一株副溶血弧菌噬菌体生理特性的研究*

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摘要:对副溶血弧菌噬菌体的生理生化特性进行了测定,结果表明:噬菌体的最适 pH 值为 8,最适温度为 35℃,60℃以上高温下迅速失活;对紫外线敏感;对乙醚、氯仿有抗性。细菌的培养时期对裂解率的影响很大,对数早期的细菌很容易感染噬菌体,而老化的细菌抗噬菌体感染能力强。在培养基中添加 Ca^{2+} 或 Mg^{2+} 有利于噬菌体的吸附。在盐度为 20 的情况下,对副溶血弧菌的裂解能力最强。该噬菌体的最佳感染复数在 0.1 ~ 1.0 之间。

关键词:副溶血弧菌,噬菌体,裂解率,感染

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Study of the Biochemical Properties of the *Vibrio Parahaemolyticus* Bacterophages*

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Abstract: The biochemical properties of a *V. parahaemolyticus* bacterophage were investigated. The results show that when the bacterophage infects the *V. parahaemolyticus*, the optimum pH value is 8; the optimum temperature is 35℃. The bacterophage is inactivated quickly when treated with upper 60℃. Also the bacterophage is sensitive to ultraviolet radiation and resistant to aether and chloroform. The incubation period of the *V. parahaemolyticus* has great effects to the bacterophage's bacteriolysis proportion. In Log phase, the bacterophage infects the *V. parahaemolyticus* easily. However the aged *V. parahaemolyticus* is resistant to bacterophage's infection. It's favorable to the bacterophage attaching to the *V. parahaemolyticus* when Ca^{2+} or Mg^{2+} was added into the culture medium. The ability of lysis of the bacterophage is strongest when the salinity of the water is about 20. The best multiplicity of infection is 0.1 ~ 1.0.

Key words: *Vibrio parahaemolyticus*, Bacterophage, Bacteriolysis proportion, Infection

噬菌体广泛地存在于海洋环境中,电子显微镜研究结果表明,海水中噬菌体的数量可达 10^4 pfu/mL ~ 10^7 pfu/mL[1],海洋中的细菌,蓝细菌及真核微生物都发现有它们的噬菌体存在;它们对海洋环境中的细菌的群落、类型和数量等的变化产生重要的影响[2]。此外,噬菌体作为指示微生物,在监测海洋环境,指示污染程度等方面的研究也有了一些进展。因此,分离和研究海洋噬菌体,弄清噬菌体与其宿主之间的生态学关系,对于监测海洋环境,控制海洋污染等方面的工作无疑具有指导意义。

我们对采自湛江东风海产品市场的副溶血弧菌噬菌体的生理生化特性进行测定,这些研究数据为进一步将噬菌体裂解技术用于生产实践中副溶血弧菌的灭活研究奠定了基础。

1　材料与方法

1.1　菌种

副溶血弧菌(*Vibrio parahaemolyticus*)由患红体病凡纳滨对虾体内分离获得。副溶血弧菌噬菌体为本实验室分离并保存,浓度为 10^{10} pfu/mL。

1.2　副溶血弧菌噬菌体生理特性的检测

1.2.1　不同 pH 值对噬菌体裂解活性的影响:参照文献[3 ~ 5]的方法,将 pH 值分别为 3、4、5、6、7、8、9、10、11、12 的蛋白胨水溶液 4.5mL,加入口径一致

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的试管中，放在 25℃，当温度平衡后加入 0.5mL 效价为 10^4 pfu/mL ~ 10^5 pfu/mL 的噬菌体液，放在 25℃作用 1h 后，用 pH7.0 的 1% 海水蛋白胨水稀释 10 倍，取 0.1mL 用双平板法，做噬菌体记数，同时做 3 个平板，做为对照。培养 12h ~ 18h 后观察计数噬菌斑。取对照平皿最高的噬菌斑数目为 100%，其他值与其比较所得的百分比即为相对出斑率。

1.2.2 噬菌体最适培养温度的测定：取稀释好的约 10^3 pfu/mL 的噬菌体液 1mL 放于小试管中，于 10℃、20℃、25℃、30℃、35℃、40℃，待温度平衡后，各取 0.1mL 用双平板法做噬菌斑实验，每个温度条件设 3 个重复，然后重新将平板放于相应的温度下培养，12h ~ 18h 后观察结果。

1.2.3 噬菌体的热稳性实验：参照文献[4,6]的方法，调整噬菌体悬液浓度为 10^4 pfu/mL ~ 10^5 pfu/mL。

1.2.4 噬菌体的紫外线灭活实验：参照文献[5,8,7,3]的方法，调整噬菌体悬液浓度为 10^4 pfu/mL ~ 10^5 pfu/mL。

1.2.5 噬菌体和细菌的混合时间对裂解率的影响[9] 为保证准确计数，要求每平皿噬菌斑数目在 30 ~ 150，确定以下稀释倍数。取约 10^3 pfu/mL 的噬菌体 100μL，加入 200μL 培养了 8h 到对数生长期的菌液，吸附 0min、5min、10min、15min、20min、25min、30min、35min、40min、45min、50min、55min、60min 后立即做双平板，培养 12h ~ 16h 后记数噬菌斑。

1.2.6 乙醚敏感实验：参照文献[10 ~ 12]的方法，其中采用的噬菌体液浓度为 10^{10} pfu/mL。

1.2.7 氯仿敏感试验：根据文献[10,13]的方法，其中氯仿处理的终浓度为 5%。

1.2.8 细菌培养时期对裂解率的影响[14]：将副溶血弧菌接种于 20mL 培养菌液，于 35℃培养 2 h、4 h、6 h、8 h、10 h、12 h、14 h、16 h、18 h、24h，取 0.2mL 和 10^3 pfu/mL 的噬菌体液 0.1mL，用双平板法测裂解情况，每组做 3 个平行。

1.2.9 Mg^{2+} 对裂解率的影响：在下层固体琼脂和上层半固体琼脂中分别加入 Mg^{2+}，使终浓度分别为 0 mmol/L、5 mmol/L、10 mmol/L、15 mmol/L、20 mmol/L、25 mmol/L、30 mmol/L、40mmol/L，然后取 10^3 pfu/mL 的噬菌体液和培养 8h 的菌液，用双平板法，测定出斑情况[4,15]。

1.2.10 Ca^{2+} 对裂解率的影响：方法同 1.2.9.。

1.2.11 柠檬酸钠对噬菌体裂解率的影响：利用浓度分别为 0%、0.25%、0.5%、1.0%、2.0%、3.0%、4.0% 的柠檬酸钠混合于上下层琼脂培养基中，然后取 10^3 pfu/mL 的噬菌体液 0.1mL，用双平板法测定在各种不同浓度柠檬酸钠平板上的噬菌斑数目[9,16]，每组做 3 个平行。

1.2.12 盐度对裂解率的影响：配制盐度分别为 0、5、10、15、20、25、30、35 的蛋白胨上层和下层培养基，观察出斑情况。

1.2.13 噬菌体最佳感染复数的测定：参照文献[14]的方法。

2 结果

2.1 不同 pH 值对噬菌体裂解活性的影响

实验结果(图 1)表明，该噬菌体在 pH8 左右时最稳定，存活率最高，活性最强。所以其最适 pH 值为 8。在 pH6 ~ 11 时(除 pH9 外)裂解活性都在 80% 以上，说明噬菌体对 pH 的适应范围比较广泛。pH 小于 4 或大于 11 时，噬菌体的裂解活性迅速下降。

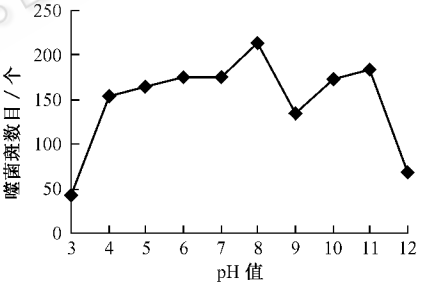

图 1 不同 pH 值对噬菌斑数目的影响

2.2 噬菌体最适培养温度的测定

结果(图 2)表明：噬菌体在 35℃时，产斑量最高，即活力最强。在 20℃以下，活力迅速降低。

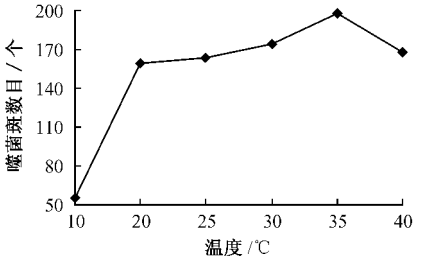

图 2 温度与噬菌体产斑量的关系

2.3 噬菌体的热稳定性实验

结果(图 3)表明，该噬菌体在 60℃处理 20min，仍有 64.9% 的相对出斑率。说明其对 60℃以下的温度，耐受力较强；而在 65℃处理 5min，即仅剩 5%

的相对出斑率，70℃处理 10min，全部失活，说明噬菌体对 60℃以上的高温敏感。

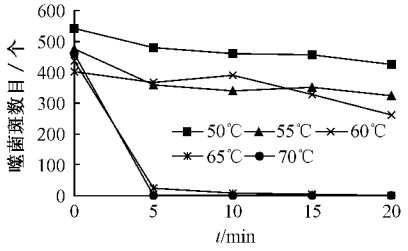

图 3　噬菌体的热稳定性实验结果

2.4　噬菌体的紫外线效应实验结果

图 4 显示在较短时间内紫外线对噬菌体具有较强的影响。在 1min 内相对出斑率就下降到 11.3%，2min 后即下降到 0.2%，在紫外线照射 18min 后，平板上噬菌斑又重新出现，相对出斑率达到 20%以上。

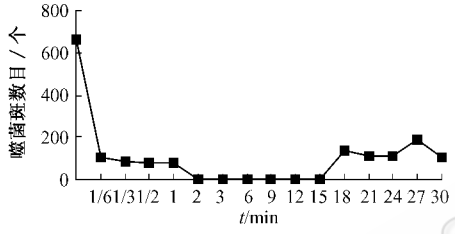

图 4　紫外线对噬菌体出斑的影响

2.5　噬菌体和细菌的混合时间对裂解率的影响

结果（图 5）表明：副溶血弧菌和噬菌体混合 35min 之内，出斑数量没多大变化，既在这段时间内不会引起裂解，而混合 40min 以上，噬菌斑数量明显上升，发生了裂解，60min 时，噬菌斑数量急剧增加。

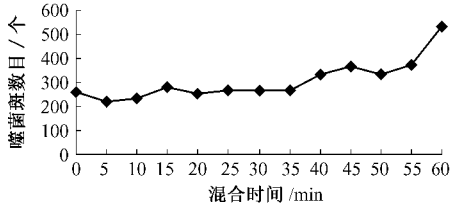

图 5　混合时间对噬菌体产斑量的影响

2.6　副溶血弧菌噬菌体对理化因子敏感性测定结果

实验结果（表 1）表明，该噬菌体在乙醚、氯仿处理后，对宿主的感染与裂解无明显变化，对乙醚和氯仿不敏感。

2.7　细菌培养时期对裂解率的影响

由图 6 看出：该实验条件下，细菌培养 18h 内对噬菌体的敏感程度没多大变化，而培养 24h 后，对噬菌体不再敏感，未见有斑出现。

表 1　理化因子对噬菌体的影响

理化因子	实验组	对照组	对数差值
乙醚	10.24	10.08	－0.16
氯仿	10.33	10.50	0.17

注：表内数值为噬菌斑数量对数值

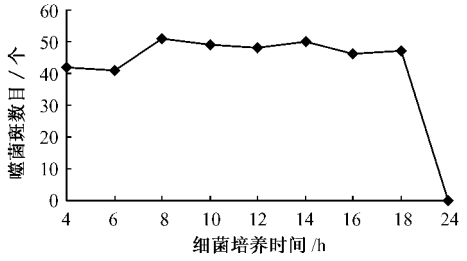

图 6　副溶血弧菌培养时间对噬菌体出斑的影响

2.8　Mg^{2+}、Ca^{2+} 对噬菌体裂解率的影响

由结果（表 2）可知噬菌体的相对出斑率受二价金属离子（Ca^{2+} 和 Mg^{2+}）的影响，在培养基中添加 20mmol/L 的 Mg^{2+} 或 25mmol/L Ca^{2+} 有利于噬菌体的吸附，比不添加的出斑率分别高出 78%和 53%。

表 2　离子浓度与噬菌体产斑的关系

浓度	0	5	10	15	20	25	30	35	40
Mg^{2+} (mmol/L)	165	183	254	282	293	278	256	272	230
相对出斑率(%)	100	111	154	171	178	168	155	165	139
Ca^{2+} (mmol/L)	178	208	267	248	252	272	265	232	247
相对出斑率(%)	100	117	128	139	142	153	149	130	139

2.9　柠檬酸钠对噬菌体裂解率的影响

不同浓度的柠檬酸钠对噬菌体的相对出斑率有明显影响，随着柠檬酸钠浓度的增高而相对出斑率明显下降。当柠檬酸钠浓度达到 1.0%时，噬菌体既全部失活。见表 3。

表 3　柠檬酸钠对噬菌体产斑的影响

柠檬酸钠浓度(%)	0	0.25	0.5	1.0	2.0	3.0	4.0
相对出斑率(%)	100	62	18	0	0	0	0

2.10　盐度对裂解率的影响

由实验结果（图 7）看出：盐度对相对出斑率有明显的影响，在盐度为 20 的情况下相对出斑率最高。

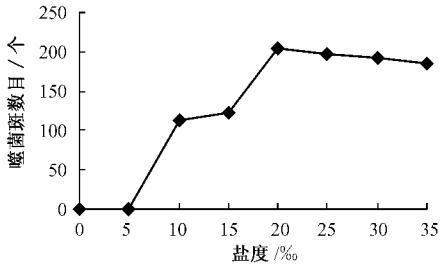

图 7 盐度对噬菌体产斑的影响

2.11 噬菌体最佳感染复数的测定

经倍比稀释后通过噬菌斑计数测得其噬菌体浓度为 5.4×10^{10} pfu/mL。按照表 4 加入噬菌体和宿主菌,测定最佳感染复数,结果见表 4。

表 4 噬菌体最佳感染复数的测定

管别	细菌数 (cfu/mL)	噬菌体数	感染复数	6h 后浓度	6h 后 *OD* 值
1	1×10^8	1×10^5	0.001	2.8×10^7	0.630
2	1×10^8	1×10^6	0.01	6.5×10^8	0.346
3	1×10^8	1×10^7	0.1	5.8×10^{10}	0.161
4	1×10^8	1×10^8	1	6.2×10^{10}	0.076
5	1×10^8	1×10^9	10	4.8×10^{10}	0.370
6	1×10^7	1×10^9	100	8.2×10^9	0.344
7	1×10^6	1×10^9	1000	7.6×10^8	0.492
对照	1×10^8	–	–	–	1.247
	1×10^7	–	–	–	1.171
	1×10^6	–	–	–	1.128

根据表 4 的实验结果,感染复数 MOI = 0.1、1 和 10 时,噬菌体感染其宿主菌产生的子代噬菌体浓度比较高。其中,当 MOI = 1 和 0.1 时,噬菌体感染其宿主菌产生的子代噬菌体浓度分别为 6.2×10^{10} pfu/mL和 5.8×10^{10} pfu/mL,确定噬菌体感染其宿主菌的最佳感染复数在 0.1 ~ 10 之间。由 *OD* 变化可知,菌体生长明显受到影响,菌体明显溶解,*OD* 值急剧下降。

3 讨论

pH 对噬菌体相对出斑率的实验结果表明其存活的 pH 范围较宽,对碱性环境具有较强的耐受力,当 pH 值达到 11 时,相对出斑率达到 80% 以上,pH8 左右时最稳定,相对出斑率最高,活性最强。对 pH4 以下的酸性环境的耐受性较差,明显影响存活率。这与其宿主副溶血弧菌的 pH 适应范围相一致。pH 为 9 时噬菌体存活率有所下降,可能是与噬菌体的等价点有关。本实验结果与林业杰等(1998)所测的 7 株副溶血弧菌噬菌体中的 PP1、PP3、PP6 相似[9]。

噬菌体在 35℃时,相对出斑率最高,活力最强。在 20℃以下,活力迅速降低。温度影响相对出斑率,可能是与噬菌体的吸附活性和噬菌体浓度有关,也与宿主细胞的生理状态、受体的数量有关。本实验的噬菌体在 60℃ 以下时,耐受力较强,对 60℃以上的高温敏感。该噬菌体对热的抗性,比陈敏和方序[16] 描述的两株钝齿棒杆菌噬菌体 ΦG-8 和 ΦG-28 强,与李广武等(1995)[4] 研究的噬菌体 PG_3 相似。

较短时间内紫外线对该副溶血弧菌噬菌体具有强的影响。2min 时相对出斑率下降到 0.2%。紫外线 18min,重新出现较大噬菌斑。这种现象在本实验首次发现。而宁淑香等(2000)[7] 在用紫外线照射河流弧菌 II 噬菌体-VP8 30min,噬菌体全部失活。杨水云等[6] 在较短时间内用紫外线对苏云金芽孢杆菌噬菌体具有强的杀灭作用。随着照射时间的延长,存活率不随时间的延长而降低。对于本实验的现象,有待进一步研究。

副溶血弧菌和噬菌体混合时间对相对出斑率有影响,与该噬菌体的潜伏期关系密切。林业杰等(1998)[9] 所测得的噬菌体 PP4 潜伏期为 35min ~ 37min,与本实验噬菌体相似。本实验副溶血弧菌在实验条件下培养 24h 后,对噬菌体不敏感,与朱明田、颜望明(1995)的研究结果相似[14]。

李广武等的研究发现,Ca^{2+}、Mg^{2+} 的添加有利于噬菌体 PG3 的吸附,有无 Ca^{2+}、Mg^{2+} 存在对噬菌体的吸附率有着明显的差别[4]。李维泉等(1999)[15] 的研究发现,噬菌体 φMMR1 成斑大小及形态受钙离子浓度的影响很大。Mg^{2+}、Ca^{2+} 对吸附的影响可能因为 Mg^{2+}、Ca^{2+} 有助于噬菌体的吸附作用,对噬菌体-宿主菌系统有稳定作用。

本实验噬菌体对柠檬酸钠的耐受性较弱,在柠檬酸钠浓度达到 1.0% 时,噬菌体即全部失活。与林业杰等研究的副溶血弧菌噬菌体 PP2、PP4 、PP5、PP6、PP7 相似[15]。陈敏和方序(2000)的研究发现钝齿棒杆菌 T6-13 噬菌体 ΦG-8 和 ΦG-28 对该试剂的反应有很大差别[16]。柠檬酸钠阻抑噬菌体的吸附、生长和细胞的裂解,这一现象可以作为噬菌体

特性的一种指标。

盐度对噬菌体相对出斑率的影响，未见同类报道。盐度在 5 以下时，未见有噬菌斑出现，可能是在低盐度下，副溶血弧菌不生长或生长不好，不能形成菌膜，无法观察到噬菌斑的形成。实验中盐度在 5 以下时，副溶血弧菌确无法形成菌膜。同时我们的实验也发现，盐度在 20 左右时，噬菌体的相对出斑率较高。

感染复数(multiplicity of infection，MOI)是指初始感染时，噬菌体的数量与宿主菌数量的比值[19]。MOI > 1 即表示每个细菌都有 1 个以上的噬菌体感染机会；MOI < 1 则相反，表示一个细菌平均得不到一个噬菌体感染的机会。不同噬菌体的感染复数是不同的，存在一个最佳用量和最大产出问题，即最佳感染复数。本实验测得的最佳感染复数在 0.1 ~ 10 之间。Lu 等[21]等测得乳酸菌噬菌体 φJL-1 感染宿主菌的最佳感染复数在 0.01 ~ 0.03 之间；Wang 等[19]用高 MOI 的噬菌体 D3112 去感染铜绿假单胞菌，与本实验测得的最佳感染复数相差甚远。可能原因主要有：①噬菌体本身特性相差甚远。②宿主菌特性的差异。③实验条件的差异。如 Lu 等[19]是在 30℃培养条件下进行研究的，而本项目在 35℃培养的；另外，Lu 等使用的是 MRS 肉汤和 MRS 平板[19]，而本实验用的是蛋白胨水培养基。

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Fermentation Kinetics of Media Optimization for the Production of Alpha Amylase by a New Isolate of *Aspergillus Oryzae*

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Abstract: The present study is concerned with the isolation and screening of different strains of *Aspergillus oryzae* for the production of alpha amylase. Ninety strains were isolated from soil and tested for the production of alpha amylase in shake flasks. Of all the strains tested, *Aspergillus oryzae* GCB-32 and *Aspergillus oryzae* GCB-35 gave maximum production of alpha amylase. Different culture media were screened for the production of alpha amylase by these two strains. M1 medium containing starch, yeast extract, NH_4Cl, $MgSO_4 \cdot 7H_2O$ and $CaCl_2$ gave the maximum production of alpha amylase by both the strains *Aspergillus oryzae* GCB-32 and *Aspergillus oryzae* GCB-35. Kinetic analysis revealed that the values of product yield coefficient ($Y_{p/x}$) and specific product yield coefficient (q_p) were found highly significant ($p \leqslant 0.05$) when medium M1 was used for the enzyme production.

Key words: Alpha amylase, Kinetics, *Aspergillus oryzae*, Fermentation media.

The starch-degrading enzyme 'α-amylase' is widely distributed in nature. This extracellular enzyme randomly hydrolyzes α 1~4 glucosidic linkage throughout the starch molecule in an endo-fashion producing oligosaccharides and monosaccharides including maltose, glucose and alpha limit dextrin.[1,5,32]

Alpha amylase is one of most important enzymes and is of great significance in present day biotechnology. It is extensively used in many industries including starch liquefaction, brewing, food, paper, textile, sugar and pharmaceuticals.[3,5,28] Although alpha amylase can be derived from several sources such as plants, animals and microorganisms, yet the enzyme from microbial sources generally meet the industrial demand. Different fungal and bacterial strains have been extensively used for the biosynthesis of alpha amylase[13,14,25,29]. However the amylase of fungal origin was found superior than the bacterial enzyme due to high stability and heat sensitivity[10].

The commonly used fungi for the production of alpha amylase include *steptomyces* sp., *Themomyces lanuginosus*, *Rhizopus* sp., *Trichoderma* sp., *Penicilium griseoroseum*, *Fusarium moniliformis* and *Alternaria* sp.[22,24,33]. However species of *Aspergillus* such as *Aspergillus niger*, *Aspergillus tamarii*, *Aspergillus awamori* and *Aspergillus oryzae* have received most attention because of their high productivity[12,23,37,38]. *Aspergillus oryzae* is well known fungus which has been widely used to obtain many kinds of hydrolytic enzymes like alpha amylase, lipases and protease. To prepare these extracellular enzymes on commercial scale, many attempts have been made to select superior strains of the fungi. Therefore it is worthwhile to isolate a potent strain of *Aspergillus oryzae* and to optimize the fermentation profile to get higher production rate of alpha amylase.

Selection of suitable fermentation medium is very important for the enhanced production of alpha amylase[9]. All microorganisms require energy and certain minerals for growth and metabolism[15] and the energy for growth comes from the oxidation of medium components. The presence of carbon and nitrogen sources and mineral

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nutrients such as P, K, Mg, and Ca are essential for the growth of fungi as well as the enzyme production[15]. So media fermentation or selection is one of most important parameters to be optimized for enzyme production from microorganisms.

The objective of this study was the selection of potent strain of *Aspergillus oryzae* and a superior and cheap fermentation medium for the production of alpha amylase.

1 MATERIALS AND METHODS

1.1 Isolation of organism

Aspergillus oryzae strains were isolated from different soil samples by serial dilution method[8]. About 0.5 mL of the diluted soil suspension was transferred to the petriplates containing malt extract -starch- agar medium. These petriplates were incubated at 30℃ for 2 d ~ 3 d. The young colonies forming clear zones of starch hydrolysis in petriplates were aseptically picked up and transferred to potato dextrose starch agar slants. The slants were then incubated at 30℃ for 3 d ~ 4 d for maximum growth and were stored at 4℃ in refrigerator for further use.

1.2 Culture Media

Different culture media were tested for the production of alpha amylase by the selected strains of *Aspergillus oryzae*. The compositions (g/L) of the media were as under:

M1: Starch, 20; yeast extract, 8.5; NH_4Cl, 1.3g; $MgSO_4 \cdot 7H_2O$, 0.12; $CaCl_2$, 0.06 (pH5.0).

M2: Starch, 10; yeast extract, 3.0; $MgSO_4 \cdot 7H_2O$, 0.005; $CaCl_2 \cdot 2H_2O$, 0.2; $FeSO_4$, 0.1; Peptone, 20 (pH 6).

M3: Starch, 10; $MgSO_4 \cdot 7H_2O$, 0.005; $CaCl_2 \cdot 2H_2O$, 0.2; $FeSO_4$, 0.1; $(NH_4)_2SO_4$, 20 (pH 6).

M4: Wheat bran, 100; $ZnSO_4 \cdot 7H_2O$, 0.062; $FeSO_4$, 0.068; $CuSO_4 \cdot 7H_2O$, 0.0008 (pH 4.5).

M5: Glucose monohydrate, 4.86; NaCl, 1.80; $(NH_4)_2SO_4$, 4.86; $CaCl_2 \cdot 2H_2O$, 1.2; trace metal solution, 0.12 mL; KH_2PO_4, 3.87 (pH 5).

M6: Starch, 25; Meat extract, 5; KH_2PO_4, 1.7; NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 1.0 (pH 5).

M7: Starch, 30; pepton, 20; KH_2PO_4, 5.0; $MgSO_4 \cdot 7H_2O$, 2.50 (pH5.5).

1.3 Inoculum preparation

In the present studies conidial suspension was used prepared in 0.005% sterilized Monoxal O T to inoculate the fermentation flasks. Ten milliliter of sterilized Monoxal O T. was transferred to a 72h old slant having profused conidial production on its surface. The conidia were scratched from the surface and test tube was shaken vigorously for breaking the clumps of conidia. The number of conidia was counted with the help of Haemacytometer, which were about 2.6×10^6 conidia per milliliter.

1.4 Fermentation technique

Twenty-five milliliter of the fermentation medium in 250 mL cotton wool plugged conical flask was used to carry out fermentation for the production of alpha amylase. The flasks containing culture media were sterilized in an autoclave at 1×10^5 Pa (15lbs/inch^2) pressure for 15 min and cooled at room temperature. One milliliter of inoculum containing 2.6×10^6 conidia was aseptically transferred to each flask and the flasks were placed in the orbital shaking incubator (SANYO Gallenkamp PLC, UK) for incubation at 30℃ with shaking speed of 200 r/min. After fixed period of incubation, the contents of the flasks were filtered and filtrate was used for the estimation of enzyme and biomass.

1.5 Enzyme assay

Alpha amylase activity was estimated according to the method of Rick and Stegbauer[34]. The one-milliliter of enzyme sample was incubated with 1 ml of 1% soluble starch solution (pH 5.5) at 40℃. The reducing sugars were measured by adding 3, 5 dinitro salicylic acid reagent boiling for 5 min cooling and measuring the *OD* at 546nm in the spectrophotometer. One unit of activity is defined as the amount of enzyme, which liberates reducing group from 1% Litners' soluble starch corresponding to 1mg maltose hydrate in 10 min.

All the fermentation experiments and enzyme assay were carried out in triplicate using analytical grade reagents and mean values were presented in the results.

1.6 Kinetic and statistical analysis

Kinetic parameters for batch fermentation experiments were determined according to the methods described by Pirt[31] and Lawford and Rouseau[19]. The following parameters of kinetics were studied:

1.6.1 Maximum specific growth rate μ_m per hour-The value of μ_m was calculated from plot of lnx vs. time of fermentation.

1.6.2 Product yield coefficient ($Y_{p/x}$) U/mL/mg-The value of $Y_{p/x}$ was determined by the equation: $Y_{p/x} = dp/dx$

1.6.3 Specific product yield coefficient (q_p) U/mL/h-The value of q_p was determined by the equation: $Y_{p/x}$. (μ) max

The experimental data was statistically analyzed by the method of Snedecor and Cochrane[35]. Duncan's multiple range test was applied under one way ANOVA. Significance has been presented in the form of probability ($p \leqslant 0.05$) values.

2 RESULTS AND DISCUSSION

Isolation and screening of a potent strain is the primary step for the production of alpha amylase at a large scale. Ninety strains of *Aspergillus oryzae* were isolated from soils of different areas of Lahore by serial dilution method[8] The amylolytic strains were isolated on the basis of formation of clear zones of starch hydrolysis in petri plates. However, zonation cannot in any way be correlated quantitatively with amount of alpha amylase produced because of hydrolytic activity of other enzymes such as gluco amylase[18]. Therefore screening of starch hydrolyzing alpha amylase using starch plate can only be qualitative and partially selective (Table1a). So these strains were further screened for alpha amylase production in 250 mL shake flasks. Of all the strains tested the strain No.32 and strain No.35 gave maximum production of alpha amylase. (Table 1) These strains were selected for further studies and assigned the codes as *Aspergillus oryzae* GCB-32 and *Aspergillus oryzae* GCB-35.

Suitable fermentation media play a very critical role in the production of enzymes as the biosynthesis of enzyme by microorganisms is directly influenced with the composition of fermentation medium. Seven different media were tested for the production of alpha amylase by the both selected strains of *Aspergillus oryzae* out of which M1 medium (g/L: Starch, 20; yeast extract, 8.5; NH_4Cl, 1.3g; $MgSO_4 \cdot 7H_2O$, 0.12; $CaCl_2$, 0.06 pH = 5.0) was found best for maximum production of alpha amylase by both the strains *Aspergillus oryzae* GCB-32 and GCB-35 (Fig1, 2).

Table 1 Isolation and screening of *Aspergillus oryzae* for the production of alpha amylase

Sr No	Isolate No	Enzyme activity (U/mL)	DCM (g/L)
1	32	37 ± 0.05^{a}	23.0 ± 0.02^{a}
2	35	32.0 ± 0.03^{b}	22.0 ± 0.03^{b}
3	36	31.0 ± 0.04^{c}	20.0 ± 0.02^{d}
4	33	27.0 ± 0.05^{d}	18.0 ± 0.02^{e}
5	80	25.0 ± 0.04^{e}	20.0 ± 0.04^{d}
6	81	22.0 ± 0.06^{f}	21.0 ± 0.04^{e}
7	61	22.0 ± 0.05^{fg}	20.0 ± 0.03^{d}
8	82	21.0 ± 0.04^{h}	20.0 ± 0.04^{d}
9	83	20.0 ± 0.05^{gh}	18.0 ± 0.02^{e}
10	89	19.0 ± 0.03^{i}	17.0 ± 0.04^{f}
		LSD = 1.040	LSD = 0.745

Each value is a mean of three parallel replicates. ± indicates the standard deviation among replicates. The values differ significantly at $p \leqslant 0.05$.

A good combination of both organic and inorganic nutrients in the fermentation medium is very important for better growth of the organism and enhanced biosynthesis of enzymes subsequently. It was found that both organic and inorganic nitrogen sources were essential for the maximum growth of *Aspergillus oryzae* GCB-32 and subsequent production of alpha amylase. In the present medium, yeast extract acted as organic nitrogen source while ammonium chloride was acted as inorganic nitrogen source. Yeast extract is a complex nitrogen source and therefore it contains free amino acids, which were proved as excellent sources for enzyme production. Pedersen and Nielsen[30] showed that a good combination of organic and

inorganic nitrogen sources is very important for the enhanced production of alpha amylase.

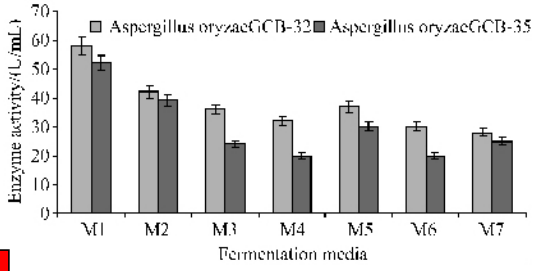

Fig 1 Screening of culture media for the production of alpha amylase by *Aspergillus oryzae* in shake flasks*
Each value is an average of three replicates. Y error bars indicate the standard error among the values.
* Medium pH 5.5, incubation temperature 30℃, incubation period, 72 h.

Table 1a Range of alpha amylase activity of isolates

Number of strains	Range of alpha amylase activity
37	0 ~ 10
40	11 ~ 20
10	20 ~ 30
3.0	30 ~ 40

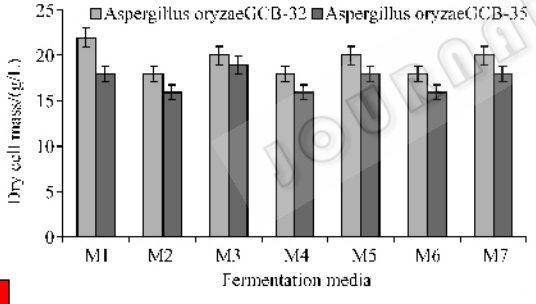

Fig 2 Screening of culture media for the production of cell mass by *Aspergillus oryzae* in shake flasks*
Each value is an average of three replicates. Y error bars indicate the standard error among the values.
* Medium pH 5.5; incubation temperature 30℃; incubation period 72 h.

The nature and amount of carbon source in culture media is important for the growth and the production of alpha amylase in microorganisms. It is empirically known that higher yield of alpha amylase can be obtained in media with complex raw material containing starch from maize barley, wheat and malt. Many workers have reported that starch is the best carbon source for the production of alpha amylase[2,11].

The production and stability of enzyme alpha amylase is greatly affected by the addition of metal ions in the fermentation medium because the metal ions act as activators for enzyme activity[21,26]. M1 medium contained the ions such as Ca^{2+}, Cl^-, Mg^{2+} and SO_4^{2-} which were essential for the growth of fungus and enzyme production. Chambert *et al*.[6] reported the maximum production of alpha amylase in the presence of Ca^{2+} because Ca^{2+} ions act as best stabilizer, binder and activator of alpha amylase. Cl^- is essential for fungal growth and it is also acts as activitor for the production of alpha amylase[27]. Levitizki and steer[20] reported a binding site for Cl^- on the alpha amylase, which affects a conformational change that enhances activity.

All the other media gave insignificant results which might be due to the lacking of the components essential for growth as well as for the production of alpha amylase or that might be due to inhibitory effect of any component of the media on the growth of organism. M2 and M3 gave insignificant results because they contained $FeSO_4$ which gave Fe^{2+} and Fe^{3+} ions in the medium which have toxic inhibitory effects on the growth of microorganism[7]. M4 medium produced less enzyme because it lacked sufficient amount of both organic and inorganic nitrogen sources which were essential both for the growth of micro organism and enzyme production. M5 medium showed less production of alpha amylase which might be due to presence of $(NH_4)_2SO_4$. Ammonium sulphate is not a good nitrogen source because ammonium ions are utilized and free acids are librated in the medium, which lowers the medium pH and makes the enzyme to be unstable[36]. M6 and M7 medium gave minimum production of alpha amylase which was due to catabolic repression because it has been reported that the addition of starch at higher concentration decreased the alpha amylase yield[16,17]. A high starch content medium, when attacked by alpha amylase during fermentation could have undergone degradation resulting in the accumulation of reducing sugars, which caused catabolite repression of alpha amylase synthesis.

The data was subjected to kinetic analysis for the calculation of product yield coefficient ($Y_{p/s}$) and specific

product yield coefficient (q_p). The kinetic analysis revealed that the values of kinetic parameters $Y_{p/x}$ and q_p were highly significant ($P \leqslant 0.05$) in the presence of M1 medium and other culture media gave relatively insignificant values (Table 2). The production of alpha amylase following growth of the organism was found to be highly significant with M1 medium and varied significantly ($P < 0.05$) with other culture media. Thus, the kinetic evaluation of experimental results confirmed that M1 culture medium was best for the production of alpha amylase by *Aspergillus oryzae* strains.

Table 2 Kinetic evaluation of different culture media for the production of alpha amylase by *Aspergillus oryzae* strains in shake flasks

Medium	$Y_{p/x}$		q_p	
	GCB-32	GCB-35	GCB-32	GCB-35
M1	2.63 ± 0.11^{a}	2.88 ± 0.02^{a}	0.658 ± 0.03^{a}	0.72 ± 0.01^{a}
M2	2.33 ± 0.02^{b}	2.43 ± 0.01^{b}	0.582 ± 0.03^{b}	0.607 ± 0.01^{b}
M3	1.8 ± 0.02^{cd}	1.26 ± 0.1^{e}	0.45 ± 0.01^{c}	0.315 ± 0.01^{e}
M4	1.77 ± 0.02^{d}	1.77 ± 0.02^{e}	0.442 ± 0.001^{c}	0.312 ± 0.005^{e}
M5	1.85 ± 0.01^{c}	1.66 ± 0.33^{e}	0.462 ± 0.001^{bc}	0.415 ± 0.001^{c}
M6	1.66 ± 0.015^{e}	1.66 ± 0.015^{e}	0.415 ± 0.005^{d}	0.312 ± 0.001^{e}
M7	1.4 ± 0.01^{f}	1.4 ± 0.01^{d}	0.35 ± 0.01^{e}	0.345 ± 0.001^{d}
LSD	0.079	0.38	0.0082	0.0075

Kinetic parameters: $Y_{p/x}$: Product yield coefficient; q_p: Specific product yield coefficient; μ_m: Maximum specific growth rate, 0.25 h^{-1}.

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