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ORIGINAL ARTICLE

Optimization and Characterization of the Depolymerase Gene from Fusarium Perfringens Expression in Pichia Pastoris

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Abstract: poly (butylene succinate) is a biodegradable plastic that can replace traditional plastics, which is helpful to solve the problem of white pollution, the key to the rapid and effective degradation of microorganisms or depolymerization Enzymes.The recombinant Pichia pastoris expression system was constructed to realize (FSC)EFFICIENT EXPRESSION.Codon Optimization Based on Pichia pastoris preferenceFSCGene codons of Pichia PastorisX33Achieving its recombinant expression in.The fermentation conditions of the strain were optimized by single factor experiments..The results show that the optimizedFSCIn gene sequence157One base changed,G + CContent59.6%Drop48.3%, Sequence homology is77.34%;Constructed recombinant expression vectorPpiczAlpha-FSCTransferred to Pichia PastorisX33, Combined with resistance plate screening,SDS-PAGEAndWestern BoltAnd the enzyme activity of shake flask fermentation was determined.1.Recombinant strain with high enzyme yieldL1;Further determine its fermentation conditions:Medium InitiationPH 6.0, Shaker speed220 r/minMethanol supplement1%, Inoculation amount8%Training time72 h, Incubation temperature30Under the optimized conditions, the enzyme activity of fermentation broth was up110 U/ml.

Keywords: Fusarium perfringens; Poly (butylene succinate); depolymerization enzyme; Pichia Pastoris

The widespread use of plastic products has led to a large consumption of petroleum resources, and the growing plastic waste is causing an inevitable[1.],

Environmental pollution, especially the problem of environmental pollution..The stability of traditional plastics makes it difficult to use chemical,Physical and biological degradation, which led to"White Pollution".Aliphatic polyester is currently regarded as the most promising biodegradable plastic polymer material that can replace traditional plastics. It can be easily accepted by the environment due to the decline of microbial role in the environment.[2]

And eventually converted into a harmless substance and returned to nature..Poly (butylene succinate (Poly(Butylene Succinate),PBS)Is its typical representative.Currently,PBSThe related researches mainly focus on its synthesis and modification.PBSAfter the product is modified or made, the degradation process is often slow in nature and vulnerable to environmental impact..So in progressPBSMaterials synthesis and Modification Research at the same time, aboutPBSThe biological degradation.Generally speaking, the microbial extracellular enzyme degradation mechanism is[3]

One of the important degradation mechanisms of biodegradable plastics.MaedaWait, fromAspergullus oryzaeThe fermentation broth was separated into a molecular weight21..6 kDaOfPBSDepolymerization EnzymePBSThe affinity of depolymerization enzyme to substrate is affected by the length of Carboxylic Acid carbon chain in Substrate.UCHIDA[4]

Wait for cloningAcidovorax delafieldii BS-3AIn the strainPBSDegrading enzyme genes, and inEscherichia coliMedium effective expression.The degrading enzyme belongs to esterase, and originated fromStreptomyces sp.

Lus amylolyticus TB-13OfPlaADepolymerase also hasPBSThe degradation ability andBacillus thermophilusIn Esterase

Ozyma AntarcticaIn Separation Purification with degradationPBSClass polymer ability of nearly horny EnzymePAEBy198A amino acid composition and horny enzyme have61%~68%Of Similarity.This study to research group after screening get of hasPBSDegradation Ability of skin Fusarium (Fusarium solani)Strain[9]And identification itsPBSDepolymerase has horny Enzyme Activity.According to, red yeast Codon Preference OptimizationPBSDepolymerase Gene construction expression vector in Pichia pastoris Fermentation[10]

Mother in implementation secretion expression And of its shake bottle of Fermentation Conditions

The preliminary Optimization.Related Research will help to achievePBSFor representative of biological biodegradable plastic of effective biological degradation andPBSDegradation EnzymePBSWaste follow-up processing and use of research lay the foundation.

1. Material and Methods

1.1 Material

1.1.1 Strain PUCm-T,PPICZαAndP. Pastoris

X33For laboratory save.

1.1.2 MediumYPD,BMGYAndBMMY MEDIUMMedium were in accordanceInvitrogenThe companyMulti-copy Pichia Expression KitOperation manual preparation.

1.1.3 Reagent and instrument Restriction Enzyme,T4 DNALigase,DNA Ladder Marker,ProteinMarker,Hydrochloride bleomycin (Zeocin),BCAProtein detection kit,Yeast genomeDNARapid extraction kit,TCAPrecipitation Kit,6 × hisAntibody were purchased from Sangon biological engineering (Shanghai) Co., limited the company.

BASE,Yeast Extract,Peptone were purchased self-, Beijing, ding guo prosperity biological technology limited responsibility the company;HisLabel protein purification pre-installed column purchase self-kings rui sheng wu Science and Technology Limited the company;PBS(Number of were molecular weight

160 000Acid alcohol1.1 1)Purchase from Anhui Anqing hexing chemical limited the company.PBSEmulsion based on the literature [10] Preparation.

VE180Micro-vertical electrophoresis Tank,HE120Multi-function level electrophoresis Tank,EPS300Electrophoresis apparatus,2500RGel imaging analysis system Shanghai, day science and technology limited the company;KTA purifier 10Protein Purification System,GE HealthcareThe company;DNP-9052Electric Constant Temperature Incubator,ZDP-250Constant Temperature training oscillator Shanghai, jing hong Experimental Equipment limited the company;BCN-1360BStyle Biological Clean workbench, Beijing, East Hal Device Manufacturing Limited the company;UV-2600UV visible spectrophotometer real Colonia(Shanghai)Instrument limited the company;Sci-entz-2CGene gene pulser transfection apparatus Ningbo xinzhi biological science and technology shares limited the company;2720StylePCRAmplification instrument American application Biological System

(ABI)The company.

1.2 Methods

1.2.1 PBSDepolymerase GeneFSCOf re-design and optimization

F.SolaniInPBSDepolymerase gene sequence(NCBI Accession Number:GI | 2493916)For basic reference, red

Yeast preference codon in don't change gene amino acid sequence of premise. On gene sequence the re-design and arrangement optimization after the gene5'End and3'End respectively joinECORI,Not IEnzyme cut site and will its namedFSC(Fusarium solani Cutinase)Gene.Finally according to re-arrangement of sequence by Sangon biological engineering (Shanghai) shares limited the company the all sequence Gene Synthesis synthesis of gene to

connectPUCm-TCarrierPUCm-T-FSC.1.2.2 FSCGene yeast expression vector of ConstructionWillPUCm-T-FSCAnd expression carrierPPICZαRespectively"ECORIAndNot IDouble Enzyme cut recovery enzyme cut after the objective geneFSCAnd carrier FragmentPPICZαWillFSCGene and load body fragmentPPICZαLinked, identified by enzyme digestionPpiczAlpha-FSCRecombinant Expression Vector.1..2..3 Competent Pichia PastorisX33Preparation of pickP.Pastoris x33Single colony, access25 ml YPDMedium (250 MLTriangle bottle),30,220 r/minOscillation Cultivation18 h;Take2 mLCultures, access100 YPDMedium (500Triangle bottle),30,220 r/minOscillatory incubationOd_{600}For1..3.1..5.;3 000 r/minCentrifugal (4.)5 minCollect bacteria, add20 mlPre-cooling aseptic water, shaking suspension of bacteria;Above centrifugal, plus10 mlPre-cooling aseptic water, shaking suspension of bacteria;Above centrifugal, plus10 mlPrecooling1 mol/LSterile sorbitol solution, shaking and resuspting the cell;Above centrifugal, add0.5 mLPrecooling1 mol/LSterile sorbitol solution, suspension of cell;80μL/Pipe distribution for use or 80Preservation.

1..2..4 FSCIdentification of transformed Pichia Pastoris and its positive transformants

Sac IRestriction Enzyme LinearizationPpiczAlpha-FSCPlasmid

After, take20μLLinearized plasmid Addition80μL x33Competent Cells and gently mix, transfer2mmPre-cooling power cup, ice placed5 minElectric shock, voltage1 500 V, Shock time4..2 MS, Immediately after the electric shock is completed, add pre-cooling1 mol/LSorbitol1 mLMix,30Static1.~2 h, Every

μLRespectively coated with differentZeocinConcentrationYPDMedium

Tablet.Selection from high antibiotic concentrations TabletsPpiczAlpha-FSCPositive transformants labeledL1~L11, Cultivate and use the yeast genomeDNARapid extraction kit for genomic ExtractionDNA, UsePpiczAlphaVector Universal Primer5'aoxPrimer sequence:

GactgttccaattgacaagcAnd3'aoxPrimer sequence:Ggatgtcagaatgccatttgc, Carry onPCRAmplification, testing the correct target positive trans for mants.Adopted15μLStandard PCRA mplification System. PCRProgram: 94Pre-Degeneration 5 min 94 Degeneration30 s 52 Annealing45 s72Extension2 min

Cycle30Times;72 Extension10 min;4 Save.

Load strain (Negative control)Were cultured in5 ML YPDLiquid Medium,30,220 r/minTraining12 h.Respectively take200MuLBacteria inoculation inBMGYMedium (PH 6.0)30,220 r/minTrainingOD_{600}For4.5About,3 000 r/minCentrifugal5 minIntoBMMY MEDIUMMedium (PH 6.0)Every12 hJoin0.5%Methanol the induced,30,220 r/minInduced Expression

72 H.Shake bottle of fermentation end after take fermentation broth centrifugal harvest Supernatant,

200MuLSupernatantTCAPrecipitation kit precipitation protein take protein heavy SuspensionNatural 20MuLTheSDS-PAGE.Further

6 × HisAntibody A anti-Western blotDetection.Another supernatant for enzyme activity determination and protein concentration and further basis protein concentration determine than vitality.

1.2.6 Enzyme Activity and protein concentration determination take supernatant (crude enzyme liquid)1 mLJoin3 mLBy40PreheatingPBSEmulsion (0.1%Volume fraction)In rapid mixing,630 nmThe measurement its absorption value (OD_1)InsulationNatural 20 minAfter measurement its absorbance value (OD_2).To boiling crude enzyme liquid for control.Enzyme Activity Unit

Definition:A enzyme activity unit (U)For everyMinThe light absorption value

WhichOd_1.For the initial absorbance value of the reaction,Od_2.For the absorbance value at the end of the reaction,T(Min)For reaction time,V(ML)Liquid Product for crude enzyme.

1..2..7 Preliminary Optimization of fermentation induction conditions of recombinant enzyme in shake flask Bacteria

Body Culture and induction1..2..5..To medium initialPH,

Shaker speed,Effects of methanol addition and inoculation amount on the Expression of Recombinant Proteins.

Code usage frequency (Table1.)In order to effectively express the target gene in Pichia pastoris

Ala),Ggg(Gly),CTC(LEU),CCG(Pro),CGA

ARG),CGG(ARG),CGC(ARG),AGC(Ser),TCG

Ser)AndACG(THR)Used in Pichia pastoris at a low frequency, while in naturalFSCIn genes, the frequency at which these codons are used

Note:P.PastorisSaid from websiteHTTP:/WWW.Kazusa.Or.Jp/codonOf, red yeast Codon Preference table

2.2 PPICZα-FSCExpression Vector of Construction

Objective GeneFSCAnd carrier FragmentPPICZαConnection after get the recombinant expression vectorPPICZα-FSC.Plasmid of construction results as shown in Figure1Shown in.PPICZα-FSCBySac ILinearization of agarose electrophoresis detection results as shown in Figure2Shown in.By figure2Visible linearization fragment about4 300 bp.WhichPPICZαCarrier3 593 BPAnd objective gene711 bp(With6His tag)Both the sum4 304 BPAnd detection results basic same (recombinant expression vector has been fully achieved the linearization can the follow-up transformation experiment.

2 ppiczα-FSCRecombinant Expression VectorSac IEnzymatic Hydrolysis verify

M:DNA ladder(BP):Natural 20 000,10 000,7/000,5 000,4 000,3000,2 000,1 500,1 000, 750, 500, 400, 300, 200, 75;1:Not linearization carrier;2:SACIEnzyme cut Linearization PPICZα-FSC M:DNA ladder(BP):20 000,10 000,7 000,5 000,4 000,3 000,2 000,1 500,1 000,750,500,400,300,200,75;1.:PpiczAlpha-FSC;2.:Digeston of ppiczAlpha-FSC by SAC I

2.3 Construction and screening of recombinant Pichia Pastoris

Sac ILinearizedPpiczAlpha-FSCExpression Vector ElectroporationP.Pastoris x33After the competent cells, the conversion liquid is coatedZeocinOfYPDMedium,30,220 r/minCulture72 hResults As shown in Figure3Shown in.By figure3The recombinant bacteria in Experimental"ZeocinConcentrationYPDFlat on the can be growth.From the Most High ConcentrationZeocinOfYPDMedium on the selected transformation sub-colony extraction genomeDNAAfterAOXGeneral primers amplification electrophoresis results as shown in Figure4Shown in.Transformation sub-L1~L11Of genome and positive of control were only amplification1A strip for objective gene strip (About711 bp)And carrier onAOX1Part sequence (About546 BP)Of sum about1 257 BPSo,L1~L11Are positive transformation sub-.The results show that linearization PlasmidPPICZα-FSCHas success integrationP.Pastoris X33Genome.

2.4 RecombinantPBSDepolymerase of secretion expression Identification

By figure5Visible in Molecular Weight24 kDAboutL1~L11Were1Article expression is high protein bands.To6HisAntibody A anti-Western blotDetection this high expression protein whether for objective protein results see Figure6.By figure6The the protein, which is for objective protein further the transformation Zi Chan enzyme of Enzyme Activity

The analysis compare the results as shown in Figure7/Shown in.By figure7/The,11 PBSDepolymerase activity whichL1Strain of enzyme activity and than vitality

Of recombinant strain of Induced by fermentation broth of centrifugal supernatant in have Are the highest follow-up selectedL1Strain for further study object.

2.5 Shake bottle of Fermentation ProductionPBSDepolymerase of Conditions Optimization

To selected of recombinant strainL1For study object on its main fermentation conditions were investigated to improvePBSDepolymerase expression results as shown in Figure8Shown in.Figure8For fermentation medium initialPHThe strain Enzyme Production Ability of influence results display with the medium initialPHOf increase,PBSDepolymerase vitality was first rise after decreased of trend and inPHFor6.0When has the highest enzyme activity.Figure8 BFor shaker speedL1Strain Enzyme Production Ability of influence with the shaker speed of increase enzyme activity was rise trend in

To220 r/minAfter again continue to increase speed enzyme activity of increase variable slow therefore select220 r/min.PPICZαInduced Expression Vector in containingAOX1Promoter so methanol of add can be[14-15]

Promote recombinant protein of expression figure8 CFor medium initialPH 6.0And shaker speed220 r/minConditions under methanol supplement of recombinant bacteriaL1The condition of enzyme production of the Influence.Methanol supplement on Enzyme Activity of influence was first rise after decreased of trend methanol supplement1%When recombinant bacteriaL1Has is high Enzyme Production Ability.By figure8 dThe inoculation amount of recombinant bacteriaL1Enzyme Production Of the activity with the inoculation amount of increase and rise inoculation amount Increase so the inoculation amount8%.This conditions under fermentation on To8%After continue to increase inoculation production enzyme activity no obvious Clear Liquid enzyme activity110 U/mL.

3 Please On

This research use, red yeast expression system implementation the skin Fusarium poly succinic acid succinic ester depolymerase Gene(FSC)The secretion expression and further the influence recombinant bacteria Enzyme Production Ability of key parameters the Optimization.Follow-up to further implementation recombinant bacteria of batch fermentation and further optimization enzyme of purification technology to implementation recombinant enzyme of Scale Production.Related Research will help to achievePBSFor representative of biological biodegradable plastic of effective biological degradation and implementationPBSBiological biodegradable plastic waste of follow-up processing and use.

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