Recombinant *E. coli* expressing *Vitreoscilla* haemoglobin prefers aerobic metabolism under microaerobic conditions: A proteome-level study

BINI RAMACHANDRAN[1], KANAK LATA DIKSHIT[2] and KUPPAMUTHU DHARMALINGAM[3,*]

[1]*Department of Genetic Engineering, School of Biotechnology, Madurai Kamaraj University, Madurai 625 021*

[2]*Institute of Microbial Technology, Sector 39A, Chandigarh 160036*

[3]*National Facility for Clinical Proteomics, School of Biotechnology, Madurai Kamaraj University, Madurai 625 021*

**Corresponding author (Fax, +0091-452-2459280; Email, dharmalingam.k@gmail.com)*

Vitreoscilla haemoglobin (VHb) expression in heterologous host was shown to enhance growth and oxygen utilization capabilities under oxygen-limited conditions. The exact mechanism by which VHb enhances the oxygen utilization under oxygen-limiting conditions is still unknown. In order to understand the role of VHb in promoting oxygen utilization, changes in the total protein profile of *E. coli* expressing the *vgb* gene under its native promoter was analysed. Two-dimensional difference gel electrophoresis (2D DIGE) was employed to quantify the differentially expressed proteins under oxygen-limiting conditions. Overexpression of proteins involved in aerobic metabolic pathways and suppression of proteins involved in non-oxidative metabolic pathways shown in this study indicates that the cells expressing VHb prefer aerobic metabolic pathways even under oxygen limitation. Under these conditions, the expression levels of proteins involved in central metabolic pathways, cellular adaptation and cell division were also found to be altered. These results imply that *Vitreoscilla* haemoglobin expression alters aerobic metabolism specifically, in addition to altering proteins involved in other pathways, the significance of which is not clear as of now.

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1. Introduction

Haemoglobin is an oxygen-binding protein that enhances the oxygen utilization capability of organisms expressing this protein (Bavis *et al.* 2007). Haemoglobins or haemoglobin-like proteins are found in vertebrates, invertebrates, plants, fungi and bacteria (Hardison 1998). Prokaryotic haemoglobin-like protein was reported first in *Vitreoscilla*,

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Abbreviations used: 2D DIGE, two-dimensional difference gel electrophoresis; 2DE, two dimensional electrophoresis; ACTH, adenocorticotrophic hormone; AMP, ammonium phosphate; BP, band pass; BVA, biological variation analysis; CHAPS, 3-[(3-cholamidopropyl) dimethylamoniol]-1-propanesulphonate; CHCA, α-cyano 4-hydroxycinnamic acid; DIA, differential in gel analysis; DMF, dimethyl formamide; DTT, dithiothrietol; FDR, false detection rate; FNR, fumerate/nitrate reduction transcriptional regulator; IAA, iodoacetamide; IEF, isoelectric focusing; IPG, immobilized pH gradient; KEGG, Kyoto Encyclopedia of Genes and Genomes; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PMSF, phenyl methyl sulphonyl fluoride; TCA, trichloro-acetic acid; TFA, trifluoro acetic acid; VHb, *Vitreoscilla* haemoglobin

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a Gram-negative obligate aerobic bacterium, found in oxygen-poor environments (Wakabayashi *et al.* 1986). Expression of this homodimeric protein (subunit molecular weight 15.8 kDa) is under the control of an oxygen-regulated promoter, which is activated by oxygen-sensing global transcriptional activator, Fnr. (Tsai *et al.* 1995). Haemoglobin found in unicellular organisms show a great deal of chemical reactivity towards oxygen (Geckil *et al.* 2003) and are predicted to perform a wider variety of functions traditionally associated with animal and plant haemoglobins, such as scavenging oxygen, serving as terminal electron acceptor during respiration, performing a possible role in anaerobic metabolism and gas metabolism during denitrification (Erenler *et al.* 2004; Hardison 1998).

Expression of *Vitreoscilla* haemoglobin in *E. coli* cultures grown under oxygen limitation has resulted in higher specific growth and considerably higher cell densities (Khosla and Bailey 1988; Tsai *et al.* 1996). The presence of VHb improved cellular energetics under oxygen-limiting conditions, but not under oxygen surplus (Chen and Bailey 1994). Indirect evidence suggests that the net effect of VHb expression in *E. coli* is to improve the efficiency, rather than the kinetics, of aerobic metabolism under oxygen limitation (Chen and Bailey 1994). It was proposed that the expression of VHb under microaerobic conditions in *E. coli* increases the efficiency of ATP production (Khosla *et al.* 1990), and NMR studies have demonstrated that the net accumulation rate of ATP is enhanced in VHb-expressing *E. coli* under microaerobic conditions (Chen and Bailey 1994). It was suggested that the presence of VHb directs microaerobic *E. coli* to utilize its respiratory pathways instead of the fermentative pathways (Tsai *et al.* 1996). A close interaction of VHb with the terminal oxidases had been demonstrated (Park *et al.* 2002) and it has been proposed that this interplay might enhance the respiratory activity and energy production. Recent studies have demonstrated that VHb expression eliminates oxidative stress in recombinant *E. coli* in an OxyR-dependent manner (Frey and Kallio 2003; Anand *et al.* 2010). Several studies have demonstrated an improvement in the overall growth of the organism expressing haemoglobin under oxygen stress (Khosla and Bailey 1988; Chen and Bailey 1994; Tsai *et al.* 1996; Akbas *et al.* 2011). However, the exact molecular mechanism behind the improvement in oxygen utilization under oxygen limited conditions on VHb expression is still vague and has to be explored further using modern technologies.

The effect of *Vitreoscilla* haemoglobin expression in *E. coli* had been analysed using genomic and proteomics approaches (Roos *et al.* 2004; Isarankura-Na-Ayudhya *et al.* 2008). Gene expression profiling of *E. coli* expressing *Vitreoscilla* haemoglobin identified the differential expression of a number of genes involved in central intermediary metabolism, cell processes, nucleic acid biosynthesis and translation machinery in response to the expression of haemoglobin (Roos *et al.* 2004). Proteomic analysis of *E. coli* expressing VHb as a fusion protein demonstrated that proteins such as tryptophanase, aldehyde dehydrogenase and isocitrate dehydrogenase were differentially expressed in the recombinant host (Isarankura-Na-Ayudhya *et al.* 2008). Both these studies examined the effect of VHb expression under aerobic conditions.

In this study, we have examined the proteins expressed by a recombinant *E. coli* expressing VHb from its native promoter under oxygen-limited conditions. The total proteome was analysed using high-resolution two-dimensional gel electrophoresis (2DE) and the proteins of interest were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF). Quantification of differential expression was carried out using two-dimensional difference gel electrophoresis (2D DIGE) analysis of biological replicates. This is the first report on the proteome-wide analysis of recombinant *E.* coli exposed to oxygen stress.

2. Materials and methods

2.1 *Strains and culture conditions*

E. coli strain DH5α (*supE*44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1*endA*1 *gyrA*96 *thi*-1 *relA*1) was used as the host for the study. Full-length *Vitreoscilla* haemoglobin gene, *vgb*, cloned in pSET152 integration vector (Bierman *et al.* 1992) under its native promoter was used for expression studies. Amp^r was used as selection marker for recombinant plasmid and the plasmid was referred as pKD152. DH5α/pSET152 was grown in Luria broth (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L pH 7.2) supplemented with 50 μg/mL apramycin and DH5α/pKD152 was grown in Luria broth supplemented with 50 μg/mL apramycin and 50 μg/mL ampicillin. Both aerobic and microaerobic cultures were inoculated with mid log phase inoculum (5%) and incubated at 37°C throughout the study. DH5α/pKD152 and plasmid control - DH5α/pSET152 were cultured under aerobic conditions in 50 mL medium taken in a 250 mL culture flask and incubated in a shaker at 180 rpm. These bacteria were grown under microaerobic conditions in 200 mL medium taken in a 250 mL culture flask incubated in a shaker at half the rotations per minute used for aerobic culture (Narro *et al.* 1990). Optical density of the cultures at 600 nm was checked at every 1 h interval until the onset of stationary phase. The number of colony forming units was calculated by plating, serially diluted 100 μL culture on Luria agar plates (Luria broth containing 18 g/L agar).

2.2 *Preparation of total cell lysate*

Late log phase cultures were used for the proteomic studies. For 2D PAGE analysis, the protein samples were prepared as reported earlier (Miethke *et al.* 2006) with certain modifications. In brief, four OD cells were washed twice in sonication buffer (10 mM tris, 1 mM EDTA, 1.4 mM PMSF) to remove the culture medium, resuspended in 400 µL of sonication buffer and disrupted by sonication on ice. The lysate was clarified by centrifugation (12,000 rpm, 4°C for 20 min) and the supernatant was used for further experiments. The supernatant was mixed with 10% (w/v) TCA in chilled acetone containing 20 mM DTT and the proteins were precipitated by centrifugation (12,000 rpm, 20 min, 4°C). The pellet was washed twice with chilled acetone to remove the residual TCA and resuspended in 2 DE sample buffer (6 M urea, 3 M thiourea, 8% (w/v) CHAPS) and stored at −70°C as aliquots. Protein content was estimated by Bradford method using bovine serum albumin as the standard (Bradford 1976).

Total cell lysate for one dimensional analysis was prepared as reported earlier (Vandahl *et al.* 2005). In brief, the cell pellet was sonicated in lysis buffer I (0.3% (w/v) SDS, 65 mM DTT) and the lysate was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was saved and the pellet was boiled for 5 min in lysis buffer II (0.3% (w/v) SDS, 40 mM tris HCl pH 8.0). After centrifugation at 12,000 rpm, 15 min, 4°C, the supernatant was pooled with the first supernatant and used for one dimensional analysis.

2.3 *Two-dimensional electrophoresis of total cell lysate*

The samples were resolved by two-dimensional electrophoresis as reported earlier (Gupta *et al.* 2007). Analytical electrophoresis was carried out using 30 µg of *E. coli* proteins whereas, 200 µg of proteins were used for the preparatory gels. All the experiments were carried out in triplicates to minimize experimental variations.

IPG strips of pH range 4–7 (18 cm) (GE Healthcare, Sweden) were rehydrated for 16 h with 340 µL rehydration mix containing protein in UTC rehydration Buffer (7 M urea, 2 M thiourea 4% (w/v) CHAPS, 50 mM DTT, 1% (v/v) IPG buffer (pH 4-7), 0.01% (w/v) bromophenol blue). Isoelectric focusing was carried out as mentioned here: 500 V step-n-hold for 1 h, 1000 V gradient for 1 h, 8000 V gradient for 3 h and 8000 V step-n-hold for 8 h. Reduction and alkylation of focused proteins were carried out as described previously (Gupta *et al.* 2007). Second dimensional separation was carried out on a 12.5% polyacrylamide gel using the preset program of maintaining 2 W/gel for 45 min followed by 10 W/gel till the completion of electrophoresis. Five micrograms of protein low-molecular-weight standard marker (GE Healthcare, Sweden) was separated on every gel in the second dimension for molecular weight calibration.

Analytical gels were stained with 0.1% (w/v) silver nitrate using modified Blum method (Mortz *et al.* 2001). In brief, the gels were fixed in 40% (v/v) ethanol/ 10% (v/v) acetic acid for 1 h, rinsed with 30% (v/v) ethanol for 20 min and washed thrice with water at room temperature. The gels were sensitized with 0.02% (w/v) sodium thiosulfate and stained with pre-chilled 0.1% (w/v) silver nitrate for 30 min at 4°C. The gels were then developed using 2% (w/v) sodium carbonate containing 0.04% (v/v) formalin and the staining reaction was terminated using 5% (v/v) acetic acid.

The preparatory gels were stained with colloidal coomassie blue stain (Candiano *et al.* 2004) as reported previously (Gupta *et al*. 2007). The gels were fixed for 2 h in 40% (v/v) methanol/ 10% (v/v) acetic acid, washed thrice with water to remove the residual alcohol, stained with colloidal coomassie blue staining solution (0.12% (w/v) Coomassie G250 in 10% (v/v) orthophosphoric acid, 10% (w/v) ammonium sulfate, 20% (v/v) methanol) overnight. The gels were destained with water.

The gels were scanned and the digitized images were taken for image analysis. The spots were detected and matched using Image Master Platinum 6 (GE Healthcare, Sweden) software as per the manufacturer's instructions. Triplicate gels (experimental replicates) of control and experimental group were imported into workspace as '.mel' files. The saliency value was set above 5 in all the cases for spot detection. The spots were edited manually wherever it was necessary. The relative intensity (the intensity of the spot taken against the total intensity of all the spots in the gel) of the spots were used for comparison. The values obtained were further confirmed by visual inspection.

2.4 *Peptide mass fingerprinting using MALDI-TOF mass spectrometry*

The spots, showing comparable differences across aerobic and microaerobic conditions, were cut out manually from a preparatory gel using a 1 mm diameter metal punch. Spot processing was carried out as described earlier (Gupta *et al*. 2007). In brief, the spots were washed with water and destained using 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile, dehydrated in 100% (v/v) acetonitrile and dried completely under vacuum for 30 min. The dried gel pieces were rehydrated with 400 ng of trypsin (Promega, USA) for 30 min and tryptic digestion was carried out at 37°C for 16 h. The peptides were extracted from the gel pieces in three steps as described previously (Gupta *et al*. 2007). The extracted peptides were dried in vacuum completely for 1–2 h and were reconstituted in 5 µL of 0.1% (v/v)

TFA in 60% (v/v) acetonitrile. The samples were desalted using C-18 ZipTips (Eppendorf, USA), and the peptides were eluted in 5 µl of 0.1% (v/v) TFA in 50% (v/v) acetonitrile.

CHCA matrix (10 mg/mL in 50% (v/v) acetonitrile and 0.1% (v/v) TFA) or CHCA-AMP mixed matrix (CHCA 2 mg/mL, 50 mM AMP, 49% (v/v) acetonitrile, 0.021% (v/v) TFA) were used for MALDI analysis. In brief, 0.5 µL of the analyte was allowed to crystallize between two matrix layers of 0.5 µL each employing sandwich method for CHCA matrix (Beavis *et al.* 1992). Also, 1 µL of digested peptides, diluted 10-fold in matrix was spotted onto the target plate by dried droplet method for CHCA-AMP mixed matrix (Zhu and Papayannopoulos 2003; Smirnov *et al.* 2004). The peptide mass fingerprint was recorded using Kratos PC Axima CFR Plus MALDI-TOF mass spectrometer (Schimadzu, Japan) in reflectron mode. Calibration of the instrument was done using a mixture of known standard peptides (bradykinin fragment (1-7) 757.39 Da; angiotensin II 1046.54 Da; $P_{14}R$ 1533.85 Da; ACTH fragment (18-39) 2465.19 Da) spotted using the same matrix preparation. A minimum of 200 profiles were collected for each sample using a laser power of 60–65 and 68–74 for CHCA and CHCA-AMP spots respectively. The monoisotopic peaks were generated without using smoothing function and the peak filter was applied to exclude masses lower than 750 Da and higher than 3500 Da. Contaminating peaks from keratin, autolysed trypsin and other known contaminants were removed manually.

Peptide mass fingerprinting was done using MASCOT (*http://www.matrixscience.com/*) and/or MS-Fit (*http://prospector.ucsf.edu/*) database search algorithm. The search was carried out against Swissprot or NCBInr database for entries under the taxonomy *Escherichia coli*. A variation of 0.15–0.5 Da in the *m/z* values was tolerated. Carbamidomethyl modification of cysteine was assigned as a fixed modification whereas oxidation of methionine and propionamide modification of cysteine was assigned as variable modifications. A missed cleavage of 0–1 was tolerated. A protein was regarded as identified when it had a significant MASCOT probability score or, for certain cases, when the theoretical and experimental M_r and pI were matching for a hit with a low MOWSE score.

2.5 *Two-dimensional difference gel electrophoresis and quantification of differential expression of proteins under microaerobic conditions*

Two-dimensional difference gel electrophoresis was carried out using three independent biological replicates of haemoglobin-expressing DH5α/pKD152 and plasmid control DH5α/pSET152 to quantify the differential expression of proteins under microaerobic conditions. Microaerobic culture was established as mentioned in section 2.1. 2D DIGE was carried out as reported earlier with minor modifications (Unlu *et al.* 1997; Tonge *et al.* 2001; Alban *et al.* 2003; Viswanathan *et al.* 2006). In brief, the bacterial cells were harvested from the microaerobic shake flask cultures by centrifugation at 12,000 rpm for 10 min at 4°C. The cells were washed twice with cell wash buffer (10 mM tris pH 8.0, 0.5 mM magnesium acetate) at 12,000 rpm, 10 min, 4°C, and resuspended in cell lysis buffer (30 mM tris, 7 M urea, 2 M thiourea, 4% CHAPS, pH 8.5). The cells were lysed by sonication on ice and the cell lysate was separated from debris by centrifugation (12,000 rpm, 4°C, 20 min). The pH of cell lysate was adjusted to 8.5 with 1–2 µL of 50 mM NaOH. The protein concentration was estimated by Bradford method. The final concentration of each of the 6 samples was adjusted to 5 µg/µL using cell lysis buffer.

Cyanine dyes Cy2, Cy3 and Cy5 (GE Healthcare, Sweden) were reconstituted to a final stock concentration of 1 mM in 99.9% anhydrous DMF (Aldrich, USA) as per the manufacturer's instructions. The dyes were diluted to 250 pmol/µL in DMF for labelling. The dye to protein labelling ratio was kept constant at 250 pmol of cyanine dye for 30 µg of protein for all labelling reactions. The biological replicates of control and experimental samples were labelled with either Cy3 or Cy5. Reciprocal dye labelling was employed to avoid the influence of labeling variability, if any, in the results. A pooled internal standard was incorporated in the experimental design to allow inter-gel comparison and quantification. Internal standard was prepared by pooling equal concentration of protein from each of the 6 samples prior to labeling. The pooled internal standard (90 µg) was labelled with 3 µL of Cy2 (250 pmol/µL) by incubating on ice for 30 min in dark. The labelling reaction was quenched by the addition of 3 µL of 10 mM lysine. Cy3 and Cy5 labelling was carried out under the same conditions as for Cy2 labelling. An equal volume of 2 DE sample buffer was added to all the samples and those samples that are to be separated on the same gel were pooled and loaded onto a pre-rehydrated IPG strip of pH range 4–7 (18 cm) using cup loading method. The sample was applied to anodic end of the strip and focusing was carried out using Ettan IPG Phor as per the following protocol: 500 V for 1 h (step-n-hold), 1000 V for 1 h (gradient), 8000 V for 3 h (gradient) and 8000 V for 5 h (step-n-hold). The focused strips were frozen overnight at −70°C. The strips were brought to room temperature and reduction, alkylation and second dimensional separation were carried out as mentioned in section 2.3. The dye front was allowed to run out of the gel to remove the unbound dyes.

The gels were taken for scanning immediately using multichannel laser scanner Typhoon 9400 (GE Healthcare, Sweden). The Cy2 images were scanned using a 488 nm

blue laser and emission was recorded using 520 nm BP 40 filter. Cy3 images were acquired using a 532 nm green laser and an emission filter of 580 nm BP 30 and Cy5 images were acquired using a 633 nm red laser and an emission filter of 670 nm BP 30. The pixel sizes for the gels were chosen to be 200 microns and the image analysis mode was set to be floursep.

The spots from the gels were detected and matched using DeCyder™ 2D software, version 6.5 (GE Healthcare, Sweden) as per the manufacturer's instructions. Intra-gel comparison was carried out using DeCyder™ DIA module. A maximum of 2500 spots were detected from each of the three biological replicates by DIA module. The gel boundaries were specified and the exclusion filter option was used to remove gel artifacts. The cut-off values used for exclusion were as follows; area<180, slope >1.3, peak volume <20000 and peak height<150 and >65000. The threshold volume ratio was set to be 1.5. The biological variation analysis was carried out using DeCyder™ BVA module. All the three biological replicates for control and experimental cases were opened in a single workspace and the gel with the maximum number of spots detected was selected as 'Master' gel. VHb recombinant and plasmid control maps were classified into 'experimental' and 'control' groups respectively. To improve the accuracy of gel-to-gel matching, approximately 10 spots were assigned as landmarks and were manually matched among the standard gels before matching the entire spot maps. Spot matching was further assessed manually for mismatching and the mismatches, if any, were corrected before statistical analysis. The statistical analysis was performed using

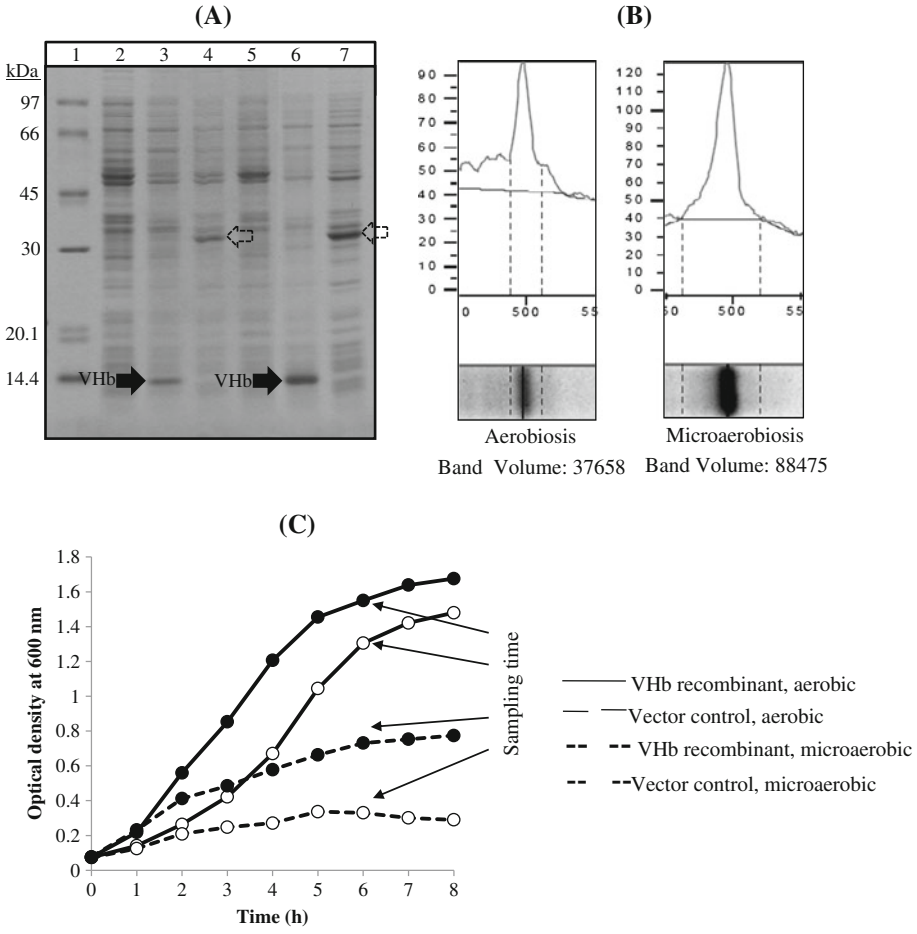


Figure 1. Expression of recombinant haemoglobin in *E. coli* under aerobic and microaerobic conditions. (**A**) One-dimensional PAGE of total cell lysate of DH5α (lanes 2 and 5), DH5 α/pKD152 (lanes 3 and 6) and DH5 α/pSET152 (lanes 4 and 7) grown under aerobic (lanes 2–4) and microaerobic (lanes 5–7) conditions. Ten micrograms of total cell lysate from each case was separated on a 12 % polyacrylamide gel and stained with colloidal coomassie blue to visualize the bands. Expression of haemoglobin is highlighted in lanes 3 and 6. The arrows in lanes 4 and 7 represent aminoglycoside acetyltransferase. (**B**) Image Quant™ TL analysis of the gel in figure 1A. The band intensity of haemoglobin under aerobiosis and microaerobiosis is depicted. (**C**) Growth curve of *E. coli* expressing haemoglobin and vector control. The sampling time point for proteome analysis is indicated in the graph.

unpaired Student's *t*-test between the control and experimental group enabling false discovery rate (FDR) correction. Differentially expressed proteins with a *p*-value less than 0.05 were considered as significant.

3. Results

3.1 *Expression of Vitreoscilla haemoglobin under oxygen-limiting and non-limiting conditions*

Total cell lysate of *E. coli* carrying recombinant VHb grown under aerobic and microaerobic conditions was analysed by 1D PAGE. A protein band corresponding to 15 kDa was found only in cell lysate of DH5α/pKD152, which harbour the recombinant haemoglobin gene but not in the lysate of *E. coli* carrying plasmid vector (figure 1A). This protein was identified as haemoglobin using mass spectrometry (supplementary section 1). It was also observed that the expression level of this protein was comparatively high under oxygen-limited conditions (compare lanes 3 and 6 in figure 1A). Quantification of band volume using Image Quant™ TL software (GE Healthcare, Sweden) demonstrated that there was a 2.3-fold increase in the expression level of recombinant haemoglobin under oxygen-limiting conditions (figure 1B). In the lanes where total cell lysate of DH5α/pSET152 was separated, an additional protein of ~30 kDa was observed (lanes 4 and 7 in

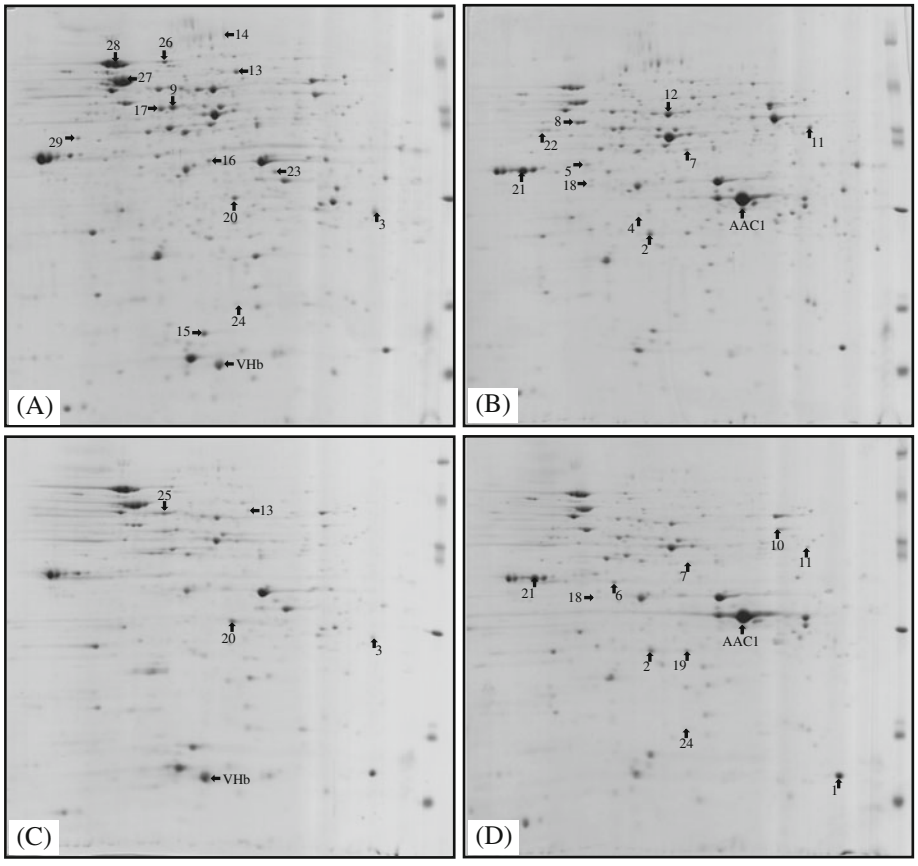


Figure 2. Expression of proteins in *E. coli* grown under different conditions of oxygen availability. The total cell lysate of *E. coli* (200 µg) grown under aerobic and microaerobic conditions were subjected to 2D PAGE analysis using IPG strips of pH range 4–7 (18 cm) for first dimension and 12.5% polyacrylamide gels for second dimension. The gels were stained with colloidal coomassie blue and the spots were identified by MALDI-TOF mass spectrometry. A representative image from each group is shown here. (**A**) DH5α/pKD152 grown under aerobic conditions, (**B**) DH5α/pSET152 grown under aerobic conditions, (**C**) DH5α/pKD152 grown under microaerobic conditions, (**D**) DH5α/pSET152 grown under microaerobic conditions. The proteins showing ±1.5-fold or more change in expression levels are indicated. The spot marked as 'VHb' in (**A**) and (**C**) represent haemoglobin and the spot marked as 'AAC1' in (**B**) and (**D**) represent aminoglycoside acetyltransferase. The proteins that are up-regulated under aerobic conditions in response to VHb expression are marked in (**A**) and those down-regulated are marked in (**B**). Likewise, proteins that are up-regulated under microaerobic conditions in response to VHb expression are marked in (**C**) and those down-regulated are marked in (**D**). The numbers correspond to the spot IDs denoted in table 1.

Table 1. Identification of differentially expressed proteins by mass spectrometry

ID[a]	Protein	Gene	Seq. cov.[b] (%)	Score[c]	Mass matched/ mass searched[d]	Observed[e]		Theoretical[f]		Fold change[g]	
						MW	pI	MW	pI	Aerobiosis	Micro aerobiosis
	Proteins involved in central intermediary metabolism[h]										
1	Phosphonate metabolism protein (PhnG)	phnG	36	47	4/29	16.5	6.75	16.5	6.51	-	−2.16
2	2-Deoxy-D-gluconate 3-dehydrogenase	kduD	44	92	7/27	27	5.35	27	5.24	−3.9	−2.54
3	Succinate dehydrogenase iron-sulfur protein	sdhB	21	55	4/24	28.5	6.7	26.7	6.32	+1.5	+1.62
4	Phosphoribosylaminoimidazole succinocarboxamide synthase	purC	31	61	5/25	28.5	5.1	27.3	5.16	−3.8	-
5	Ketodeoxygluconokinase	kdgK	22	72	5/23	38.5	4.95	33.9	4.92	−3.5	-
6	Transaldolase	talA	16	40	3/23	37.5	5.1	37.4	5.29		−1.53
7	Glycine cleavage system aminomethyltransferase T	gcvT	17	45	4/26	41.5	5.6	39.9	5.36	−2.0	−1.99
8	ATP synthase subunit B	atpF	40	150	11/30	50	4.9	50.2	4.9	−2.0	-
9	Isocitrate dehydrogenase	icdA	35	142	12/25	47	5.2	42.7	5.06	+2.3	-
10	Tryptophanase	tnaA	28	114	11/27	54	6.3	52.7	6.14	-	−1.73
11	Serine hydroxymethyltransferase	glyA	17	38	5/24	43	6.4	45.3	6.03	−2.1	−3.74
12	Chain G, Glycerol kinase	glpK	21	104	8/26	54	5.35	56.0	5.36	−2.1	
13	Phosphoenolpyruvate carboxykinase	pckA	25	115	8/22	63	5.65	59.6	5.46	+1.5	+1.5
14	Biotin sulfoxide reductase	bisC	7	38	4/23	83	5.55	81.9	5.58	+3.7	-
15	L-Rhamnose isomerase	rhaA	30	45	5/27	18	5.4	17.2	6.08	+2.8	-
16	Thioredoxin reductase	trxB	23	54	4/23	37.5	5.45	34.4	5.3	+2.3	-
17	6-Phosphogluconate dehydrogenase	gnd	30	130	8/24	51	5.1	48.8	5.14	+2.2	-
18	Chain A, malonyl-CoA-[acyl-carrier-protein] transacylase	fabD	20	45	3/30	34	5.0	32.2	4.95	−1.9	−2.11
	Proteins involved in nucleic acid metabolism										
19	Chain A, Purine nucleoside phosphorylase	deoD	45	103	6/25	27	5.6	25.6	5.54	-	−1.57
	Proteins involved in cellular transport mechanisms										
20	Outer membrane protein 3b (a), protease VII	ompT	27	73	5/24	30.5	5.5	35.5	5.55	+2.6	+4.57
21	OmpF porin mutant	ompF	19	54	4/28	38.5	4.6	36.3	4.69	−1.8	−4.95
22	Maltoporin	lamB	19	68	5/25	47	4.6	47.3	4.7	−1.8	-
23	PTS system mannose-specific EIIAB component	manX	16	43	3/22	34.5	5.9	34.8	5.74	+2.0	-
24	Outer membrane protein W precursor	ompW	40	92	5/24	21	5.6	25.8	5.93	+2.1	−2.25
	Proteins involved in adaptation process										
25	Aldehyde dehydrogenase A	aldA	22	100	7/27	55	5.1	52.2	5.07	-	+1.76
26	Heat shock protein 90	htpG	12	62	6/30	70.5	5.15	71.3	5.09	+1.6	-
27	GroEL	mopA	17	60	4/25	54	4.85	52	4.79	+3.4	-
28	Molecular chaperone DnaK	dnaK	17	73	6/26	66	4.8	66	4.84	+3.1	-
	Proteins involved in other cell processes										
29	Cell division protein FtsZ	ftsZ	27	90	6/25	41	4.5	40.2	4.65	+2.1	-

[a] The ID given in column 1 corresponds to the spot IDs given in figure 2.

[b] Sequence coverage. The percentage of amino acid sequences covered by the matched peptides in whole protein sequence.

[c] Probability based MOWSE score, that was considered significant ($p < 0.05$) for identification. For identifications with MOWSE score below. 60, theoretical and observed MW and pI were compared and in such cases the protein was considered identified only if the theoretical and observed MW and pI matches.

[d] The number of *m/z* values matches with the protein identified by database search.

[e] Apparent molecular weight and pI of the identified proteins obtained from 2D gel. Molecular weight was calculated based on the R_f value of the molecular weight standards and pI value was calculated by dividing the length of the strip with the pH intervals.

[f] Molecular weight and pI of the protein as given in the database.

[g] Proteins showing a fold change of ±1.5 or more were considered significant. Only significant fold changes are listed in the table.

[h] Classification of the identified proteins based on their function annotated in EcoCyc database.

figure 1A), which was not seen in plasmid free DH5α and DH5α/pKD152 lysates. This protein was identified as aminoglycoside acetyltransferase (a vector-encoded protein that confers apramycin resistance). The MASCOT score was 58 and sequence coverage was 28% indicating the accuracy of identification. Even though apramycin resistance gene was undisturbed in pKD152, its expression was down-regulated on haemoglobin expression in the heterologous host under both aerobic and microaerobic conditions (figure 1A).

The growth pattern of *E. coli* recombinants carrying the VHb gene under its native promoter and the vector control was examined under oxygen-limiting and non-limiting conditions. Expression of haemoglobin gene had shown to enhance the growth rate of recombinant *E. coli* under both aerobic and microaerobic conditions (figure 1C). Comparison of the colony-forming units under both aerobic and microaerobic conditions indicated that the haemoglobin recombinants reached higher cell densities than the vector control (data not shown).

3.2 *Proteome profile of E. coli expressing Vitreoscilla haemoglobin*

The total cell lysate of control and recombinant *E. coli* was collected at different time points and analysed by one-dimensional electrophoresis. It was observed that the maximum level of expression of the recombinant protein was achieved at late log phase (data not shown). Both aerobic and microaerobic cultures of DH5α/pKD152 and DH5α/pSET152 reached the maximum cell density approximately 6 h post inoculation. Therefore, proteomic analysis was carried out using cultures grown for 6 h at 37°C. Medium range IPG strips of pH range 4–7 (18 cm) were used for the separation of total cell lysate in the first dimension and 12.5% polyacrylamide gels for second dimension in 2D PAGE and 2D DIGE experiments. A set of three independent gels were run for samples from each condition. The reproducibility of two-dimensional separation was confirmed by comparing the replicates. Representative gels showing proteins expressed under different experimental conditions are depicted in figure 2. A

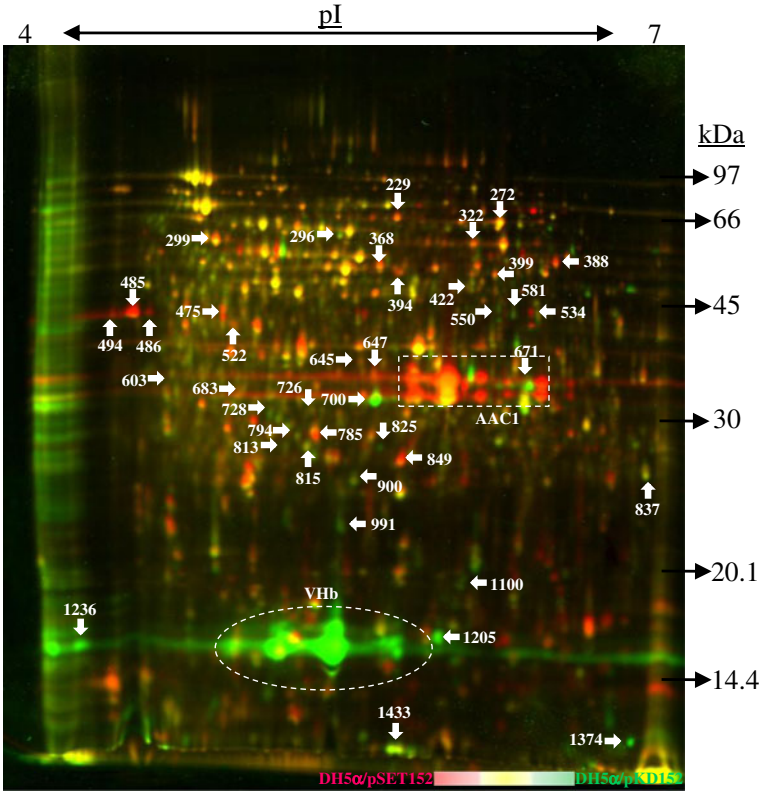


Figure 3. 2D DIGE analysis of haemoglobin expressing *E. coli.* Representative 2D DIGE gel image of *E. coli* expressing VHb under microaerobic conditions is given. Total cell lysate of plasmid control was labeled with Cy5 (red) and the VHb recombinant *E. coli* with Cy3 (green). VHb, which was expressed only in the experimental cultures, appeared green (dotted ellipse) and the aminoglycoside acyltransferase, which was seen overexpressed in the control cultures, appeared red (dotted rectangle) in the 2D DIGE image. The numbers denoted corresponds to the proteins shown to be differentially expressed ($p<0.01$) in experimental sample under microaerobiosis. The details are provided in table 2.

Table 2. List of proteins showing differential expression ($p<0.01$) in response to hemoglobin expression under microaerobic conditions

No	Spot ID[a]	Identification[b]	Fold change[c]	*p*-value	Experimental MW[d]	Experimental pI[d]
1	825	ND[e]	+5.68	4.8e-005	27.4	5.9
2	494	ND	−4.18	6.8e-005	41.6	4.35
3	663	ND	−12.65	0.00017	33.4	6.5
4	272	ATP synthase subunit A	−1.29	0.00024	60.3	6.3
5	666	ND	−9.41	0.00028	31.7	6.1
6	1374	ND	+26.01	0.00043	<14.4	6.9
7	383	ND	−1.79	0.00055	51.7	6.15
8	388	Serine hydroxymethyl transferase	−3.74	0.00063	51.7	6.55
9	991	ND	+4.7	0.00070	22.7	5.45
10	1205	ND	+22.9	0.00094	18.7	6.0
11	726	ND	+1.56	0.0017	30.0	5.45
12	689	ND	−3.75	0.0018	31.7	5.9
13	700	Outer membrane protein 3b(a) protease	+4.57	0.0019	31.7	5.7
14	849	ND	−3.37	0.0019	26.1	5.85
15	690	ND	−9.77	0.0024	31.7	6.2
16	422	ND	+1.51	0.0026	47.8	6.15
17	837	ND	+1.46	0.0027	27.4	6.9
18	628	ND	−4.05	0.0032	35.1	6.0
19	296	ND	+2.34	0.0032	58.4	5.5
20	813	ND	+2.05	0.0033	27.4	5.25
21	815	ND	+2.03	0.0034	27.4	5.35
22	886	ND	+1.88	0.0039	24.9	5.4
23	785	2-deoxy D-gluconate-3-dehydrogenase	−2.54	0.0042	28.5	5.4
24	394	ND	−2.1	0.0045	50.7	5.8
25	368	ND	−1.92	0.0048	51.7	5.7
26	475	ND	−2.73	0.0048	40.4	4.9
27	794	ND	+2.78	0.0051	24.4	5.3
28	399	ND	−1.77	0.0051	50.7	6.2
29	1433	ND	+2.15	0.0055	<14.4	5.8
30	486	ND	−4.57	0.0055	41.6	4.55
31	299	ATP synthase subunit B	−1.49	0.0056	60.3	4.9
32	1100	ND	+4.46	0.0057	19.2	6.2
33	485	OmpF porin	−4.95	0.0060	41.6	4.4
34	671	ND	+1.94	0.0061	33.4	6.4
35	645	ND	−3.95	0.0063	39.3	5.6
36	322	ND	−2.11	0.0065	55.5	6.1
37	522	ND	−1.4	0.0065	40.4	5.0
38	581	ND	+4.29	0.0067	41.0	6.45
39	424	ND	+1.46	0.0068	45.0	5.0
40	550	ND	+1.42	0.0075	41.6	6.3
41	654	ND	−4.77	0.0076	35.1	5.8
42	701	ND	−3.44	0.0076	30.0	5.6
43	728	ND	+4.27	0.0079	30.0	5.2
44	1236	ND	+17.96	0.0083	17.4	4.35
45	900	ND	+3.37	0.0084	24.9	5.6
46	683	ND	−2.00	0.0086	31.7	5.0
47	534	ND	−1.6	0.0088	41.0	6.4
48	603	ND	+1.5	0.0089	39.3	4.75
49	647	ND	−3.4	0.0092	35.1	5.6
50	427	ND	+1.62	0.0092	45.0	4.85
51	229	ND	−1.72	0.0093	66.0	5.9

[a] Spot IDs are as depicted in figure 3.

[b] Protein identification was carried out by MALDI-TOF mass spectrometry from a representative preparatory gel.

[c] Fold change was calculated using the standardized volume ratios of control and experimental spots. Proteins showing a fold change of ±1.5 or more and with *p* value <0.01 were listed.

[d] Apparent MW and pI in 2D gel. Experimental MW was calculated by comparing the R_f values. pI value was calculated by dividing the length of the strip with its pH interval.

[e] ND, identification not done.

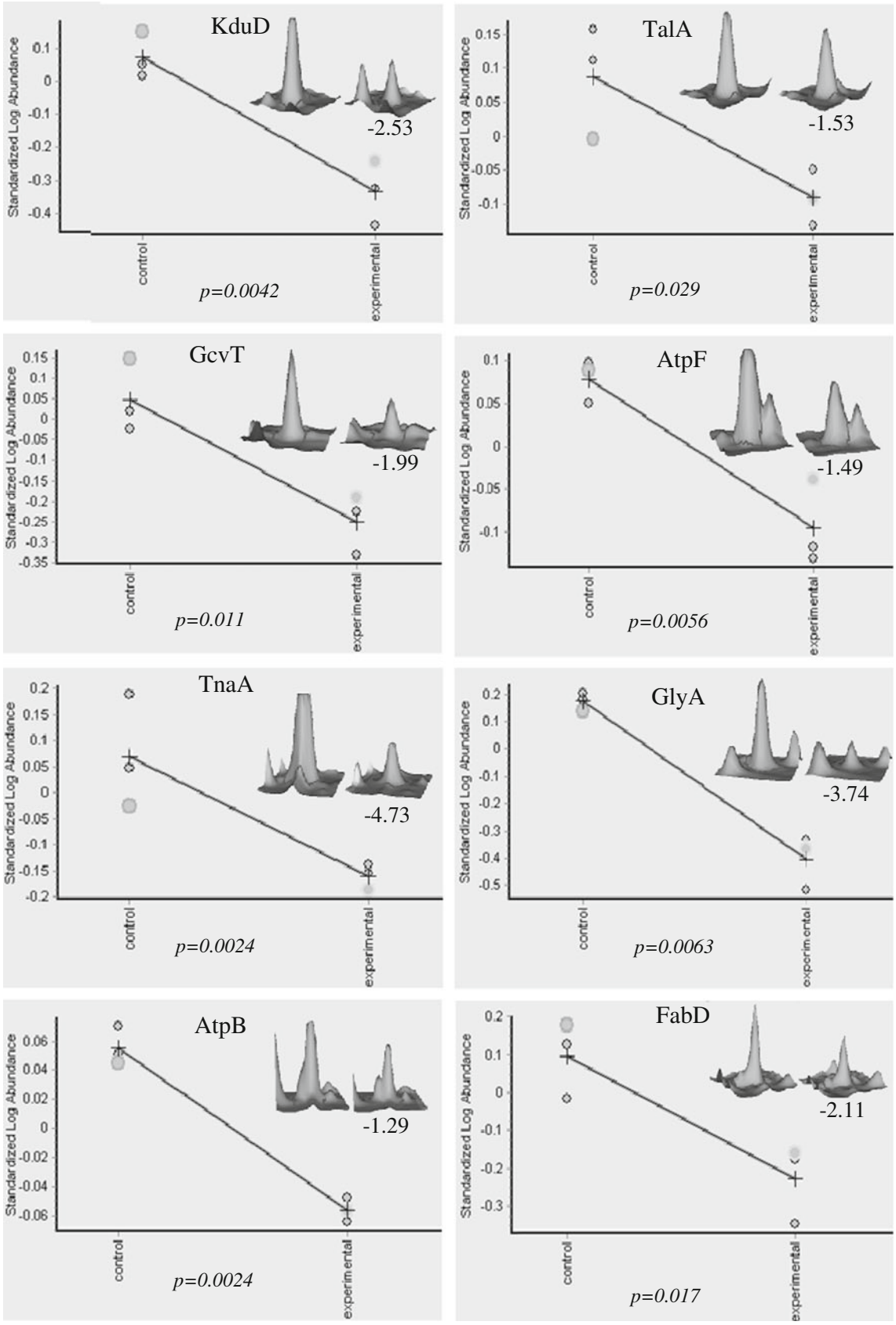
set of 29 proteins, which were shown to be differentially expressed (±1.5-fold) in response to VHb expression under aerobic and/or microaerobic conditions by gel matching, could be identified by MALDI-TOF mass spectrometry. The proteins up-regulated on haemoglobin expression under aerobic conditions are marked in figure 2A and those down-regulated are marked in figure 2B. Likewise, the proteins up-regulated on haemoglobin expression under microaerobic conditions are marked in figure 2C and those down-regulated in figure 2D. The list of these proteins and their expression patterns are provided in table 1. The protein marked as 'VHb' in figure 2A and C was found only in the lysate of DH5α/pKD152. This protein was identified as *Vitreoscilla* haemoglobin by matching the *m/z* values obtained from peptide mass fingerprinting of the tryptic digests with theoretical values obtained by *in silico* tryptic digestion (supplementary section 1), since database search algorithms could not identify the protein conclusively. The protein marked as 'AAC1' in figure 2B and D was seen only in the lysate of DH5α/pSET152. This protein was identified as aminoglycoside acetyltransferase by mass spectrometry. The probable function was assigned for each protein based on compilations from earlier references and on the entries in EcoCyc database (Karp *et al.* 2002; Han and Lee 2006). These proteins are grouped into five groups: proteins involved in intermediary metabolism, proteins involved in nucleic acid metabolism, proteins involved in cellular transport mechanisms, proteins involved in adaptation process and proteins involved in cell division.

Data depicted in figure 2 demonstrate that expression of *Vitreoscilla* haemoglobin in heterlogous host changes the cellular protein profile under aerobic and microaerobic conditions. Most of the proteins upregulated under aerobiosis in the recombinant *E. coli* were also up-regulated under microaerobiosis and vice versa. However, expression of outer membrane protein W was found to be up-regulated (2.1-fold) under aerobic conditions, but was down-regulated (2.25-fold) under oxygen-limited conditions.

3.3 *Differential expression of E. coli proteins under aerobiosis in response to VHb expression*

Expression of haemoglobin in *E. coli* grown under aerobic conditions altered the levels of 24 proteins. Of these, 14 proteins were found to be up-regulated and 10 were found to be down-regulated. Mapping the differentially expressed proteins in KEGG pathways using KEGGmapper (Ogata *et al.* 1999) showed that the proteins that were up-regulated generally include those involved in metabolic pathways, citrate cycle, glycolysis/gluconeogenesis and synthesis of secondary metabolites (supplementary section 2). Succinate dehydrogenase (1.5-fold), isocitrate dehydrogenase (2.3-fold), phosphoenolpyruvate carboxykinase (1.5-fold) and 6-phosphogluconate dehydrogenase (2.2-fold), involved in carbohydrate metabolism and energy production were found to be up-regulated. At the same time, certain other proteins grouped under metabolic pathways by KEGGmapper, such as, ketodeoxygluconokinase (−3.5-fold), 2-deoxy-D-gluconate 3-dehydrogenase (−3.9-fold), phosphoribosylaminoimidazole succinocarboxamide synthase (−3.8-fold), serine hydroxymethyltransferase (−2.1-fold), glycerol kinase (−2.1-fold) and glycine cleavage system aminomethyltransferase (−2.0-fold) were found to be down-regulated under aerobic conditions in recombinant *E. coli*. 6-phopshogluconate dehydrogenase, an enzyme in pentose phosphate pathway, was found to be up-regulated (2.2-fold) while another enzyme, ketodeoxygluconokinase, which is known to have an indirect role in pentose phosphate pathway by mediating the interconversion of pentose and gluconate, was found to be down-regulated (−3.5-fold). Transaldolase, another protein involved in pentose phosphate pathway was up-regulated only 1.2-fold on VHb expression under aerobiosis. There was no significant increase in the expression levels of ATP synthase under aerobic conditions even though VHb expression is known to enhance the efficiency of ATP production (Khosla *et al*. 1990). There was a notable increase in the expression levels of proteins involved in cellular adaptations like HSP 90 (1.6-fold), GroEL (3.4-fold) and DnaK (3.1-fold) under aerobic conditions. Cell division protein, FtsZ, was found to respond positively to intracellular VHb expression. It was found to be up-regulated 2.1-fold in response to VHb expression under aerobic conditions when compared to the vector control. OmpW, a protein responding to oxygen limitation (Partridge *et al*. 2007), was found to be up-regulated (2.1-fold) under aerobiosis, and OmpF, a protein responding to osmotic stress, was found to be down-regulated (−1.8-fold).

Figure 4. Differential expression of *E. coli* proteins in response to VHb expression under microaerobic conditions. Regulatory pattern of proteins as obtained by 2D DIGE biological variation analysis is depicted. The scatter plot represents the normalized spot volume in the control (DH5α/pSET152) and experimental (DH5α/pKD152) replicates for each spot. The 3D view of the corresponding spots is given in the inset. Fold change and *p*-value for respective proteins are provided in the graph. Abbreviations used: KduD, 2-deoxy-D-gluconate-3-dehydrogenase; TalA, Tranaldolase; GcvT, Glycine cleavage system aminomethyltransferase T; AtpF, ATP synthase subunit B; TnaA, Tryptophanase; GlyA, Serine hydroxymethyltransferase; AtpB, ATP synthase subunit A; FabD, Chain A, malonyl-CoA-[acyl-carrier-protein] transacylase; DeoD, Purine nucleoside phosphorylase; OmpT, Outer membrane protein 3b(a) protease; OmpF, OmpF porin.


KduD
-2.53
p=0.0042
TalA
-1.53
p=0.029
GcvT
-1.99
p=0.011
AtpF
-1.49
p=0.0056
TnaA
-4.73
p=0.0024
GlyA
-3.74
p=0.0063
AtpB
-1.29
p=0.0024
FabD
-2.11
p=0.017
Standardized Log Abundance
control
experimental

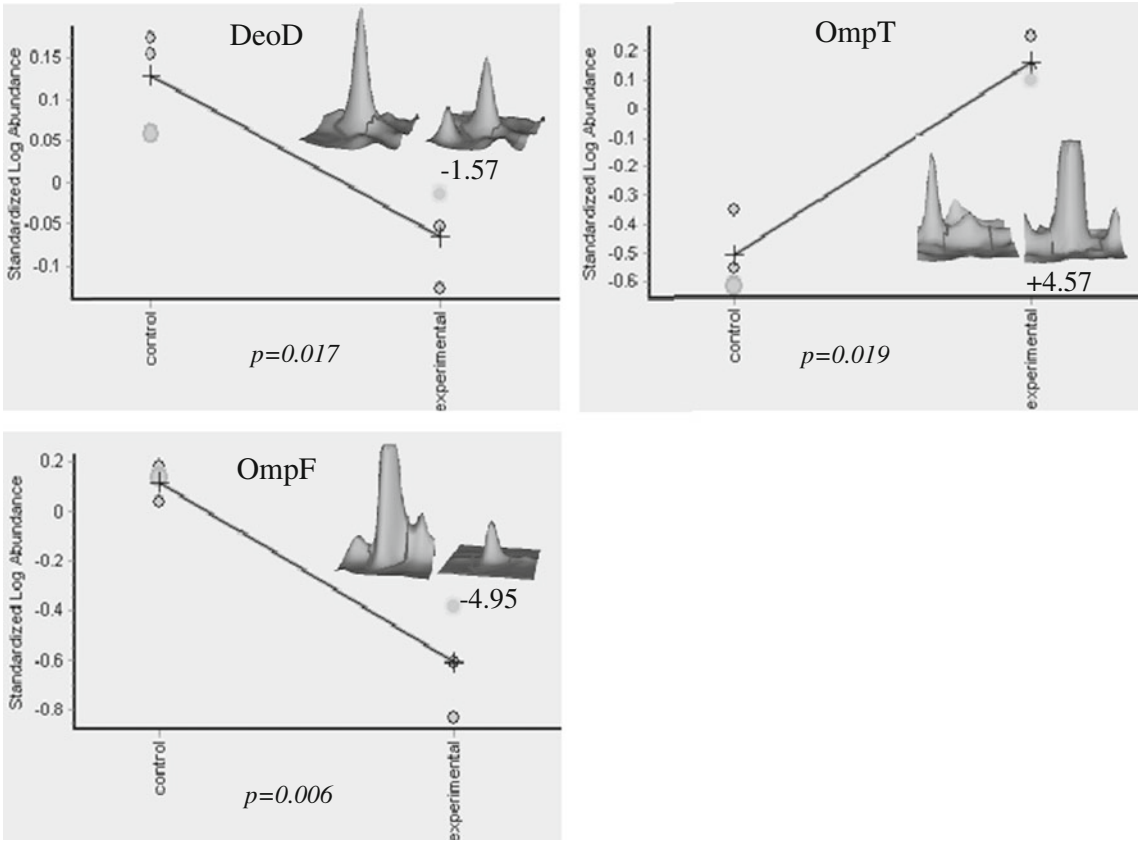


Figure 4. (continued).

3.4 *Differential expression of E. coli proteins under microaerobiosis in response to VHb expression*

Proteomic analysis of VHb expressing *E. coli* grown under microaerobic conditions was carried out by 2D PAGE and quantification of differential expression was carried out by 2D DIGE. Fourteen proteins showing differential expression under microaerobic conditions by 2D PAGE analysis could be identified by mass spectrometry. Of this, four proteins were found to be up-regulated and 10 proteins were found to be down-regulated (figure 2C and D; table 1). Statistical significance of differential expression of these proteins was analysed using three biological replicates by 2D DIGE as described in materials and methods. A representative 2D DIGE gel image is given in figure 3. DeCyder™ DIA analysis detected an average of 1200 spots from each gel, of which an average of 460 spots were up-regulated while an average of 500 spots were down-regulated on VHb expression under microaerobic conditions. However, statistical analysis of the differentially expressed proteins using DeCyder™ BVA module identified significant alterations in the expression levels of 51 proteins (±1.5-fold) at 99% confidence interval in response to VHb expression under microaerobiosis (Fig 3). Of these, 24 proteins were up-regulated and 27 proteins were down-regulated (table 2). The number of differentially expressed proteins within 95% confidence interval was 271. Peptide mass fingerprinting could identify 11 of these differentially expressed proteins and the remaining proteins could not be identified using our lab standardized methods. Even though the spots such as 6, 10 and 44 were shown to be up-regulated (marked as 1374, 1205 and 1236, respectively in figure 3), in the Coomassie blue stained gels the quantity of these proteins were found to be too low and hence attempts to identify these proteins using MALDI failed. The identified proteins include serine hydroxymethyltransferase, outer membrane protein 3b(a) protease, OmpF porin, 2-deoxy-D-gluconate 3-dehydrogenase, transaldolase, glycine cleavage system aminomethyltransferase T, tryptophanase, purine nucleoside phosphorylase, Chain A, malonyl CoA (acyl carrier protein) transacylase and ATP synthase subunits A and B. Although the differential expression of ATP synthase subunits was found to be statistically significant, the subunits A and B were down-regulated by only 1.29- and 1.49-folds, respectively, in the biological replicates. The regulatory pattern, fold change and *p*-values for these 11 proteins are depicted in figure 4.

Serine hydroxymethyltransferase, which catalyses the interconversion of serine and glycine, was found to be down-regulated by 3.74-fold (p=0.00063) on VHb expression under microaerobiosis. The membrane proteins OmpT was up-regulated by 4.57-fold (p=0.0019), while OmpF was down-regulated by 4.95-fold (p=0.0060). Expression of native haemoglobin under microaerobiosis was shown to reduce the expression of tryptophanase by 4.73-fold (p=0.0024). The expression of transaldolase was found to be down-regulated by 1.53-fold (p=0.029) under microaerobiosis. Aminomethyltransferase of glycine cleavage system was down-regulated by 1.99-fold (p=0.011), indicating a probable reduction in degradation rate of glycine on VHb expression. Purine nucleoside phosphorylase, which catalyses the conversion of guanosine or inosine to their base and sugar phosphates, and a protein involved in fatty acid biosynthesis, malonyl coA acyl carrier protein transacylase, were also found to be down-regulated by 1.57-fold (p=0.017) and 2.11-fold (p=0.017), respectively. Aminoglycoside acetyltransferase, a plasmid encoded protein conferring apramycin resistance was shown to be down-regulated in the recombinant *E. coli* carrying VHb gene by 1D PAGE and 2D PAGE analysis. DeCyder™ analysis has shown that the downregulation is statistically significant (p<0.01) and there was a 3.77-fold down-regulation in the expression levels.

4. Discussion

Effect of *Vitreocilla* haemoglobin expression in recombinant *E. coli* had been examined by transcriptome and proteome profiling previously (Roos *et al*. 2004; Isarankura-Na-Ayudhya *et al.* 2008). These studies were restricted to VHb recombinants growing under aerobic conditions. In this study, we have demonstrated differential expression of *E. coli* proteins in response to VHb expression under aerobic and microaerobic conditions. Previous reports (Champion *et al.* 2003; Aldor *et al.* 2005; Wang *et al*. 2005) have shown that the overexpression of recombinant proteins lead to alterations in the proteome profile of *E. coli*. Hence, in this study, we had used VHb gene under its own promoter to avoid copy-number-dependent changes.

Accumulation of haemoglobin in *E. coli* was found to be high under microaerobic conditions when compared to aerobic growth conditions (Khosla and Bailey 1989; Khosla *et al*. 1990). This observation was confirmed in our study, and the data in figure 1B shows that the expression levels of haemoglobin was approximately threefold higher under microaerobic conditions. Recombinant *E. coli* harbouring *vgb* under its native promoter expresses haemoglobin under the control of an oxygen-sensitive regulatory mechanism mediated by *fnr* (Tsai *et al.*1995). FNR is active under oxygen-limited conditions and stimulates the expression of genes required for adaptations under oxygen scarcity. Although the intracellular concentration of FNR is the same under both anaerobic and aerobic conditions, its activity is regulated by oxygen availability (Shalel-Levanon *et al.* 2005). Oxygen-mediated inactivation of FNR is prevented under oxygen-limited conditions and hence the expression of genes regulated by FNR increases. Our results implied that FNR-mediated regulation of *Vitreoscilla* haemoglobin expression is operational in *E. coli* also, indicating the functional similarity in *Vitreoscilla* and *E. coli*. It was reported earlier that the presence of a plasmid places a metabolic burden to the host and there is an inverse relationship between the cell growth rate and plasmid size (Zund and Lebek 1980; Cheah *et al.* 1987). It has been suggested that the energy requirement of recombinant organism to maintain the plasmid and express encoded genes might be insufficient to allow maximum growth rate in shake flask cultures (Khosravi *et al.* 1990). This study demonstrates an approximately two-fold reduction in the doubling time of *E. coli* expressing plasmid encoded VHb under aerobic conditions and approximately threefold reduction in doubling time under oxygen-limited conditions (figure 1C), and its growth rate was found to be high compared to plasmid-free cells (data not shown). This implies that expression of VHb-enabled recombinant *E. coli* to relieve the metabolic stress due to plasmid replication and expression of plasmid encoded genes. Moreover, cell division protein FtsZ was also shown to be up-regulated in VHb recombinants in this study. Expression of haemoglobin is suggested to enhance the oxygen utilization properties of the recombinant host, thereby enhancing the growth and cell density under oxygen stress (Khosla and Bailey 1988; 1989; Dikshit *et al.* 1990; Liu *et al.* 1995).

This study suggests that haemoglobin-expressing *E. coli* adapts to microaerobic conditions by differential expression of proteins involved in various metabolic pathways. A schematic diagram depicting the affected pathways and proteins is given in figure 5. It had been shown that VHb directs *E. coli* growing under oxygen-limited conditions to utilize more of its respiratory pathways and less of its fermentative pathways (Tsai *et al.* 2002). VHb expression has been shown to aid aerobic respiration under hypoxic conditions in its native host (Chi *et al.* 2009), *Saccharomyces cerevisiae* (Chen *et al.* 1994) and *E. coli* (Dikshit *et al.* 1992). Transcriptome analysis by microarrays had shown that nearly all citrate cycle genes were induced in VHb-expressing cells (Roos *et al.* 2004). However, isocitrate dehydrogenase, a key enzyme in the citrate cycle, was shown to be down-regulated in VHb-fusion-protein-expressing *E. coli* under aerobic conditions (Isarankura-Na-Ayudhya *et al*. 2008). Proteome analysis of VHb recombinant under microaerobic conditions correlated with the transcriptome analysis and the proteins involved in citrate cycle such as succinate dehydrogenase (involved in the conversion of succinate to fumarate) and isocitrate

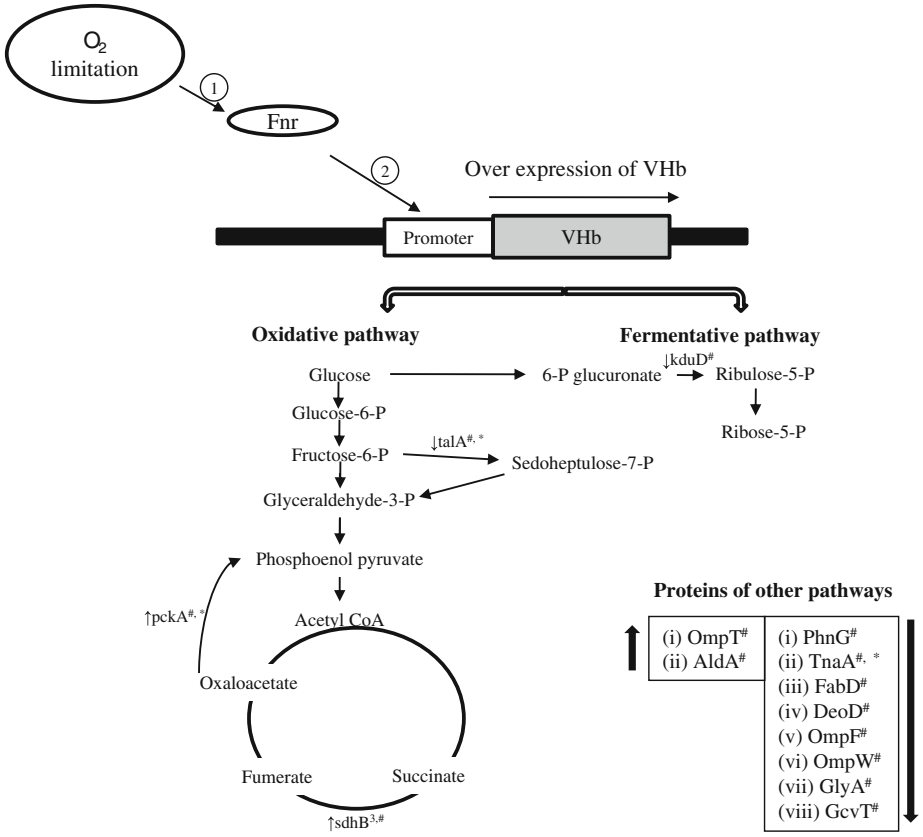


Figure 5. Pathways affected by *Vitreoscilla* haemoglobin in *E. coli* cultures exposed to oxygen stress. Proteins found to be up-regulated in oxidative pathways and those down-regulated in fermentative pathways are shown. Proteins involved in other pathways, affected by VHb expression under oxygen stress are also shown. The arrow head pointing up represents upregulation and pointing down represents down-regulation of the proteins. Apart from this data provided in this report (indicated by #), previously published data (indicated by *) showing similar regulation are discussed in the text.

dehydrogenase (involved in the conversion of isocitrate to 2-oxoglutarate) were found to be up-regulated in VHb-expressing *E. coli*. Up-regulation of succinyl CoA synthetase (1.38-fold), involved in the conversion of 2-oxoglutarate to succinyl CoA, could be demonstrated. However, the biological variation was not statistically significant. Other proteins involved in citrate cycle could not be identified from 2D gels using our methods, and hence their expression pattern could not be compared. Up-regulation of enzymes involved in citrate cycle and down-regulation of transaldolase A and 2-deoxy-D-gluconate 3-dehydrogenase, enzymes involved non-oxidative branch of glucose metabolism (pentose phosphate pathway), demonstrating the utilization of aerobic metabolic pathways in VHb expressing *E. coli* under oxygen-limited conditions.

It had been demonstrated that the ATP content in VHb-expressing cells was twice as high as that of control cells under microaerobic conditions (Kallio *et al.* 1994). ATP synthase catalyses synthesis of ATP primarily under aerobic metabolism and half of the ATP synthase genes were found to get up-regulated in haemoglobin-expressing cells by transcriptome analysis (Roos *et al*. 2004). However, our proteomic study had shown that ATP synthase subunits A and B were down-regulated in recombinants under micro-aerobiosis. ATP synthase subunit B was found be down-regulated even under aerobic conditions. The rest of the ATP subunits could not be identified from 2D gel in the current study. A positive relationship between protein abundance and transcript abundance during the exponential growth of *E. coli* was reported (Corbin *et al.* 2003). However, the amount of mRNA and the amount of protein present in the cell does not relate directly in all the cases (Gygi *et al.* 1999). Hence, both the protein and transcript levels need to be examined using multiple approaches before drawing a conclusion on expression levels.

This study demonstrated up-regulation of aldehyde dehydrogenase (AldA), a protein involved in adaptation process, in VHb-expressing *E. coli* under oxygen-limited conditions. However, no significant changes in the expression level of AldA were observed under aerobic conditions. AldA expression is controlled by ArcA (anoxic redox

control protein A), which represses the expression of numerous operons under redox conditions of growth (Alexeeva *et al.* 2003). Crp-mediated down-regulation of AldA was proposed under aerobic conditions in VHb fusion protein expressing *E. coli* (Isarankura-Na-Ayudhya *et al*. 2008). Our studies demonstrate that Crp-mediated repression of AldA could be alleviated under microaerobic conditions in VHb-expressing *E. coli.* Up-regulation of these proteins in VHb-expressing cells implies that the cells are utilizing the respiratory pathways and are not exposed to redox growth conditions even though oxygen supply was limited in the culture conditions.

Outer membrane protein W, which was shown to get induced on reduction in oxygen availability by FNR system (Constantinidou *et al.* 2006; Partridge *et al*. 2007), was found to be down-regulated under microaerobiosis in VHb expressing *E. coli*, indicating that the recombinant cells were not experiencing a reduction in oxygen availability. Induction of OmpW in plasmid control under microaerobiosis can be interpreted based on oxygen limitation experienced by the cells. On the contrary, its expression was found to be increased under aerobiosis in VHb recombinants. Osmotic stress response protein OmpF was down-regulated under microaerobiosis, unlike under aerobiosis, as demonstrated by transcriptome analysis (Roos *et al.* 2004). In an earlier report, it was shown that tryptophanase expression was completely abolished in cells expressing VHb fusion protein (Isarankura-Na-Ayudhya *et al*. 2008). Our study confirms the reduction in tryptophanase levels, in addition the relative levels of VHb under aerobic and microaerobic conditions directly correlated with the down-regulation of tryptophanase. Other proteins involved in amino acid metabolism were also found to be down-regulated. For example, glycine cleavage system aminomethyltransferase T (GcvT), an enzyme which catalyses oxidative cleavage of glycine to CO_2 and NH_3 (Kikuchi 1973), and serine hydroxymethylmethyltransferase (GlyA), a major source of one-carbon units used in the synthesis of purines, thymine, methionine and aminoacyl tRNAs (Lorenz and Stauffer 1996), were found to be down-regulated in haemoglobin-expressing *E. coli* under microaerobic conditions. Down-regulation of GlyA had been shown to be associated with over expression of recombinant proteins in previous studies (Lorenz and Stauffer 1996). Protein involved in fatty acid biosynthesis, Malonyl CoA-ACP transacylase (FabD), was shown to be down-regulated under microaerobic conditions. Repression of *fabHDG* operon had been demonstrated under amino acid starvation in *E. coli* (Podkovyrov and Larson 1996). Other gene products of *fab* operon could not be identified from recombinant *E. coli*, and hence their regulation could not be demonstrated. OmpT, a protease, was found to be significantly up-regulated in VHb recombinants. The role of OmpT as a stress response protein and its temperature-regulated expression had been demonstrated elsewhere (Rupprecht *et al.* 1983). However, the effect of oxygen deprivation or haemoglobin expression on OmpT up-regulation needs to be explored further. Purine nucleotide phosphorylase (DeoD), an enzyme involved in nucleotide synthesis, was shown to be down-regulated under microaerobiosis in this study. However, *deoC,* a gene located in its overlapping operon, was shown to be up-regulated in VHb-expressing *E. coli,* under aerobic conditions (Roos *et al*. 2004). Oxygen-limited conditions might impose a metabolic load on VHb recombinants, which might direct the cellular machinery to operate energy-efficient pathways to adapt to the stress condition. Expression of VHb allows the recombinant to adapt better to such hostile environments.

5. Conclusion

Expression of *Vitreoscilla* haemoglobin directly or indirectly influences the expression of proteins involved in aerobic metabolism even in the heterologous host under oxygen-limited growth conditions. Proteomic analysis of haemoglobin-expressing *E. coli* have shown that the proteins, especially enzymes in aerobic respiration and energy metabolism are up-regulated compared to controls, thereby enabling effective utilization of oxygen under microaerobic conditions. The growth enhancing properties of *Vitreoscilla* haemoglobin can be exploited as a method for metabolic engineering to enhance the production of recombinant products from heterologous host.

Acknowledgements

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Corresponding editor: SUDHA BHATTACHARYA