTCAGTAGGC-3' *Sal*I	
BIO2 (AF212161)	1.19	For:5'-AAACCCGGGATGTCGTTTATATTGACTG-3' *Sma*I	pWS35-BIO2
		Rev:5'-AAACCCGGGTCAAGCACCGAATGCAAC-3' *Sma*I	
L41 (E11620)	1.27	For:5'-CGGATCCTTGGTGGACCGGGTGTAGG-3' *Bam*HI	pMCC series
		Rev:5'-CGGTACCTACAGGATACCGAACCAAGT-3' *Kpn*I	
BIO2 (AF212161)	1.84	For:5'-GCATATGCTTGGTGGACCGGG-3' *Nde*I	pMCC series
		Rev:5'-CGGTACCGGTAGAGGTGTGGTC-3' *Kpn*I	
URA3-1 (E11619)	0.5	For:5'-CGGATCCAAATAGCTCTCTACTTGC-3' *Bam*HI	pMCC33U pMCC35U
		Rev:5'-CGAATTCGATATCAATATGCGTCTTTC-3' *Eco*RI	
URA3-2 (E11619)	0.59	For:5'-GCATATGATATCGGCAACACCGTCAA-3' *Nde*I	pMCC33U pMCC35U
		Rev:5'-GCATATGCAATCTACAACTTCCGAAA-3' *Nde*I	
HIS3-1 (Y12658)	0.54	For:5'-CGAATTCAAGGAGGCCTTGGCATATA-3' *Bam*HI	pMCC34H pMCC36H
		Rev:5'-CGGATCCACCAGTGGTCGTAGAGTC-3' *Eco*RI	
HIS3-2 (Y12658)	0.44	For:5'-GCATATGCGCCACTTCCGGGTGCGTG-3' *Nde*I	pMCC34H pMCC36H
		Rev:5'-GCATATGCCAAGGCCTCCTTGACGGC-3' *Nde*I	

the biotin-operon-deficient *E. coli* strain R901 and turn red on the indicator plates (Shiuan and Campbell 1988).

Site-directed mutagenesis of the L41 gene of *C. utilis*

The selectable marker used in the present integrative plasmids is the cycloheximide (CYH)-resistant *mL41* gene encoding the mutated ribosomal protein L41 of *C. utilis* (Kawai et al. 1992). The L41 gene with its regulatory region was first obtained by PCR and subcloned into plasmid M13mp18 for site-directed mutagenesis as described (Kunkel 1987; Farh et al. 2001). The 56th amino acid of L41 protein was converted from Pro (nt 166–168, CCA) into Gln (nt 166–168, CAC) and the mutated L41 gene *mL41* was obtained. Since the gene was derived from the host, the gene was directly used with its own promoter and terminator sequences for the expression. The L41 gene (GenBank accession number E11620) has one intron (nt 1,115–1,481) between the very short exon 1 (atgg, nt 1,111–1,114) and exon 2 (nt 1482–1798). Therefore, the forward primer to clone the mutated L41 DNA fragment contains the designed restriction site, with the exon 1 sequence connected directly with the 5′ end of the exon 2 sequence.

Construction of the integrating vectors

A number of plasmids have been constructed in the present study to facilitate the stable integration and expression of the BIO2 gene. The integrative plasmids include pMCC31S, pMCC32H, pMCC33U, pMCC35U, pMCC36H, pMCC38S, and pWS35 (Figs. 1 and 3). Each plasmid contains the fragments for integrating into the chromosome, the promoter, and the terminator sequences, the BIO2 gene, the selectable marker *mL41* gene, the *E. coli* replication origin, and the ampicillin-resistant gene. The primer sequences used to clone various DNA fragments for assembling the integrative plasmids are listed in Table 1. The construction flow charts of plasmid pMCC31S and pWS35 are described as examples for the plasmid construction in the present work (Figs. 2 and 3).

Transformation of *C. utilis* by electroporation

The transformation of *C. utilis* was carried out by a modified electroporation method (Becker and Guarente 1991). The wild-type *C. utilis* stock (30 μl) was mixed with 3 ml YPD solution (2% glucose, 1% Bacto yeast extract, 2% Bacto peptone) and incubated at 30°C, 280 rpm overnight. Then, the 2-ml culture was added to 200 ml YPD–CYH solution (40 μg cycloheximide/ml) and incubated again for 16 h, followed by centrifugation at 6,500 rpm at 4°C for 10 min. The pellets were rinsed with 10 ml 1 M cold sorbitol solution and then resuspended in 2 ml sorbitol solution. To transform the yeast, 1 ml *C. utilis* solution was mixed with 5 μl cold salmon sperm DNA solution (50 μg/ml, as DNA carrier) and 50 μl linearized and purified plasmid DNA solution (approximately 2 μg DNA) in a 0.2-cm cuvette and electroporated at 0.75 kV, 800 Ω, and 25 μF (BioRad Gene Pulser II electroporator). After electroporation, the solution was recovered in 20 ml YPD medium (containing 1 M sorbitol) and incubated at 30°C, 280 rpm, for 6–8 h, then centrifuged at 8,000 rpm for 15 min. The pellets were resuspended in 1 ml YPD medium and plated on the YPD–CYH medium and incubated at 30°C, 280 rpm, for 3 days. The successfully transformed *C. utilis* would grow on the plates.

Assaying the biotin level of the transformed recombinant *C. utilis*

The colonies growing on the selection plates were picked and transferred to 3 ml YPD–CYH medium and incubated at 30°C and 280 rpm overnight. Each culture (200 μl) was centrifuged at 6,000 rpm for 5 min, and the supernatants were assayed for biotin concentration by the competitive enzyme-linked immunosorbent assay (ELISA) method (Shiuan et al 1997).

Southern hybridization analysis of the integrated genes

The copy number and the stability of the integrated plasmids can be estimated by Southern hybridization analysis (Southern 1975). The genomic DNA of the transformed *C. utilis* was isolated (Phillippsen et al. 1991), digested (with *Bam*HI and *Xba*I, in the case of *C. utilis* transformed with pMCC31S), and electrophoresed on a 0.8% agarose gel. Then the gel was blotted onto a nylon membrane (Hybond N membrane, Amersham), hybridized with probe, and washed according to the manufacturer's instruction. The 1.3-kb *Bam*HI/*Xba*I fragment was labeled with [α-^{32}P] dATP (3,000 Ci/mmol, New England Biolabs) by nick translation as described (Sambrook et al. 1989). The membranes were subjected to X-ray film autoradiography, and the band intensities were compared after film development and scanning. Similar Southern hybridization analysis was also performed to identify the copy number of the integrated BIO2 gene using the 315-bp DNA probe as mentioned in the section "Molecular cloning and sequencing of the BIO2 gene of C. utilis."

Results

Cloning, sequencing, and characterization of the BIO2 gene encoding biotin synthase of *C. utilis*

Our strategy to construct a recombinant strain of the food yeast *C. utilis* capable of producing a high level of biotin includes cloning the BIO2 gene (codes for biotin synthase, the enzyme catalyzing the rate-limiting step of the biotin biosynthetic pathway) and integrating the gene with

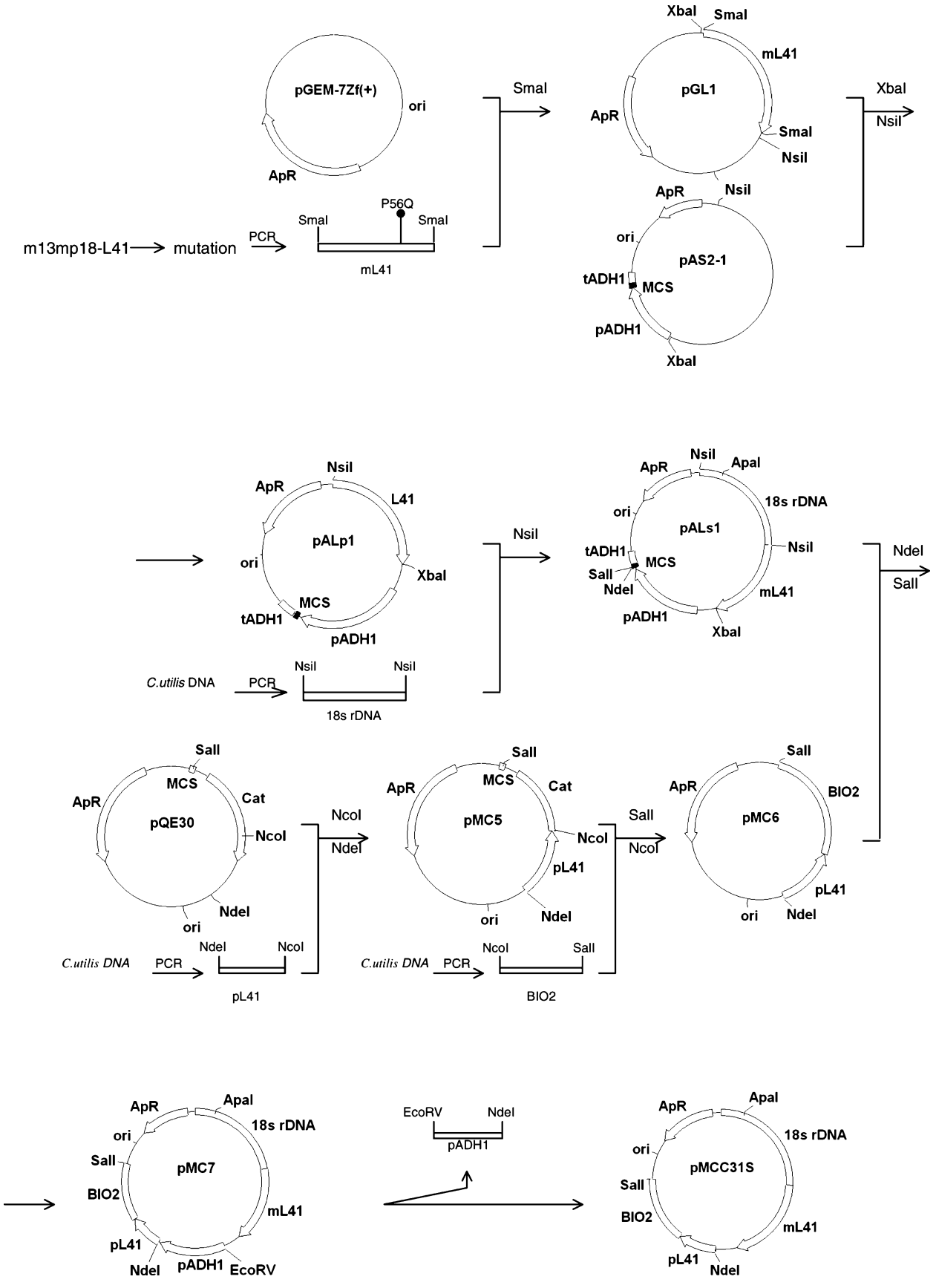

Fig. 2 Construction of the plasmid pMCC31S

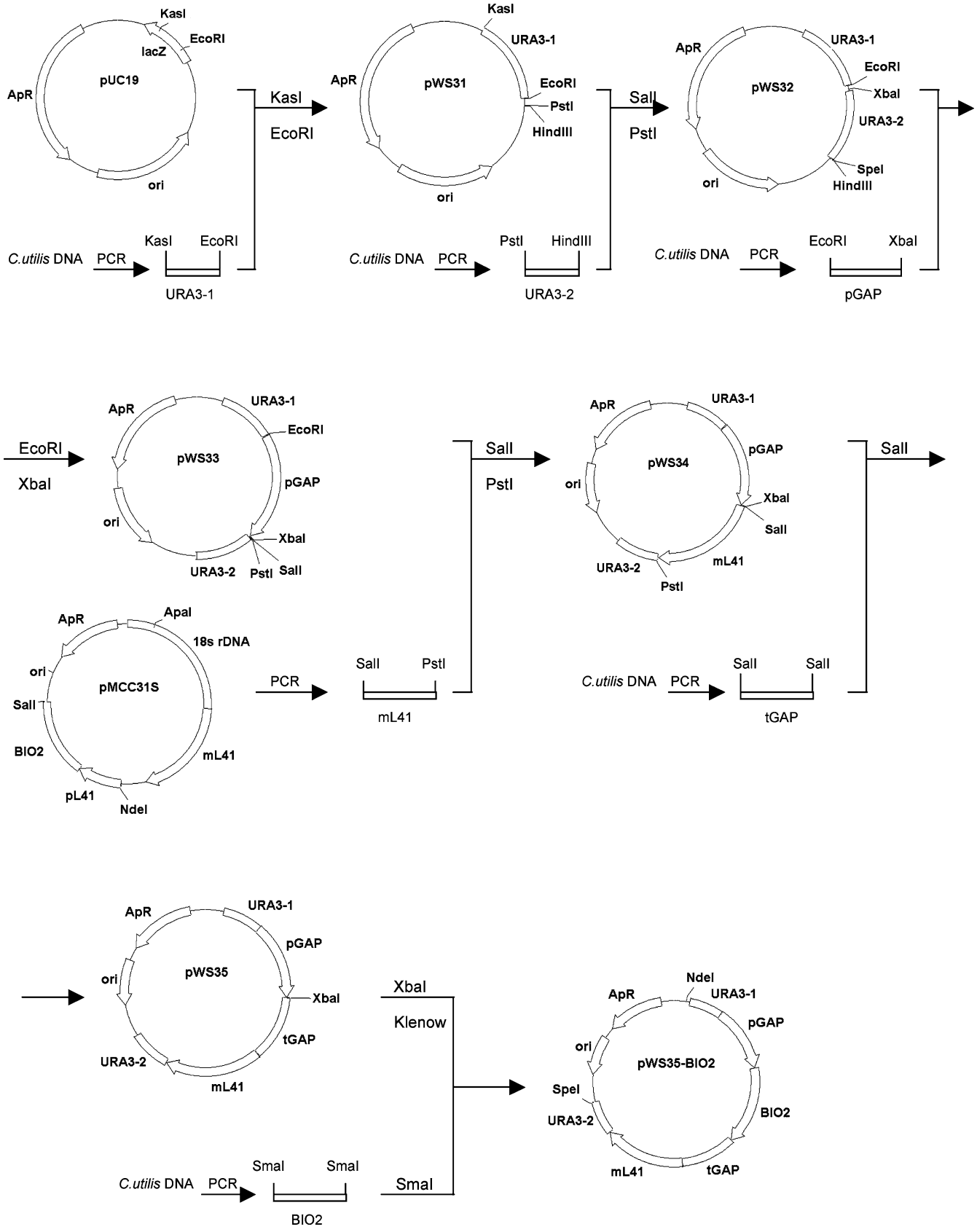

Fig. 3 Construction of the plasmid pWS35-BIO2

a strong promoter into the chromosome of *C. utilis*. As described in "Materials and methods," the lambda EMBL3 library of *C. utilis* was first constructed and screened with a 315-bp DNA probe containing the highly conserved region among the biotin synthase genes. From the 14-kb inserted DNA of the selected clones, the BIO2 gene was identified in the approximately 5-kb *Bam*HI/*Hin*dIII fragment through Southern analysis and subjected to further DNA sequencing. The entire 5,278-nt DNA sequence was submitted to GenBank (accession number AF212161) and analyzed through sequence alignment with the known biotin synthase sequences. Two ORFs, nt 3,915–5,102, and nt 4,044–5,102, were found to share high similarities and may represent the BIO2 gene. BLAST comparison (NCBI blastp) showed that the deduced amino acid sequence (395 aa) of the long-form BIO2 shares 71, 58, 55, and 48% identity with the biotin synthase of *Saccharomyces cerevisiae* (SwissPro P32451, 375 aa), *Schizosaccharomyces pombe* (SwissPro O59778, 363 aa), *A. thaliana* (PIR P54967, 378 aa), and *E. coli* K-12 (InterPro P12996, 346 aa), respectively.

The two ORFs were further subcloned into the *Bam*HI site of the expression plasmid pQE30 to obtain pQE30A and pQE30B, respectively. Plasmids pQE30A and pQE30B were then used to transform the biotin-operon-deficient *E. coli* strain R901. Both of the transformants were found to express sufficient amount of biotin, as they were able to complement the strain R901 in the minimal medium supplemented with DTB (40 μg/ml, data not shown). Although both the long form (encodes 395 aa) and the short form (encodes 352 aa) exhibited the enzyme activity of biotin synthase, the long form was used further as the BIO2 gene in the present work.

Construction of integrating plasmids

Since *C. utilis* is polyploid and does not have a sexual life cycle, the auxotrophic mutants that usually served as the hosts for transformation systems are very hard to obtain. However, a novel strategy using the mutated ribosomal protein L41 gene to confer CYH resistance as a selectable marker and a fragment of chromosomal DNA for plasmid integration constituted an efficient transformation system for *C. utilis* (Kondo et al. 1995). Based on these criteria, integrative plasmids pMCC31S, pMCC32H, pMCC33U, pMCC35U, pMCC36H, pMCC38S, and PWS35 were constructed in the present study to facilitate the stable integration and expression of BIO2 gene. As shown in Fig. 1, different gene fragments, including 18S rDNA, *URA3*, and *HIS3* DNA fragment, were constructed in linear or gapped form to direct the integration of the respective plasmid into the chromosome. Strong promoters like pL41 or pGAP were chosen to drive the expression of BIO2 gene, and the mutant L41 (*mL41*) gene was used as the selection marker. The primer sequences used to clone various DNA fragments to assemble the integrating plasmids are listed in Table 1.

The construction flow charts of plasmids pMCC31S, pWS35, and pWS35-BIO2 are described as examples for the plasmid construction work. As shown in Fig. 2, the construction of plasmid pMCC31S was started by inserting the PCR-produced *mL41* gene into the *Sma*I site of pGEM-7Zf(+) to obtain pGL1. The *Xba*I/*Nsi*I fragment containing the *mL41* gene was then obtained from pGL1 and inserted into pAS2-1 to form pAL1. Next, the 1.7-kb-long *C. utilis* 18S rDNA with *Nsi*I ends was produced by PCR and inserted into the *Nsi*I site of pAL1, obtaining plasmid pALs1. In another series (Fig. 2), the 0.7-kb DNA fragment containing the *L41* promoter region was produced by PCR and cloned into the *Sal*I–*Nco*I site of plasmid pQE30, obtaining pMC5. Next, the BIO2 gene of *C. utilis* was obtained by PCR and subcloned into the *Nco*I–*Sal*I site of pMC5, obtaining pMC6. Then the *Nde*I–*Sal*I fragment of pMC6 containing *L41* promoter and the BIO2 gene was subcloned into the *Nde*I–*Sal*I site of pALS1, obtaining pMC7. Finally, the *Eco*RV–*Nde*I fragment containing the *ADH1* promoter region was deleted from pMC7, obtaining pMCC31S.

The integrative plasmid pWS35 was designed to use the stronger pGAP promoter (to transcribe the BIO2 gene) and the *URA3-1*/*URA3-2* as the potentially more stable integrating fragments (Kondo et al. 1998). As shown in Fig. 3, the construction process was started by inserting the *URA3-1* fragment into the *Kas*I–*Eco*RI site of pUC19, obtaining pWS31. Then, the *URA3-2* and pGAP fragments were inserted, obtaining pWS32 and pWS33, respectively. The *mL41* fragment was produced by PCR from pMCC31S and inserted into the *Sal*I–*Pst*I site of pWS33, obtaining pWS34. The terminator tGAP fragment was then assembled into the *Sal*I site of pWS34, obtaining pWS35. Finally, the BIO2 gene was inserted into the *Xba*I site (treated with Klenow fragment) to obtain the plasmid pWS35-BIO2.

Biotin-producing capability of various recombinant yeasts

The BIO2 gene of *C. utilis* was assembled into each of the integrative plasmids and transformed into yeast *C. utilis* in order to increase its biotin-producing capability. The plasmids were linearized first with proper restriction enzymes before electroporation. Successful transformants that appeared on the YPD–CYH plates after 2–3 days revealed that the plasmids had been integrated onto the yeast chromosome through homologous recombination, and the mutated L41 gene on the plasmids was expressed to confer resistance to CYH. The copy number of the integrated plasmids, however, may be varied in different transformants, and this resulted in different expression levels of the cloned genes. Several colonies were picked from each plate (or different transformation process) to assess the biotin-producing capability. The biotin levels reached by each plasmid under the same electroporating and assaying conditions are listed in Table 2. As expected, the biotin

Table 2 Assay of the biotin production capability of the recombinant *C. utilis* transformed with various integrating plasmids

C. utilis transformed with plasmid	Biotin level (μg/ml)				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
pMCC31S	0.87	1.23	1.89	1.20	0.94
pMCC32H	0.23	0.24	0.17	0.22	
pMCC33U	0.13	0.15	0.16		
pMCC35U	0.62	1.07	0.70		
pMCC36H	0.63	0.48	0.37		
pMCC38S	0.73	0.77	0.83	0.82	0.70
Background					
C. utilis	0.03	0.02	0.05	0.06	0.04
YPD–CYH	0.06	0.03	0.05	0.03	0.06

The yeast was transformed and grown in YPD–CYH medium for 72 h and assayed for biotin level using the competitive ELISA method. The detection limit of the method is 5 pg biotin/ml, and the accuracy is approximately 5% of each measurement as described (Shiuan et al. 1997). The varied biotin levels may be attributed to different integration copies of the BIO2 gene even under the same transformation and growth conditions

levels were varied from different colonies transformed with the same linearized plasmid. For example, the recombinant *C. utilis* transformed with plasmid pMCC31S produced biotin varying from 0.867 to 1.885 μg/ml. The level may be elevated in principle by adjusting the electroporating conditions, including more linearized DNA and higher selection pressure. On the other hand, the stability of the integrated plasmid was also taken into consideration. It was observed that the biotin-producing levels dropped severely after 3 days in the assaying medium without the addition of CYH (data not shown). As we examined the stability of the plasmid pMCC31S through Southern analysis, the copy number of the integrated BIO2 gene dropped from 5–10 copies per genome under the selection pressure to less than 2 copies as CYH was removed from the assaying medium. However, the stability of the integrated gene using different integration sites was not examined in the present study. No direct correlation between the biotin level and the colony size, or the time required for the colonies to appear on the selection plates and the colony size, was observed in the study.

Discussion

Currently, the biotin chemical industry is driven by a sizable market of several hundred million US dollars per year. It is estimated that about 30 tons of biotin are produced annually (DeBaets et al. 2000). To overcome the many disadvantages faced by the biotin industry (Fleckenstein et al. 2001; Streit and Entcheva 2003), efficient fermentation processes to produce biotin are in great demand. The consensus is that any fermentation process has to be able to produce more than 1 g biotin per liter and with a cheap substrate in order to be cost-effective (Streit and Entcheva 2003). Through overexpression of extra copies of biotin-biosynthesis genes and selection for mutants resistant to analogues or other chemicals, various high-biotin-producing strains had been generated during the past decades. In *E. coli*, biotin synthesis has reached 10 mg/l by transforming a multicopy plasmid harboring biotin operon and deregulating at least one enzyme of the fatty acid biosynthetic pathway in the host (Furuichi et al. 2000). In *Serratia marcescens*, overexpression of various *bio* genes has led the recombinant strain to produce biotin at the 500-mg/l level (Sakurai et al. 1994). A strain of *E. coli* resistant to threonine analogue can produce biotin even up to 970 mg/l in the fermentation broth (Kanzaki et al. 2001). The best results (more than 1 g biotin per liter of culture medium) were achieved in *B. subtilis* with strains resistant to 5-(2-thienyl) pentanoic acid and overexpression of various *bio* genes (Bower et al. 2001). Although the biotin yields had almost achieved the profitable margin, none of the patented technologies has been cost-effective enough to reach the production line. The bottlenecks in the above-mentioned microbial biotin production processes could be that the fermentation processes still relied on complex media including expensive biotin precursors, the plasmid instability of the recombinant strains, the possible toxic side effects of some metabolites, and finally, the costly purification process.

In the present study, we decided to bypass the procedures that rely on overproducing biotin in microorganisms and purifying biotin as the product. Instead, we chose yeast as both the biotin-producing strain and as the end product. The yeast of the genus *Saccharomyces* or the yeast *C. utilis* have been established by the Food and Drug Administration (FDA) to be of high biological safety when used for the production of useful substances with recombinant DNA technology (Kessler et al. 1992). In addition to the biosafety concerns, yeast can generally be cultured at a cell density higher than that of bacteria. Unlike the yeast of the genus *Saccharomyces*, *C. utilis* does not produce ethanol under aerobic conditions; therefore, its cell growth is not self-inhibited. Consequently, it is possible to produce metabolites efficiently through the continuous culture of *C. utilis* under a high cell density, even at much cheaper substrates such as molasses. In the present study, we have cloned, sequenced, and characterized the biotin synthase (BIO2) gene from the food yeast *C. utilis*. By integrating

the biotin synthase gene into the chromosome of *C. utilis*, the biotin level reached above 1.8 mg/l of the growth medium (Table 2). This level (1.8 mg/l) was obtained by using the integrating plasmid pMCC31S and culturing in a 30-ml flask for 72 h. The level is about 200-fold of that of the wild type *C. utilis*, and it is generally expected that through well-controlled fermentation processes the yield could be improved ten times more. Although the biotin level of the present work is not comparable with any of the best patented technologies, the YPD medium we used is much cheaper than the complex medium containing expensive biotin precursors. Furthermore, the dried yeast powder with such high biotin level may have the potential to be used directly as food additive, cosmetic additive, or animal feed without further expensive processes to isolate and purify biotin from the fermentation broth.

The present setup may be improved by many ways. First, since biotin synthase is a radical *S*-adenosylmethionine (SAM)-dependent enzyme (Guianvarch et al. 1997; Layer et al. 2004), SAM may be included in the medium as a source of deoxyadenosyl radical to restore the repeated catalytic turnovers of biotin synthase. Second, as the cysteine desulfurase was suggested to supply the sulfur to regenerate the iron–sulfur cluster of biotin synthase (Flint 1996; Kiyasu et al. 2000), the *nifS* and *nifU* genes of *Azotobacter vinelandii*, or their homologs in *E. coli*, the *IscS* and *IscU* genes, can also be assembled in the integrating plasmid to enhance the production of biotin (Zheng et al. 1998; Jameson et al. 2004). In addition, the instability of high-copy integration might be resolved if reduction of the intrinsic recombination activity is possible. Alternatively, the whole work may be switched to another transformation system that uses auxotrophic mutants obtained from the yeast NRRL Y-1084 of *C. utilis* and transformed with plasmids containing the genes *URA3* and *HIS3* of *C. utilis* as the selection markers. The NRRL Y-1084 strains are defective mainly in the biosynthetic pathways of uracil and histidine (Elena et al. 1998).

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