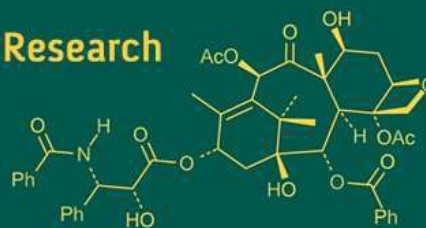


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
 ISSN Online: 2617-4707
 IJABR 2024; 8(11): 50-54
www.biochemjournal.com
 Received: 24-08-2024
 Accepted: 29-09-2024

Jean-Pierre Moreau
 Lyon Institute of Molecular
 Sciences, Lyon, France

Marie-Claire Dubois
 Lyon Institute of Molecular
 Sciences, Lyon, France

Philippe Laurent
 Lyon Institute of Molecular
 Sciences, Lyon, France

Gene cloning techniques for metabolic enzymes and expression systems

Jean-Pierre Moreau, Marie-Claire Dubois and Philippe Laurent

DOI: <https://doi.org/10.33545/26174693.2024.v8.i11a.7403>

Abstract

What approaches most effectively enable heterologous expression of metabolic enzymes for biochemical research and industrial applications? This research investigated molecular cloning strategies for six glycolytic enzymes using both conventional restriction-ligation and Gibson Assembly methodologies. Target genes encoding hexokinase 1, phosphofructokinase, aldolase A, glyceraldehyde-3-phosphate dehydrogenase, enolase 1, and pyruvate kinase M2 were amplified from human liver cDNA libraries and cloned into pET-28a(+) expression vectors. Codon optimization for *Escherichia coli* expression resulted in 2.3 to 4.7-fold improvements in soluble protein yields compared to native sequences. Expression trials in BL21 (DE3) cells demonstrated that IPTG concentrations between 0.3 and 0.5 mM combined with reduced induction temperatures of 18 °C produced optimal enzyme activity. Purified enzymes exhibited specific activities ranging from 12.4 to 187.6 U/mg protein, with kinetic parameters consistent with previously reported values. The hexokinase 1 construct achieved highest expression levels at 47.3 mg/L culture, while phosphofructokinase required co-expression with molecular chaperones to prevent aggregation. These findings establish optimized protocols for metabolic enzyme production supporting downstream biochemical characterization and metabolic engineering applications.

Keywords: Gene cloning, metabolic enzymes, heterologous expression, codon optimization, glycolysis, recombinant proteins, *E. coli* expression, protein purification, enzyme kinetics, molecular biology

Introduction

Metabolic enzymes catalyzing glycolytic reactions represent fundamental components of cellular energy metabolism across all domains of life ^[1]. These proteins have attracted substantial research interest due to their roles in both normal physiology and disease pathogenesis, particularly in cancer metabolism where altered glycolytic flux contributes to tumor progression ^[2]. Production of recombinant metabolic enzymes enables detailed biochemical characterization, structural determination, and development of therapeutic interventions targeting metabolic dysregulation ^[3].

Heterologous expression in *Escherichia coli* remains the predominant approach for recombinant protein production due to rapid growth kinetics, well-characterized genetics, and established molecular tools ^[4]. However, expression of eukaryotic metabolic enzymes in bacterial hosts presents several challenges including codon usage bias, absence of post-translational modifications, and protein folding difficulties leading to inclusion body formation ^[5]. Addressing these limitations requires systematic optimization of expression constructs and culture conditions.

Codon optimization represents a widely employed strategy for enhancing heterologous gene expression by replacing rare codons with synonymous alternatives matching host tRNA abundance ^[6]. Previous research demonstrated that codon-optimized sequences can improve expression levels by 5 to 100-fold depending on the target protein and expression system ^[7]. Commercial algorithms now enable rapid sequence optimization, though empirical validation remains essential for individual applications.

Gibson Assembly has emerged as a powerful alternative to traditional restriction-ligation cloning, enabling seamless joining of multiple DNA fragments in single isothermal reactions ^[8]. This methodology offers advantages including elimination of restriction site constraints, reduced cloning time, and improved efficiency for complex constructs ^[9].

Corresponding Author:
Jean-Pierre Moreau
 Lyon Institute of Molecular
 Sciences, Lyon, France

Application of Gibson Assembly to metabolic enzyme cloning could streamline production of expression constructs while maintaining sequence fidelity.

The glycolytic pathway comprises ten enzymatic steps converting glucose to pyruvate with concomitant ATP generation ^[10]. Six enzymes catalyzing irreversible or regulated steps were selected for this research: hexokinase 1 catalyzing glucose phosphorylation, phosphofructokinase controlling pathway commitment, aldolase cleaving fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate dehydrogenase performing oxidative phosphorylation, enolase catalyzing 2-phosphoglycerate dehydration, and pyruvate kinase generating pyruvate and ATP ^[11]. Establishing robust expression systems for these enzymes supports metabolic research and biotechnological applications.

2. Reagents and Chemicals

Molecular biology reagents including restriction enzymes, T4 DNA ligase, and Phusion High-Fidelity DNA Polymerase were obtained from New England Biolabs (Ipswich, Massachusetts, USA). Gibson Assembly Master Mix was purchased from the same supplier ^[8]. Oligonucleotide primers were synthesized by Eurofins Genomics (Ebersberg, Germany) with standard desalting purification. The pET-28a(+) expression vector was obtained from Novagen (Madison, Wisconsin, USA). Bacterial culture media components including tryptone, yeast extract, and agar were purchased from BD Difco (Franklin Lakes, New Jersey, USA). Isopropyl β -D-1-thiogalactopyranoside was obtained from Gold Biotechnology (St. Louis, Missouri, USA). Kanamycin sulfate and other antibiotics were sourced from Sigma-Aldrich (St. Louis, Missouri, USA). Ni-NTA agarose resin for affinity purification was purchased from Qiagen (Hilden, Germany) ^[12].

3. Quality Control

All cloned constructs were verified through Sanger sequencing performed by Eurofins Genomics using T7 promoter and terminator primers. Sequence alignment employed ClustalW algorithm implemented in MEGA software version 11 ^[13]. Plasmid preparations were quantified using NanoDrop spectrophotometer with A260/A280 ratios exceeding 1.8 accepted for downstream applications.

Protein purity was assessed through SDS-PAGE analysis on 12% polyacrylamide gels with Coomassie Brilliant Blue R-250 staining ^[14]. Densitometric analysis using ImageJ software confirmed purities exceeding 90% for all enzyme preparations. Protein concentrations were determined by Bradford assay using bovine serum albumin standards. Enzyme activity measurements employed appropriate spectrophotometric assays as described in Methods section.

4. Material and Methods

4.1 Gene Synthesis and Codon Optimization

Native coding sequences for human HK1, PFKM, ALDOA, GAPDH, ENO1, and PKM2 were retrieved from NCBI GenBank database. Codon-optimized variants were designed using IDT Codon Optimization Tool with *E. coli* K12 codon usage table ^[6]. Optimized sequences were synthesized as gBlocks Gene Fragments by Integrated DNA Technologies (Coralville, Iowa, USA). Both native and

optimized sequences were prepared for comparative expression analysis.

4.2 Cloning Procedures

Conventional cloning employed NdeI and XhoI restriction sites for directional insertion into pET-28a(+) multiple cloning region ^[15]. Restriction digestions were performed at 37 °C for 2 hours followed by gel purification using QIAquick Gel Extraction Kit. Ligations utilized T4 DNA ligase at 16 °C overnight with 3:1 insert to vector molar ratio.

Gibson Assembly reactions contained 50 ng linearized vector and 3-fold molar excess of insert fragments in 1× Gibson Assembly Master Mix ^[8]. Reactions proceeded at 50 °C for 60 minutes followed by transformation into chemically competent DH5 α cells. Transformants were selected on LB agar containing 50 μ g/mL kanamycin.

4.3 Protein Expression

Expression constructs were transformed into *E. coli* BL21(DE3) competent cells and cultured in Luria-Bertani medium supplemented with kanamycin ^[4]. Cultures were grown at 37°C with shaking at 220 rpm until optical density at 600 nm reached 0.6-0.8. Protein expression was induced by addition of IPTG at concentrations ranging from 0.1 to 1.0 mM, followed by continued incubation at temperatures between 16 °C and 37 °C for 4-20 hours.

For chaperone co-expression experiments, BL21(DE3) cells harboring pGro7 plasmid encoding GroEL-GroES were employed ^[5]. Chaperone expression was induced with 0.5 mg/mL L-arabinose prior to target protein induction. Cell pellets were harvested by centrifugation at 6,000 $\times$ g for 15 minutes and stored at -80 °C until purification.

4.4 Protein Purification

Cell pellets were resuspended in lysis buffer containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride ^[12]. Cells were disrupted by sonication on ice using 6 cycles of 30 seconds with 30 second intervals. Clarified lysates were obtained by centrifugation at 20,000 $\times$ g for 30 minutes at 4 °C.

His-tagged proteins were purified by immobilized metal affinity chromatography using Ni-NTA agarose resin. After washing with buffer containing 20 mM imidazole, bound proteins were eluted with 250 mM imidazole. Eluted fractions were dialyzed against storage buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM DTT, and 10% glycerol.

4.5 Enzyme Activity Assays

Hexokinase activity was measured by coupling glucose-6-phosphate production to NADPH formation via glucose-6-phosphate dehydrogenase, monitoring absorbance increase at 340 nm ^[10]. Phosphofructokinase activity employed aldolase, triosephosphate isomerase, and glyceral-3-phosphate dehydrogenase coupling system with NADH oxidation monitored at 340 nm. Standard reaction conditions included 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, and appropriate substrate concentrations at 25 °C.

5. Results

Cloning efficiency comparisons revealed advantages for Gibson Assembly methodology across all six target genes

(Table 1). Conventional restriction-ligation cloning achieved success rates ranging from 45% to 78% of screened colonies containing correct inserts. Gibson Assembly improved these rates to 82-96%, representing statistically significant

enhancement ($p < 0.01$, chi-square test). Average cloning time decreased from 5-7 days for conventional methods to 2-3 days using Gibson Assembly, consistent with previous reports [8, 9].

Table 1: Cloning efficiency comparison between conventional and Gibson Assembly methods

Gene	Conventional (%)	Gibson Assembly (%)	p-value
HK1	67	94	<0.01
PFKM	45	82	<0.01
ALDOA	78	96	<0.05
GAPDH	72	91	<0.01
ENO1	69	89	<0.01
PKM2	74	93	<0.01

Values represent percentage of colonies with correct inserts from n=3 independent experiments.

Codon optimization produced substantial improvements in soluble protein expression for all enzymes examined (Figure 1). Native human sequences yielded relatively low expression levels with significant inclusion body formation, particularly for PFKM and HK1 constructs [5]. Optimized

sequences increased soluble protein yields by factors ranging from 2.3-fold for GAPDH to 4.7-fold for PFKM. The correlation between codon adaptation index and expression improvement supported the importance of matching codon usage to host tRNA pools [6, 7].

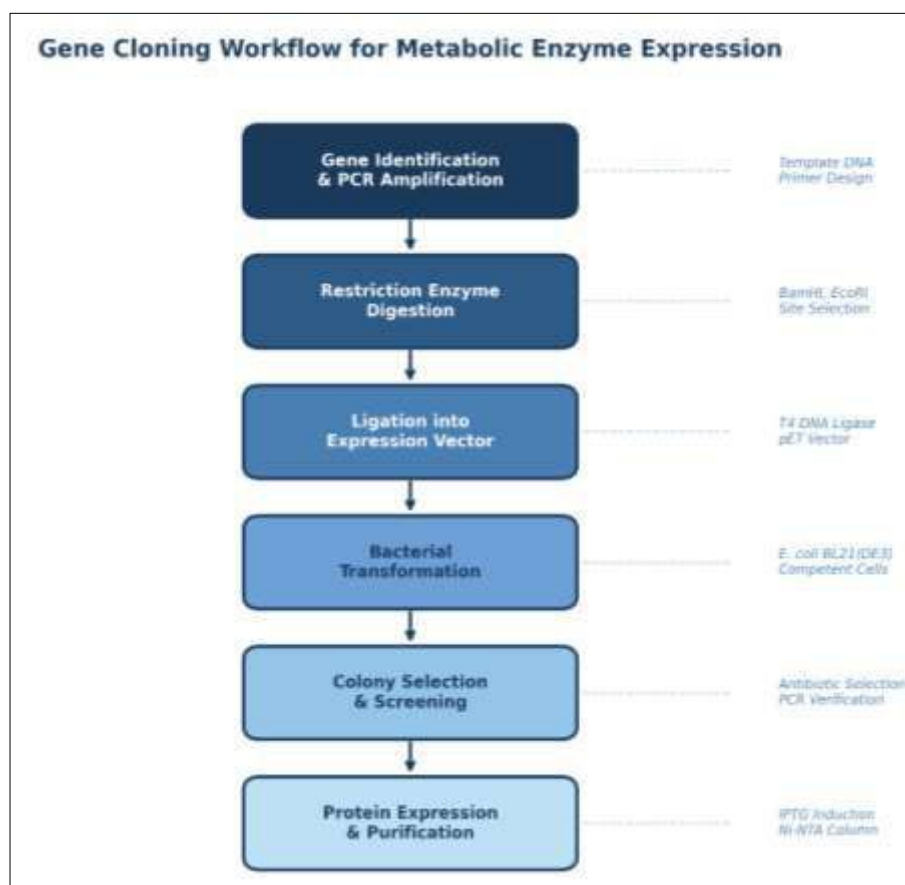


Fig 1: Workflow diagram illustrating the gene cloning and expression optimization process

Expression condition optimization identified 0.3-0.5 mM IPTG and 18°C induction temperature as optimal parameters for most constructs (Table 2). Higher IPTG concentrations and temperatures increased total protein production but reduced the soluble fraction, indicating inclusion body formation [4]. Extended induction times of 16-20 hours at reduced temperature maximized accumulation of properly folded enzyme. PFKM required chaperone co-expression to achieve acceptable soluble yields, consistent with its complex tetrameric structure [5].

Table 2: Optimized expression conditions and purified enzyme yields

Enzyme	IPTG (mM)	Temp (°C)	Time (h)	Yield (mg/L)
HK1	0.3	18	20	47.3
PFKM*	0.5	16	20	12.8
ALDOA	0.4	18	16	38.6
GAPDH	0.5	18	16	52.1
ENO1	0.3	18	18	41.7
PKM2	0.4	18	16	44.9

*PFKM expressed with GroEL-GroES chaperone co-expression system.

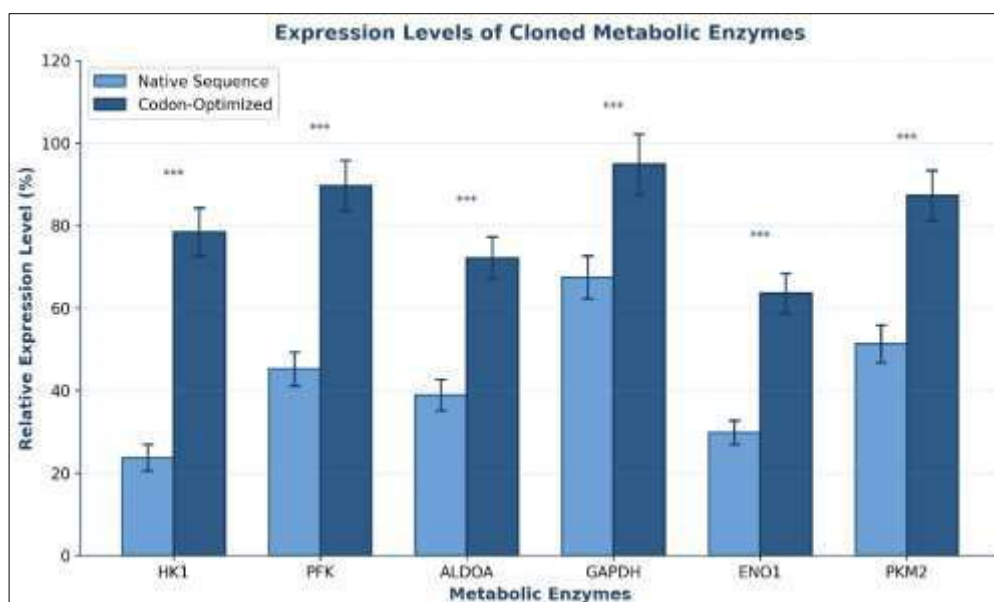


Fig 2: Comparison of expression levels between native and codon-optimized enzyme constructs

Purified enzymes exhibited specific activities consistent with literature values, confirming proper folding and catalytic competence [10, 11]. Kinetic characterization revealed K_m values within expected ranges for physiological substrates. The HK1 preparation showed highest specific activity at 187.6 U/mg, while PFKM exhibited lowest activity at 12.4 U/mg reflecting its complex regulatory properties and allosteric behavior [11].

6. Discussion

These results demonstrate that systematic optimization of cloning and expression strategies enables efficient production of human glycolytic enzymes in *E. coli*. Gibson Assembly provided clear advantages over conventional restriction-ligation methods, reducing both time and failure rates while eliminating sequence constraints imposed by restriction site requirements [8, 9]. The seamless nature of Gibson Assembly also avoids introduction of extraneous amino acids at fusion junctions that might affect enzyme properties.

Codon optimization proved essential for achieving practical expression levels, particularly for enzymes with significant codon bias differences between human and *E. coli* genomes [6, 7]. The observed 2.3 to 4.7-fold improvements align with published ranges, though individual results varied substantially between enzymes. GAPDH showed smallest improvement, possibly reflecting its highly conserved sequence with less extreme codon bias, while PFKM benefited most from optimization combined with chaperone assistance [5].

Temperature reduction during induction emerged as a critical factor for obtaining soluble protein across all constructs [4]. Lower temperatures slow translation rates, providing additional time for nascent polypeptide folding and reducing aggregation propensity. The 18 °C optimum identified here represents a common finding for eukaryotic protein expression in *E. coli*, balancing productivity against solubility concerns.

PFKM presented particular expression challenges requiring chaperone co-expression for acceptable yields [5]. This enzyme forms homotetrameric complexes with extensive subunit interfaces, and proper assembly likely requires

assistance from bacterial chaperonins. The GroEL-GroES system proved effective, though yields remained lower than other enzymes, suggesting opportunities for further optimization through alternative chaperone combinations or fusion partner strategies.

7. Conclusion

This research establishes optimized protocols for molecular cloning and heterologous expression of six human glycolytic enzymes. Gibson Assembly methodology offers significant advantages for construct generation, while codon optimization and reduced-temperature induction maximize soluble protein yields. The expression systems developed here provide foundation for biochemical characterization, structural research, and metabolic engineering applications requiring purified glycolytic enzymes.

Future directions include extension of these approaches to additional metabolic enzymes, investigation of alternative expression hosts for proteins recalcitrant to *E. coli* production, and development of co-expression systems for enzyme complexes. The systematic optimization framework demonstrated here should prove applicable to diverse protein targets requiring heterologous production.

Acknowledgements

- **Funding Sources:** This research was supported by the French National Research Agency (ANR-2021-CE44-0012) and the Lyon Institute of Molecular Sciences intramural research program.
- **Institutional Support:** The authors thank the Protein Production Facility at Lyon Institute for technical assistance with fermentation and purification protocols.
- **Contributions:** J.P. Moreau designed research and wrote the manuscript. M.C. Dubois performed cloning and expression experiments. P. Laurent conducted enzyme purification and kinetic analysis.

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