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Emoleila Itoandon
Department of Research and Development, Almon Research Foundation, India

Ferdinand Okougha
Science Laboratory Technology and Research Department, Nigerian Building and Road Research Institute, Nigeria

Femi Adams
Production Analytical and Laboratory Management Department, Federal Institute of Industrial Research, Nigeria

Azeez Tijani
Production Analytical and Laboratory Management Department, Federal Institute of Industrial Research, Nigeria

Steve Osasere Erimwioghae
Department of Nursing, Angel Staffing, Texas, USA

Correspondence
Emoleila Itoandon
Department of Research and Development, Almon Research Foundation, India

Effect of hyper active amylase produced by *Bacillus* species consortium

Emoleila Itoandon, Ferdinand Okougha, Femi Adams, Azeez Tijani and Steve Osasere Erimwioghae

Abstract
An investigation was carried out to determine the effect of amylase activities from *Bacillus licheniformis* (Bl), *Paenibacillus polymyxa* (Pp) and a consortium of both (BlPp). The results showed growth density of 3.58 g/ml and 3.38 g/ml for (Bl) at 9th hour and (Pp) at 7th hour respectively. Amylase activities of 0.45 U/ml and 0.43 U/ml were recorded at optimal pH 7.0 for Bi and Pp, while an increased activity of 0.55 U/ml was recorded for the consortium of (BlPp) at optimal pH 5.0. Similarly, secretome activities were recorded at optimal 50ºC with values of 0.48 U/ml and 0.40 U/ml for (Bl) and (Pp), while a higher activity of 0.56 U/ml was observed for the consortium of (BlPp). Although there have been various developments of synthetic co-cultures, the results from this experiment have shown a fundamental and unique approach of natural microbial consortium application for synthesis of refined industrial byproduct.

Keywords: microbial interaction, substrate, fermentation, biocatalyst

Introduction
The starch hydrolyzing amylase can be synthesized from bacteria, fungi and other microorganisms by solid state and submerged fermentation techniques. Its high impact industrial relevance has led to the development of various techniques involving; genome modifications and construct of novel strains (Bittihn *et al.* 2018, Shong and Collins, 2013) [5, 22], improving formulation of substrate conditions (Hellman *et al.* 2021) [12] and currently development of microbial co-cultures (Che and Men, 2019) [6]; towards achieving more valuable and improved end products. Amylase deriving substrates are obtained from various foods and this substrate has been classified as complex or simple carbohydrate. It has been reported that complex carbohydrates contains longer chain and takes longer time to hydrolyze, while the simple carbohydrates contain shorter chain and lesser time to degrade. Recent studies have shown that utilization of microbial co-culture over mono-culture in biosynthesis and bioprocessing has several advantages such as reducing metabolic burden and overcoming stressful regulatory mechanisms in complex reactions (Roell *et al.* 2019) [21]. Similar investigations have further illustrated that differential activities of co-cultures enhances nutrient utilization efficiently, creating stronger dynamics against resistance and resilience to environmental changes caused by physiological factors controlling the system (Kato *et al.* 2008, de Lime *et al.* 2016) [17, 7]. Another advantage of microbial consortium is the ability to function actively on complex materials in this case, degrading complex carbohydrates at shorter time and secreting higher volume of refined simple sugars [Shong *et al.* 2012]. Although, much has been achieved in the development and application of synthetic and genetically modified microbial consortium for industrial applications, there has been overwhelming challenges as regards to understanding the selective strains' mechanisms of interrelation within a system (Johns *et al.* 2016, Zhou *et al.* 2015) [16, 25]. To overcome this challenge, several models have been designed to study the expression of co-culture subpopulations and their stability during maximal synthesis of target byproducts such as amylase (Che and Men, 2019) [6]. In any complex reaction such as fermentation, the function of microbial consortium on complex carbohydrates is expected to degrade strongly binding polymers that often appear challenging to monocultures. The aim of this investigation was to determine the effect of amylase produced by co-culture bacterial on starch hydrolysis.

Materials and Methods

Media formulation

A 100ml medium (A) was formulated with the following ingredients: Casein enzymic hydrolysates 1.0 g, Yeast extract 0.5 g, Sodium chloride 1.0 g, Agar 1.5 g, starch 0.5 g, NaCl 0.85 g. The medium was sterilized under 15psi at 110ºC for 10 minutes. The sterilized medium was allowed to cool up to 45ºC, poured into sterile Petri Dishes and left to solidify. Another medium (B) with the following ingredients: Glucose 10.0 g, Ca(PO4)2 5.0 g, $(NH4)_2SO_4$ 0.5 g, $MgSO_4.7H_2O$ 0.01 g, $MnSO_4.7H_2O$ 0.01 g, $FeSO_4$ 0.01 g, KCl 0.2 g, Yeast extract 0.5 g. The medium was sterilized under the same condition and left to cool before use. In this investigation, medium (A) was used to determine the microbial compatibility testing and quantitative analysis of starch production while medium (B) was used for synthesis of amylase by submerged fermentation. The media A and B were prepared and adjusted to pH 7.0 for the purpose of this investigation.

Microbial Selection

1 gram of Soil and 1 ml of marine water samples were collected and used for the isolation of *Bacillus licheniformis* (Bl) and *Paenibacillus polymyxa* (Pp) following standard microbiological and laboratory techniques. Each sample was suspended in separate 100ml conical flasks (A and B) containing 50 ml of sterile water and mixed thoroughly. 0.1 ml of each sample was plated on fresh culture plates containing *Bacillus* Chromo Select medium and incubated for 24 hours at 37ºC. To identify the test strains, purification of growth specimens was carried out by streak method on same medium and incubated under same condition. The morphological analysis, biochemical and genomic identification were carried out following standard methods as described by (Aruwa and Olatope, 2015, Jeong *et al.* 2019) [2, 15].

In vitro compatibility testing

Testing of selected strain compatibility was conducted with a modification by following the Disk-diffusion method as described by Tabacchioni *et al.* 2021 [24]. Colonies of *Bacillus licheniformis* (Bl) and *Paenibacillus polymyxa* (Pp) were picked with a sterile wire loop and dispensed into separate tubes containing 0.5 McFarland standard suspensions. Into a third tube containing the standard suspension, a wire loop was used to pick colonies separately and inoculated into the suspension forming a consortium (BlPp). There was gradual increase in microbial load (1.0×10^8 CFU/mL) addition until the solutions became turbid and then incubated for 15 minutes at 45ºC. Using the method of point inoculation, 4 loops full of *Bacillus licheniformis* (Bl) suspensions and another 4 loop full of *Paenibacillus poluymyxa* (Pp) suspensions were inoculated on the surface of the medium (A), also 1 loop full of mixed cultures (BlPp) suspension was inoculated onto the same medium (A). The culture medium was incubated at 37ºC for 48 hours. Positive compatibility result showed clear zones of test microorganisms growing beyond the disc border. Furthermore, zones of inhibition observed from the test above showed ability of the selected strains to hydrolyze starch within an environment under the same conditions.

Amylase Production

Overexpression of amylase by submerged fermentation technique was done using the medium (B) already prepared as described above. 100 ml of the medium (B) was dispensed into 3 separate sterilized 250 ml conical flask labeled C, D and E. 1.0 ml of *Bacillus licheniformis* (Bl) suspension was inoculated into conical flask C, 1.0 ml of *Paenibacillus polymyxa* (Pp) was inoculated into conical flask D, and 1.0 ml of the mixed strains (BlPp) was inoculated in conical flask E respectively. The culture media were further incubated at 37ºC for 48 hours using a shaker incubated (Name/Model: TAITEC/GBR-300) regulated at 150 rpm.

Enzyme purification

A modification of Bano *et al.* 2011 [4] was carried out in this investigation. Crude samples were filtered using ultrafiltration with 100,000 MrCO membranes and filtrates were collected and again re-concentrated with 30,000 MrCO membranes. The final filtrates were collected for further purification using solid ammonium sulfate at a final concentration of 40% saturation and then kept for 18 h at 4°C. The precipitated proteins were separated by centrifugation at 35,000 × g for 15 min at 0°C. Purified samples were collected in fresh sterile test tubes and used for assay.

Assay

Amylase activity was estimated following this procedure; 250µl of enzyme sample was incubated with 250µl of 1% soluble starch solution in 20mM phosphate buffer pH 7.0 at 37°C for 30 min. The reducing sugar from each sample was measured by adding 250µl of 3, 5-dinitro salicylic acid reagent to stop the reaction mixture. The tubes were boiled at 100ºC for 5 min, cooled and measured for O.D at 540nm in the UV spectrophotometer (Spinco Biotech. PVT LTD), (Miller, 1959) [19].

Effect of pH and temperature (ºC)

Different reaction mixtures prepared were adjusted to pH values of 3.0 - 11 using 6N HCl and 5N NaOH solutions intermittently. One ml of each sample was mixed with 1ml of iodine added to it and incubated for 40 minutes at 30ºC. The resulting colour (blue black) was determined for its absorbance at 540nm. Similarly, reaction mixtures prepared were incubated at various temperatures of 20ºC – 100ºC. To 1ml of the incubated samples with 0.5ml of the enzyme from the different temperature, 1ml of iodine was added and the resulting colour (blue black) was determined for its absorbance at 540nm. Optimal conditions of the samples were determined with modifications following methods as described by Hamed and Ikhram, (2012) and further confirmatory analysis was carried out according to Baltas *et al.* (2016) [3].

Results

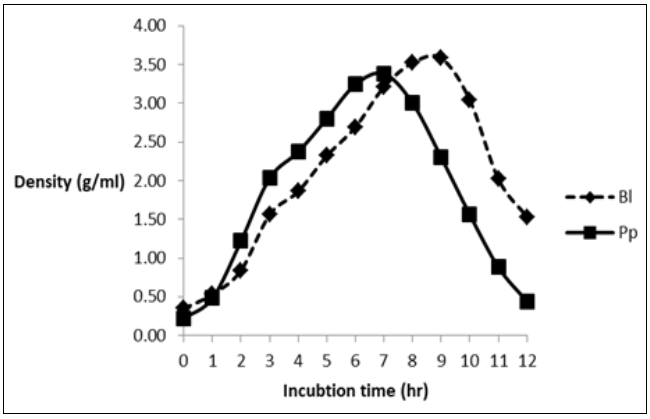

Fig 1: Graphical representation of comparative growth density between *Bacillus licheniformis* (Bl) and *Paenibacillus polymyxa* (Pp). The growth patterns showed significant level of interaction between the strains supporting them to co-exist within a specific environment while utilizing available nutrients

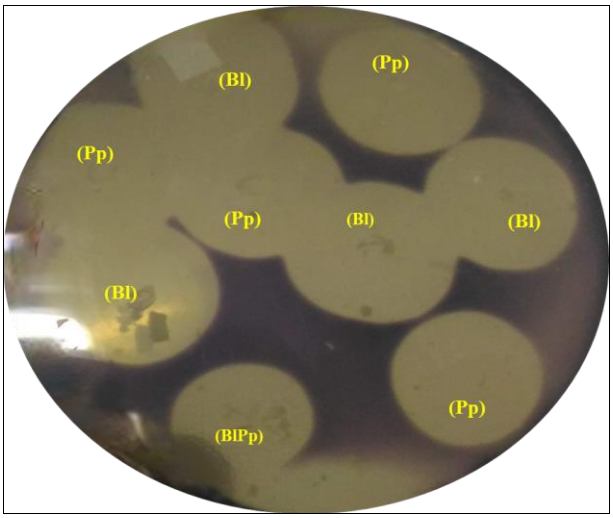

Fig 2: Image describing growth compatibility and starch hydrolytic expressions of *Bacillus lichineformis* (Bl), *Paenibacillus polymyxa* (Pp) and the consortium (BlPp) on the formulated medium (A). The medium composed of supporting nutrients such as carbon and nitrogen sources for growth and development. Co-existence adaptability within the environment was measured by utilization of Sodium (Na) and Chloride (Cl) present in the medium

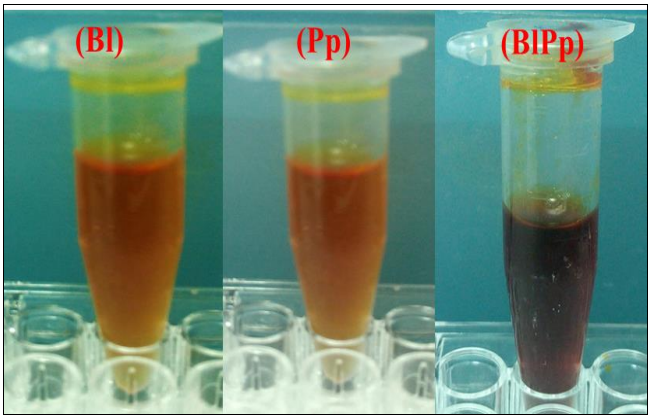

Fig 3: Colouration of hydrolytic activities by amylase from *Bacillus licheniformis* (Bl), *Paenibacillus polymyxa* (Pp) and the consortium (BlPp). Differential colouration showed hyper active effect of amylase from a consortium (BlPp) over monoculture (Bl) and (Pp) on releasing glucose from starch

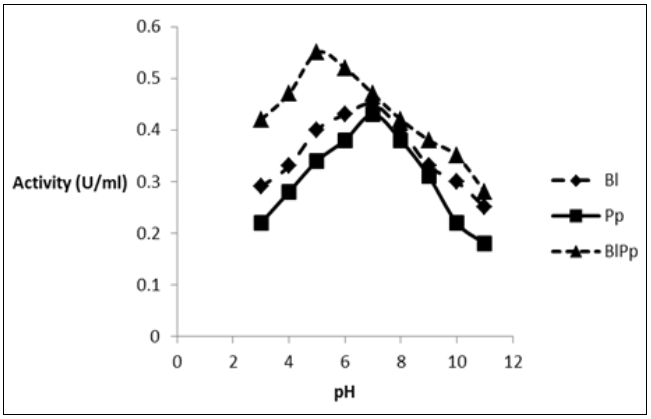

Fig 4: Optimal pH. Differential activities of amylase synthesized by *Bacillus licheniformis* (Bl), *Paenibacillus polymyxa* (PP) and a consortium (BlPp) at variable pH are illustrated in this figure respectively

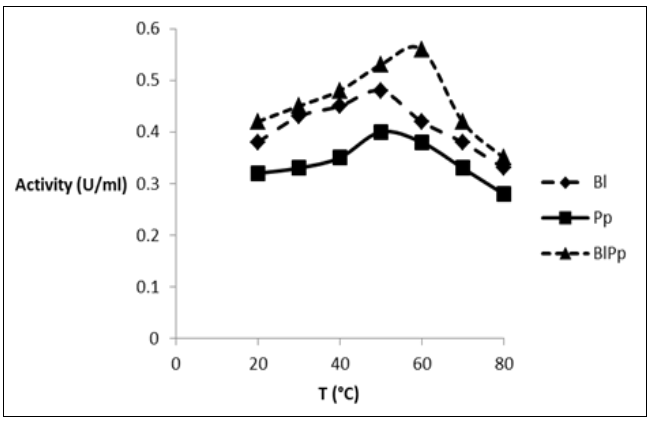

Fig 5: Optimal Temperature (°C). Differential activities of amylase synthesized by *Bacillus licheniformis* (Bl), *Paenibacillus polymyxa* (PP) and a consortium (BlPp) at variable temperature (°C) are illustrated in this figure respectively.

Discussion

The identified *Bacillus licheniformis* (Bl) and *Paenibacillus polymyxa* (Pp) from the methodology are amongst the bacteria community that have been used for the production of amylase by submerged fermentation technique as monocultures. Their growth densities are often influenced by the physiological conditions affecting the environment and this has been related to the volume of biomolecules such as amylase secreted within the environment (Lutz *et al.*, 2006). The growth patterns were measured and illustrated in Figure 1. From the figure, recorded densities of 3.58 g/ml at 9th hour and 3.38 g/ml at 7th hour from (Bl) and (Pp) respectively were used to translate the ability of co-existence in a community with favorable environmental conditions such as micronutrients, water activity, and energy sources (Gani *et al.* 2013) [10]. The effects of incubation time and the choice of medium have been reported to influence the metabolic reaction affecting sporulation at different time interval (Henshaw and Wakil, 2019) [12].

The study of growth of coexistence described above was used to estimate expressions similar to a natural habitat. In this investigation, a simple technique involving a modified formulation (Medium A) as described in the methodology was adopted to determine compatibility and starch hydrolysis as shown in Figure 2. The observed reaction on the plate translated high level compatibility between the strains and starch degrading reaction. This is similar to

reactions reported by (Razak *et al.* 2021, Irabor A and Mmbaga, 2017, Kim *et al.* 2016) [20, 13, 18]. This further describes the multifunction ability of microbial co-existence involved in a division of labour to perform complicated functions more efficiently than individual populations (Silva-Salinas *et al.* 2021) [23], furthermore exhibiting stronger dynamics of resistance and resilience against individual members within environmental (Kato *et al.* 2008) [17] Submerged fermentation has been reported to be very important for the production of amylase because of the by-product high yield and control of physiological conditions. Significant enhancement of amylase activity from the consortium (BlPp) was recorded in Figure 3 showing deeper colour reaction when compared to assay of amylase releasing glucose form the monocultures. During assay, glucose is released and the colouration is often used to determine its concentration. The result was confirmed in a report by Fossi *et al.* 2014 [9]. Qualitative analysis of amylase from *Bacillus licheniformis* (Bl), *Paenibacillus polymyxa* (Pp) and the consortium of both strains (BlPp) were measured to determine variable activities at a pH range 3 – 11. The differential patterns of optimal activities were shown in Figure 4 and Figure 5 respectively. In this investigation amylase recorded activities of 0.45 U/ml and 0.43 U/ml at optimal pH 7.0 for (Bl) and (Pp) respectively, while an increased activity of 0.55 U/ml was recorded from amylase synthesized by the consortium (BlPp) as represented in Figure 4. Similarly, activities against temperatures 20 – 100 (ºC) were measured. Activities of 0.48 U/ml and 0.40 U/ml were at optimal 50ºC for (Bl) and (Pp) respectively while an increased activity was observed at pH 5.0 for amylase secreted by the consortium as represented in Figure 5. The reports observed are in agreement with Abu *et al.* 2005 [1], Fossi *et al.* 2014 [9] confirming the hyper activity of mixed culture on conversion of starch into fine simpler sugars at pH 3 - 8 and 50 – 70ºC. The co-existence of bacteria within a habitat is dependent on the different environmental conditions present and each isolate react differently within the same environment. In this investigation, it was established that activities of amylase produced by a consortium of *Bacillus licheniformis* and *Paenibacillus polymyxa* (BlPp) was hyper active compared to amylase from monocultures.

Conclusion

In this study, amylase was synthesized by *Bacillus licheniformis* (Bl), *Paenibacillus polyyxa* (Pp) and a mixture of both strains (BlPp). Activities of enzymes from monocultures were compared to that from the mixed culture. The study showed high significant impact of mixed culture on starch degradation thus concluding that microbial consortium has high potential in industrial applications over monocultures.

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