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OPTIMAL CONDITIONS OF FERMENTATION OF THE *PICHIA PASTORIS* YEAST EXPRESSING THE RECOMBINANT PRES2-S PROTEIN (M-HBsAg) OF HEPATITIS B VIRUS (HBV)

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The aim of this study is determination of optimal fermentation conditions of the *Pichia pastoris* yeast expressing the recombinant Pres2-S protein of hepatitis B virus (HBV). For this purpose we investigated the main factors influencing the cultivation of recombinant strain of the yeast *Pichia pastoris* pPIC3.5-S-HBsAg - the composition of the nutrient medium, pH and dissolved oxygen (DO). As a result, by enzyme-linked immunosorbent assay (ELISA) the optimal concentration of methanol $\approx 1.0\%$, which is the initiator and the only source of carbon for protein expression in the *Mut⁺* phenotype *Pichia pastoris* yeast cells was determined. In the selected conditions at the end of the fermentation process, the wet biomass of yeast cells was 420 g/l.

Keywords: pPIC3.5-Pres2-S, fermentation, *Pichia pastoris*, GS115, recombinant protein, expression, HBV

ОПТИМАЛЬНЫЕ УСЛОВИЯ ФЕРМЕНТАЦИИ ДРОЖЖЕЙ *PICHIA PASTORIS*, ЭКСПРЕССИРУЮЩИХ РЕКОМБИНАНТНЫЙ БЕЛОК PRES2-S (M-HBsAg) ПОВЕРХНОСТНОГО АНТИГЕНА ВИРУСА ГЕПАТИТА В (HBV)

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Целью данной работы является определение оптимальных условий ферментации дрожжей *Pichia pastoris*, экспрессирующих рекомбинантный белок Pres2-S вируса гепатита В (HBV). Для этого исследовали основные факторы влияющие на выращивание в ферментере рекомбинантного штамма дрожжей *Pichia pastoris* pPIC3.5-S-HBsAg - состав питательной среды, показатели pH и растворенного кислорода (DO). В результате с помощью твердофазного иммуноферментного анализа (ИФА) было определено, что оптимальная концентрация метанола, который является инициатором и единственным источником углерода в экспрессии белков в рекомбинантных клетках дрожжей *Pichia pastoris* фенотипа *Mut⁺* составляет 1.0%. В конце процесса ферментации в подобранных оптимальных условиях количество влажной биомассы клеток дрожжей составляло 420 г/л.

Ключевые слова: pPIC3.5-Pres2-S, ферментация, *Pichia pastoris*, GS115, рекомбинантный белок, экспрессия, HBV

GEPATIT B VIRUSI (HBV) REKOMBINANT PRES2-S (M-HBsAg) OQSILINI EKSPRESSIYALAYDIGAN *PICHIA PASTORIS* ACHITQI SHTAMMI FERMENTATSIYA JARAYONINING OPTIMAL SHART SHAROITLARI

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Mazkur tadqiqot ishining maqsadi Gepatit B virusi (HBV) rekombinant Pres2-S oqsilini ekspressiyalaydigan *Pichia pastoris* achitqi shtammini fermenterda ko'paytirish jarayonining optimal shart-sharoitlarini aniqlashdan iborat bo'ldi. Shu maqsadda rekombinant *Pichia pastoris* pPIC3.5-S-HBsAg achitqi shtammini ko'paytirishga ta'sir etuvchi asosiy omillar - ozuqa muhitining tarkibi, pH va erigan kislorod (DO) ko'rsatkichlari tadqiq etildi. Tajribalarda olingan natijalar *Mut⁺* fenotipiga mansub rekombinant *Pichia pastoris* achitqi hujayralari uchun fermenterdagi oqsil ekspressiyasining asosiy induktori va yagona uglerod manbai bo'lgan metanolning maqbul konsentratsiyasi qattiq fazali immunoferment tahliliga (IFA) ko'ra 1.0% ni tashkil etishini ko'rsatdi. Natijada, tanlab olingan sharoitdagi fermentatsiya jarayonining oxirida achitqi hujayralari xo'l biomassasining miqdori 420 g/l ni tashkil etishiga erishildi.

Kalit so'zlar: pPIC3.5-Pres2-S, fermentatsiya, *Pichia pastoris*, GS115, rekombinant oqsil, ekspressiya, HBV

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Kirish

Pichia pastoris ekspressiya tizimiga bugungi kunda rekombinant oqsillar olish uchun eng progressiv tizimlardan biri sifatida qaralmoqda. *Pichia pastoris* – asosiy uglerod manbai sifatida metanoldan foydalanadigan metilotrofik achitqidir [1,2]. Arzon, tuzli ozuqa muhitida yuqori zichlikda o'sa oluvchi *Pichia pastoris* achitqilari (400-450 g/l gacha) va rekombinant oqsillarni yuqori unum bilan sintez qilish qobiliyati (20 mg/l dan 14.8-30 g/l gacha) *Pichia pastoris* ekspressiya tizimida biologik faol oqsillar, fermentlar, antitanalar, sitokinlar, gormonlar va boshqa ko'p turdagi geterogen oqsillarni yuqori unum bilan sanoat miqyosida ishlab chiqarishga imkoniyat yaratadi. Hozirgi vaqtda ushbu ekspressiya tizimida olingan rekombinant oqsil preparatlarining xavfsizligi tasdiqlangan bo'lib farmatsevtika bozoriga keng tavsiya etilmoqda [1-3]. Odam gepatit V virusi (HBV) sog'liqni saqlash sohasidagi eng dolzarb muammolardan biri bo'lib, dunyoda u bilan

zararlangan 329 mln dan o'triq odam aniqlangan va har yili 1,4 mln inson ushbu kasallik asoratlari tufayli vafot etmoqda [4-6]. Unga qarshi kurashning asosiy chorasi bu vaksina bilan emlashdir. Hozirgi vaqtda jahonda gen muhandisligi usullari yordamida HBV DNK ning S regionini asosida rekombinant HBsAg oqsili olingan bo'lib, gepatit B virusiga qarshi tijorat vaksinalari sifatida qo'llanilib kelinmoqda. Ushbu oqsil (226 aminokislota qoldig'idan iborat) gepatit B virusining barcha genotiplariga xos bo'lgan faqat 1 ta umumiy immunogen "a" determinanta saqlaydi va shu sababli virusga nisbatan to'laqonli himoya vazifasini bajara olmaydi [7,8]. Ma'lumki virus genomining Pres1 (108 a.q.) va Pres2 (55 a.q.) regionlari kodlaydigan antigen strukturalari uning tashqi yuzasining yuqori immunogen xossasiga ega bo'lgan qismi hisoblanadi. HBV yuza oqsilining Pres2-S (Pres2 + S) regionini (281 aminokislota qoldig'idan iborat) polialbumin-bog'lovchi faollikka ega qismi bo'lib, oxirgi yillarda olib borilgan

1-jadval
PTM1 va BSM tuzli ozuqa muhitlarining tarkibi

PTM1	
Tarkibi	Miqdori (g/l)
CuSO ₄ ·5H ₂ O	6,0
NaI	0,08
MnSO ₄ ·H ₂ O	3,0
Na ₂ MoO ₄ ·2H ₂ O	0,20
H ₃ BO ₃	0,02
CoCl ₂ ·6H ₂ O	0,50
ZnCl ₂	20,0
FeSO ₄ ·7H ₂ O	65,0
H ₂ SO ₄	5,0 mkl
Biotin	0,2
BSM	
CaSO ₄	0,93
MgSO ₄ ·7H ₂ O	14,9
K ₂ SO ₄	36,4
KOH	4,13
Glitserin	50
H ₃ PO ₄ kons.	26,5
Ko'pikka qarshi agent	0.1%

tadqiqotlar vaktsina tarkibida Pres2-S oqsilining mavjudligi uning immunogenlik xossasini tubdan oshishiga sabab bo'lishini ko'rsatdi [9-11]. Ayni vaqtda gepatit B virusi (HBV) yuza oqsillari asosidagi bir necha turdagi vaktsinalar ayrim rivojlangan mamlakatlarda achitqi ekspressiya tizimlaridan foydalangan holda ishlab chiqarilmoqda [12-14]. Ma'lumki olinayotgan rekombinant oqsillarning miqdori shtamm-produsentlarni fermenterda ko'paytirish jarayoniga uzviy bog'liq bo'lib, maqbul sharoitlarni tanlash asosida yuqori unum bilan tegishli oqsil sinteziga erishish mumkin [15-20]. Mazkur tadqiqot ishida rekombinant Pres2-S oqsilini ekspressiyalaydigan *Pichia pastoris* achitqi shtammlarini fermenterda ko'paytirishning optimal shart-sharoitlari o'rganildi.

Tadqiqot usullari

Tadqiqot ishida rekombinant Pres2-S oqsilini ekspressiyalaydigan pPIC3.5-Pres2-S *Pichia pastoris* GS115 shtammidan (O'MKI Molekulyar genetika laboratoriyasi kolleksiya); bioreaktor/ fermenter BioFlo/CelliGen 115 (New Brunswick, AQSh) qurilmasi; YPD, YPD Agar, Yeast Nitrogen Base (YNB); MGY, BMGY, PTM1, Basal salt (BS) tuzli ozuqa muhitlaridan; ko'pikka qarshi agent (HIMEDIA); fermenterni kislorod bilan ta'minlash uchun havo kompressori va kislorod ballonlaridan foydalanildi.

Pichia pastoris hujayrasidan Pres2-S oqsilini ajratish. Ko'paytirilayotgan hujayra biomassasidan 500 mkl hajmda olinib 10 min, 12000 ay/min sentrifuga qilinadi va breaking buffer (BB) (50 mM natriy fosfat, pH=7.4, 1 mM PMSF, 1 mM EDTA, 5% glitserin) bilan bir marta yuvib olinadi va yana cho'ktirilib 500 mkl BB da eritiladi. Ustiga teng miqdorda shisha sharchalar (glass beads, dm=5 mm) qo'shiladi. 30 sek davomida vorteks qilinib, 30 sek muzli hammomda ushlanadi va shu tariqa 10

marta takrorlanadi. So'ngra probirka 4°C, 12000 ay/min da 10 min davomida sentrifugalanadi. Ekstraksiya bo'lgan umumiy oqsil miqdori 5-10 mg/ml ni tashkil etadi. PTM1 va BSM tuzli ozuqa muhitlarining tarkibi quyidagi jadvalda keltirilgan.

Fermentatsiya jarayoni asosan 4 bosqichni o'z ichiga oladi: birinchi bosqich bu – kolbada hujayra kulturasi ma'lum zichlikkacha o'stirib olish (boshlang'ich inokulm) bosqichi hisoblanadi. Ikkinchi bosqich - fermenterda boshlanib hujayra biomassasini glitserinli muhitda ma'lum vaqt davomida ko'paytirishdan iboratdir. Uchinchi bosqich – kam vaqt oralig'ida (4-5 soat) 15 ml/soat/l miqdorida ozuqa muhitiga glitserin qo'shilishi orqali hujayralarning ma'lum zichligiga erishish va metanol metabolizmiga tayyorgarlik bosqichidir. To'rtinchi bosqich - induksiya bosqichi bo'lib, bunda biomassa kerakli genning initsiatori (boshlab beruvchi) va yagona uglerod manbai bo'lgan metanol bilan oziqlantirish bosqichidir. Oqsil ekspressiyasida uch xil fenotip (Mut⁺, Mut⁰ va Mut⁻) uchun uch bosqichli muhit strategiyasi amalga oshiriladi. Bizning tadqiqotimiz Mut⁺ fenotipi asosidagi jarayonni o'z ichiga oldi.

Boshlang'ich inokulum fermenterdagi kulturaning 5-10% ni tashkil etishi lozim. Buning uchun yakka holdagi Pres2-S genini saqlovchi *Pichia pastoris* GS115 shtammi minimal glitserin (MGY: 1% glitserin, 1.34% YNB, 2% pepton, 1% achitqi ekstrakti) yoki buferlangan minimal glitserin (BMGY: 100 mM kaliy fosfatli bufer, pH 6.0, 1% glitserin, 1.34% YNB, 2% pepton, 1% achitqi ekstrakti) ozuqa muhitlaridan biriga ekildi. Ekilgan rekombinant *P.pastoris* shtammi orbital silkitgichda (shaker) 30°C, 220–240 aylanmada, optik zichligi OD₆₀₀ 2-6 ga teng bo'lgunga qadar inkubatsiya qilinadi. Bu muhit keyingi fermentatsiya jarayoni uchun inokulum bilan ta'minlaydi.

Ikkinchi (glycerol batch) faza achitqi biomassasini oshirish uchun glitserinli ozuqa muhitidan iborat bo'lib, muhit tarkibidagi glitserinning miqdori 4% ni tashkil etdi. Glitserindan tashqari ozuqa muhit tarkibiga 1.34% YNB va BS tuzli muhitlar kiritildi. Dastlabki muhitning hajmi mos ravishda umumiy reaktor hajmining 65–75% yoki 45–60% ni tashkil etib, induksiya fazaga qadar bo'lgan jarayon taxminan 40–50 soatni tashkil etdi. Fermenterdagi DO (erigan kislorod) miqdori tashqaridan berilayotgan kislorod (yoki havo) va aylanish tezligi mutanosibligida fermenterning “cascade” (keyinchalik kaskad) funksiyasi orqali nazorat qilib turiladi va uning miqdori 20% dan oshib ketmasligi kerak.

Uchinchi (glycerol fed batch) fazada hujayralar glitserindan metanolli muhitga moslashishi uchun imkoniyat yaratildi. Buning uchun muhitga tarkibida 12 ml PTM1/l saqlovchi 50 % li glitserindan 18.25 ml/soat/l miqdorida qo'shib borildi. Jarayonning davomiyligi o'rtacha 4-5 soatni tashkil etdi. Berilayotgan havoning

2-jadval
Pichia pastoris fenotiplarning restriktaza fermentlariga bog'liqligi

Restriktaza fermenti	Integratsiya sohasi	Fenotip
Sal I va Stu I	his4 bo'yicha	His ⁺ Mut ⁺
Sac I	5' AOX1 qismi bo'yicha	His ⁺ Mut ⁺
Bgl II	AOX1 bo'yicha almashinish	His ⁺ Mut ^S

3- jadval
 Pres2-S rekombinant oqsil miqdorining ozuqa muhitidagi metanol konsentratsiyasiga bog'liq holda o'zgarishi

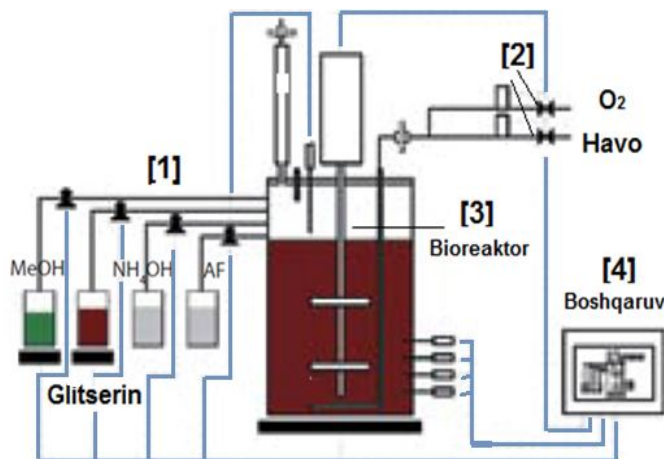
Fenotip turi	Metanol konsentratsiyasi	IFA test tahlili natijalari (OD=450 nm)
Mut ⁺	0,5%	1,0
	1,0%	1,21
	1,5%	0,92
	2,0%	0,47

umumiy oqimi 1-1.25 vvm da ushlangani maqsadga muvofiq. Aylanish tezligi o'rtacha 800-1200 ay/min oralig'ida ushlab turildi.

To'rtinchi(methanol fed batch) metanol bilan to'yintirish yoki induksiya fazasida, muhitga metanol berishni boshlanishidan oldin glitserinning ozuqa muhiti tarkibida tugaganiga iqrar bo'lish maqsadida DO ning 30% dan 100% gacha ko'tarilishi nazorat qilindi. Bunga glitserin nasosi va DO kaskad boshqaruvini o'chirish orqali erishildi. Mut⁺ shtammlarida DO ni yana stabil 30% ga qaytarish uchun 1 dan 3 soatgacha vaqt talab etildi. Shundan so'ng muhitga metanol berish boshlandi va uning miqdori 1-2 % ni tashkil etdi.

Natijalar va muhokama

Pichia Expression Kit (Invitrogen, USA) [21] ma'lumotlariga ko'ra rekombinant plazmidalarni chiziqsimon holga keltirishda ishlatilgan restriktaza fermentlarining turiga qarab Mut⁺ va Mut^S kabi fenotiplar olinadi (2-jadval).



1-rasm. Fermentatsiya jarayonining sxematik ko'rinishi. Bunda [1] turli reagentlarni reaktorga etkazib berish, [2] kislorod yoki havo bilan ta'minlash, [3] bioreaktor, aralastirish, [4] jarayonni avtomatik boshqaruv qurilmasidir.

Biz *P. pastoris*ning Mut⁺ fenotipini olish maqsadida pPIC3.5-Pres2-S rekombinant plazmidalarini *SacI* restriktaza fermenti bilan kesib chiziqsimon holga keltirdik va uni *P. pastoris* hujayrasiga elektroporatsiya usuli orqali kiritdik. Mazkur ferment bilan ishlov berish orqali asosiy uglerod manbai va maqsadli genning initsiatori bo'lgan metanolni yuqori darajada utilizatsiya xususiyatli Mut⁺ fenotipi hosil bo'ldi.

Rekombinant oqsil ekspressiyasi tegishli achitqi shtammini fermenterga yuklanadigan umumiy hajm miqdorining 5 % nisbatida hisoblanib, maxsus botiq kolbaga ekilishi bilan boshlandi. Fermenterga 5 litr hajmda 4 % glitserin tutuvchi BS tuzli muhiti yuklandi. Muhitning pH ko'rsatkichi 25% ammiak bilan 6.0 ga to'g'irlandi. So'ngra fermenterga optik zichligi OD₆₀₀ 5.0 gacha o'stirilgan hujayra kulturasi solindi. Fermenterning aylanish tezligi 600 ay/min, eruvchan kislorodning miqdori esa 25 % dan yuqori bo'lishi ta'minlandi. Vaqt o'tishi bilan hujayralarning ko'payishi sababli ularning kislorodga bo'lgan talabi ham ortib boradi buning natijasida eruvchan kislorodning (DO) miqdori 25 % dan pasayishi kuzatiladi [11]. Bunday hollarda kislorod miqdorini oshirish uchun fermenterning aylanish tezligi va berilayotgan havoning miqdorlari oshirib borilish talab etiladi. Mazkur bosqich DO ko'rsatkichi 100% bo'lgunga qadar davom ettirildi va hujayra biomassasining optik zichligi OD₆₀₀ 120 g/l ni tashkil etdi. So'ngra tarkibida 12 ml PTM1/1 saqllovchi 50 % li glitserindan 18.25 ml/soat/1 miqdorda qo'shib borildi. Navbatdagi induksiya bosqichida muhitga tarkibida 12ml/l PTM1 saqllovchi 100 % metanoldan 3.5 ml/l/soat miqdorda 4 soat davomida qo'shildi. So'ngra mazkur miqdor 7.3 ml/l/soat ga oshirilib, jarayonning oxiriga qadar davom ettirildi. Fermentatsiya jarayoni borishining sxematik ko'rinishi 1-rasmda keltirilgan.

Biz o'z tadqiqodimizda metanolning har xil (0.5, 1.0, 1.5, 2.0%) miqdorining Pres2-S oqsilining



2-rasm. Rekombinant Pres2-S oqsilini ekspressiya qiluvchi *Pichia pastoris* achitqi hujayralarining fermentatsiya jarayonidan keyingi ko'rinishi.

ekspressiyasiga bo'lgan ta'sirini o'rgandik (2-jadval). Rekombinant oqsilning hujayradan ekstraksiyasi maxsus shisha sharchalar yordamida amalga oshirildi. Pres2-S oqsilining ekspressiya darajasini baholash uchun immunoferment (IFA) tahlilidan foydalanildi.

Mazkur jadvaldan Pres2-S oqsilining optimal ekspressiyasi uchun metanolning 1.0% miqdori eng maqbul bo'lganligini ko'rishimiz mumkin. Induksiya bosqichining oxirida ko'paytirilgan nam hujayralarning miqdori 420g/l ga yetdi. Bu shuni anglatadiki, fermenterdagi ozuqa muhitning deyarli yarmi hujayra biomassasidan iborat bo'ldi (3-rasm).

Xulosa

Rekombinant Pres2-S oqsilini ekspressiyalaydigan *Pichia pastoris* achitqi shtammini fermenterda ko'paytirishning maqbul sharoitlari aniqlandi. Olingan natijalarga ko'ra

rekombinant *Pichia pastoris* Mut⁺ fenotipiga mansub achitqi hujayralarini fermenterda o'stirish jarayonida uglerod manbai va oqsil ekspressiyasining induktori sifatida metanolning miqdori 1.0% ni tashkil etishi optimal ekanligi aniqlandi. Shuningek fermentatsiya jarayonining oxirida achitqi hujayralari biomassasining miqdori 420 g/l ozuqa muhitiga nisbatanni tashkil etishiga erishilib, bu miqdor adabiyotlarda keltirilgan ma'lumotlar bilan solishtirilganda ancha yuqori zichlikka erishilganini va jarayon samarali bo'lganligini ko'rsatadi.

Olingan natijalar rekombinant Pres2-S oqsilini sintezlaydigan *Pichia pastoris* achitqi shtammlarini fermenterda ko'paytirish va tegishli oqsildan kelajakda virusli Gepatit B ga qarshi zamonaviy vaksina yoki miqdoriy immunoferment tahlili uchun antigen sifatida foydalanish imkoniyatini beradi.

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