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# MOLECULAR AND PHENOTYPIC RESISTANCE OF GROUNDNUT VARIETIES TO LEAF SPOTS DISEASE IN GHANA

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**ABSTRACT**

Groundnut yield is constrained in most cases by early and lateleaf spots diseases. Selection and breeding for resistance will enhance yields. Therefore, this study was conducted in Crop Research Institute, Fumesua-Kumasi, Ghana to screen 20 groundnut varieties for leaf spots resistance using both molecular and phenotypic tools. Differences in disease incidence among individual plants, severity score, lesion diameter and defoliation across the 20 genotypes were highly significant ($p<0.01$) under phenotypic screening. Fourteen genotypes were moderately resistant while six genotypes were susceptible. The SSR markers $pPGseq2F5_{280}$, $pPGseq2B10_{280/290}$, $pPGPseq17F6_{120/140/150}$, $PMc588_{180/220}$ and $PM384_{100}$ confirmed most resistant genotypes at the molecular level. The resistant genotypes confirmed by the markers were 'ICG7878', 'Obolo', 'Oboshie', 'Jenkaar', 'Adepa', 'Nkosour', 'Azivivi', 'Nkatekokoo', 'Behenase', 'Manipinta', 'Otuhia', 'GK7', 'Nkatiesari' and 'Sumnut22'. Genotypes '55-437', 'Yenyawoso', 'Bremawuo', 'Kumawu', 'Konkoma' and 'Shitaochi' were susceptible. Hence, resistance to leaf spots exists among commercially grown groundnuts in Ghana. Both morphological descriptors and DNA molecular could be used for identification of resistant genotypes.

**Keywords:** *Arachis hypogaea*, *Cercospora*, Phenotypic, Resistance, SSR Markers

## INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important legume crop grown globally. It is mostly grown in developing countries of Africa and Asia (**FAOSTAT, 2015**). In 2014, Ghana was the seventh largest producer of groundnut in Africa with about 426280 tonnes of unshelled groundnuts on a land area of 334000 hectares (**FAOSTAT, 2015**). The crop is mostly grown in the northern part of Ghana by resource-poor farmers, who in most cases are unable to buy and use fungicide to control diseases (**Nutsugah*et al.*, 2007**). Groundnut is a good source of minerals, vitamins, plant protein and unsaturated oil for most people in Ghana (**Asibuo *et al.*, 2008**). Low yields and quality of groundnut in developing countries have been partly attributed to early and late leaf spots disease (**Janila *et al.*, 2013a; Gaikpa *et al.*, 2015**). Early leaf spot (ELS) is caused by the fungus *Cercospora arachidicola* Hori and late leaf spots (LLS) by the fungus *Cercosporidium personatum* Berk. & M.A. Curtis (**Janila *et al.*, 2013a**). The *C. arachidicola* forms dark brown lesions that are larger and sub-circular on groundnut leaflets whiles *C. personatum forms a darker, smaller and more circular lesions on the leaflets.* The leaf spots reduce available photosynthetic area of the groundnut plant and also cause abscission of leaflets of the crop (**McDonald *et al.*, 1985**).
The use of resistant cultivars to manage leaf spots would be cheaper to farmers and environmentally safer compared to application of chemicals. Sources of disease-resistant genotypes and their successful selection are essential for improving commercial cultivars and elite breeding lines (**Fehr, 1987**). However, sources of cultivated groundnut genotypes for *Cercospora* leaf spot resistance are reported to be generally scarce (**Kishore *et al.*, 2005**). Identification of leaf spot resistant and susceptible lines using conventional screening techniques has been described as difficult, because of its partial and polygenic nature (**Dwivedi *et al.* 2002; Janila *et al.*, 2013b**). Molecular markers, such as simple sequence repeat markers (SSRs), associated with leaf spots resistance have been found to improve the process of identification of resistant genotypes (**Mace *et al.*, 2006; Shoba *et al.*, 2012**). Early and late leaf spots occur together in Ghanaian farms in most cases. Proper identification of resistant groundnut varieties is very vital for farmers and crop scientists to make informed decisions on the cultivars to grow under a particular condition.The objective of our study was to screen commercial groundnut genotypes in Ghana for *Cercospora* leaf spots (early and late combined) resistance using both phenotypic and molecular (SSR markers) tools.

## MATERIAL AND METHODS

### Plant materials

Twenty (20) groundnut genotypes comprising 10 each of *fastigiata* and *hypogaea* subspecies (Table 1) were used for the study. Genotypes'ICG7878' and '55-437' served as resistant and susceptible checks respectively.

**Table 1** Characteristics and sources of collection of groundnut genotypes used for the study

| Genotype | Sub-species | Days to maturity | Seed colour | Source of collection |
|---|---|---|---|---|
| '1CG7878' | *Hypogaea* | 120 | Dark rose | ICRISAT, Niger |
| '55-437' | *Fastagiata* | 90 | Pink seed | ICRISAT, Niger |
| 'Obolo' | *Fastagiata* | 105-110 | Brown | CSIR-CRI, Ghana |
| 'Oboshie' | *Fastagiata* | 105-110 | Brown | CSIR-CRI, Ghana |
| 'Yenyawoso' | *Fastagiata* | 90 | Dark Red | CSIR-CRI, Ghana |
| 'Bremawuo' | *Fastagiata* | 90 | Dark Red | CSIR-CRI, Ghana |
| 'Kumawu' | *Fastagiata* | 90 | Brown | CSIR-CRI, Ghana |
| 'Konkoma' | *Fastagiata* | 90 | Brown | CSIR-CRI, Ghana |
| 'Jenkaar' | *Hypogaea* | 110-120 | Brown | CSIR-CRI, Ghana |
| 'Adepa' | *Hypogaea* | 110-120 | Brown | CSIR-CRI, Ghana |
| 'Nkosour' | *Hypogaea* | 110-120 | Brown | CSIR-CRI, Ghana |
| 'Azivivi' | *Hypogaea* | 110 | Brown | CSIR-CRI, Ghana |
| 'Shitaochi' | *Fastagiata* | 86-90 | Brown | CSIR-CRI, Ghana |
| 'Nkatekokoo' | *Fastagiata* | 86-90 | Dark Red | CSIR-CRI, Ghana |
| 'Behenase' | *Fastagiata* | 90 | Dark Red | CSIR-CRI, Ghana |
| 'Manipinta' | *Hypogaea* | 110-120 | Variegated | CSIR-CRI, Ghana |
| 'Otuhia' | *Hypogaea* | 110-115 | Brown | CSIR-CRI, Ghana |
| 'GK7' | *Hypogaea* | 110-120 | Brown | CSIR-CRI, Ghana |
| 'Nkatiesari' | *Hypogaea* | 110 | Light tan | CSIR-CRI, Ghana |
| 'Sumnut22' | *Hypogaea* | 110-120 | Brown | CSIR-CRI, Ghana |

**Legend:** ICRISAT-International Crops Research Institute for the Semi-Arid Tropics, CSIR-CRI-Council for Scientific and Industrial Research-Crop Research Institute

**Phenotypic screening**

***Experimental site and design***

Phenotypic screening was conducted in a plant house at Crops Research Institute (CRI), Fumesua- Kumasi, Ghana. It is located in semi-deciduous forest zone and has rainfall of 1500-2000mm per annum. The experimental design used was completely randomised design with five pots per genotype. A sandy-loam soil rich in organic manure was steam-sterilised using barrel-steam method. Plastic containers of 60cm in diameter were filled with the soil and used. Two seeds were sown per container and later thinned to one. Plants were inoculated using hand atomizer by spraying spore suspensions of early and late leaf spots pathogens at 30 and 40 days after sowing (DAS) respectively, with a spore concentration of approximately $5.4\times10^5$ conidia per ml. The pathogens were isolated from fresh infected groundnut leaflets on plants growing on the field at CRI, Fumesua-Kumasi, Ghana. The inoculation was done in the evening, between the hours of 17.30 and 18.00. Inoculated plants were irrigated the following day in the evening and thereafter when necessary to ensure disease build up.

***SSR molecular screening***

Molecular screening of the 20 groundnut varieties was carried out in the Molecular Biology Laboratory of CRI, Fumesua- Kumasi, Ghana. Genomic DNA of the 20 genotypes was extracted using the Qiagen protocol. Hundred milligrams (100mg) of newly expanded leaves was weighed into eppendorf tubes and ground to fine powder with liquid nitrogen. Four hundred microliters (400µl) Buffer AP 1 and 4µl RNase A (100mg/ml) were added to each powered leaf sample and vortexed vigorously to mix. The mixture was incubated at 65˚C for 10min and mixed by inversion. One hundred and thirty microliters (130µl) Buffer AP 2 was added to the lysate, mixed and incubated on ice for 5min. It was then centrifuged at 14000rpm for 5min. The lysate was pipetted into a QIA shredder mini-spin coloumn and centrifuged at 14000rpm for 2min. The flow-through fraction was transferred into a new eppendorf tube without disturbing the cell debris pellet. Buffer AP3/E (1.5 volumes) was added to lysate and mixed. Six hundred and fifty microliters (650µl) of the mixture including any precipitate that was formed was pipetted into a DNeasy mini-spin coloumn and centrifuged at 8000rpm for 1min. Flow-through was discarded and collection tube reused. Six hundred and fifty microliters (650µl) of the remaining mixture was again pipetted into the DNeasy mini-spin coloumn and centrifuged at 8000rpm for 1min. Flow-through and collection tube were discarded. The DNeasy mini-spin space was placed into a new 2-ml collection tube and 500µl buffer AW added. It was centrifuged at 8000rpm for 1min, flow-through discarded and the collection tube reused. Buffer AW (500µl) was added to the DNeasy mini-spin coloumn and centrifuged at 14000rpm for 2min. Spin was emptied for 2min. DNeasy mini-spin coloum was transferred to a 2-ml tube; 50µl of Buffer AE was pipetted directly unto the DNeasy membrane, incubated at room temperature for 10min and centrifuged at 8000rpm for 1min to elute. This step was repeated. The quality of the DNA was checked using 0.8% agarose gel electrophoresis and bands compared to a DNA standard. The DNA of each genotype was primed using eight selected SSR markers (Table 2) reported to be linked to leaf spots resistance in groundnuts in previous studies (**Mace *et al.*, 2006; Shoba *et al.*, 2012**). The markers were purchased from Metabion International AG, Germany.

**Table 2** The sequences of forward (F) and reverse (R) primers and annealing temperatures of SSR markers used

| SSR primers | Sequences (5'-3') | Anealing Temperature (ºC) |
|---|---|---|
| PM384-F | GGCGTGCCAATAGAGGTTTA | 52.0 |
| PM384-R | TGAAAACCAACAAGTTTAGTCTCTCT | |
| pPGPseq5D5-F | AAAAGAAAGACCTTCCCCGA | 52.0 |
| pPGPseq5D5-R | GCAGGTAATCTGCCGTGATT | |
| PM375-F | CGGCAACAGTTTTGATGGTT | 55.0 |
| PM375-R | GAAAAATATGCCGCCGTTG | |
| PMc588-F | CCATTTTGGACCCCTCAAAT | 60.0 |
| PMc588-R | TGAGCAATAGTGACCTTGCATT | |
| pPGPseq2B10-F | AATGCATGAGCTTCCATCAA | 50.4 |
| pPGPseq2B10-R | AACCCCATCTTAAAATCTTACCAA | |
| pPGPseq2F5-F | TGACCAAAGTGATGAAGGGA | 50.4 |
| pPGPseq2F5-R | AAGTTGTTTGTACATCTGTCATCG | |
| pPGSseq13A7-F | AATCCGACGCAATGATAAAAA | 50.4 |
| pPGSseq13A7-R | TCCCCTTATTGTTCCAGCAG | |
| pPGSseq17F6-F | CGTCGGATTTATCTGCCAGT | 52.0 |
| pPGSseq17F6-R | AGTAGGGGCAAGGGTTGATG | |

The polymerase chain reaction mixtures (10µl) contained 1µl template DNA (5ng), 1µl of 10xTaq buffer, 0.9µl $MgCl_2$ (25mM), 0.2µl of dNTPs (20mM), 0.5µl of primers 10µM (Forward and Reverse), 0.12µl of Taq polymerase (Super Therm) and 5.78µl of sterile double distilled water. Amplification was performed in 0.2 ml thin-walled PCR plates (96wells/plate) in a thermal cycler (Applied Biosystems). The samples were initially incubated at 94.0ºC for 2min followed by 35 cycles of 94.0ºC for 45s, 50.4ºC-60.0ºC for 30s (optimized individually for each SSR primer as in Table 2) and 72.0ºC for 1min 30s. Final extension was 72.0 ºC for 10 min and incubated at 4ºC at infinity. Amplified products were analyzed using 6 % non-denaturing polyacrylamide gel. Hundred base pair (100bp) ladder was usedas standardladder. Electrophoresis was carried out at a constant power of 100V for 1hr 40mins and gel was silver-stained.

**Data collected**

Percentage of leaves infected by leaf spots per plant at 60 DAS and 90 DAS and the percentage of leaves defoliated at 75 DAS and 90 DAS were recorded for each plant using the expressions:

$$\text{Percentage of leaves infected per plant} = \frac{\text{Number of leaves infected per plant}}{\text{Total number of leaves per plant}} \text{x} 100$$

$$\text{Percentage defoliation} = \frac{\text{Number of leaves defoliated per plant}}{\text{Total number of leaves per plant}} \text{x} 100$$

Leaf spots disease severitywas assessed on each plant using a rating scale of 1-9 (**Subrahmanyam *et al.*,1995**). Groundnut varieties that had disease scores between 4 and 6 were considered as moderately resistant while those that had score of 7 were considered as susceptible. The diameters of 5 randomly selected lesions on the leaves of the main stem of each plant were recorded at 60 DAS for ELS and at 80 DAS for LLS.

DNA banding profiles were scored for power marker analysis as presence (+) or absence (-) for the expected leaf spots resistant and susceptible alleles (base pairs) for each groundnut genotype as reported by **Mace *et al.* (2006)** and **Shoba *et al.* (2012).** Individual bands were also scored as presence (1) or absence (0) for genetic cluster analysis.

**Statistical analysis**

Percentage data were transformed using arcsine transformation in Microsoft Excel prior to analysis of variance. Genstat statistical software (12.0 edition) was used for analysis of variance and to find correlations between means. The means were separated using Tukey's HSD at 5%. The Genstat software was also used for cluster analysis of phenotypic data to generate a dendrogram using Euclidean Similarity Matrix and Group Average Method. Summary statistics about the SSR markers were calculated using power marker software (3.25). Also, NTSYS software (2.2) was used for hierarchical cluster analysis of SSR marker data to generate a dendrogram using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and Simple Matching Similarity Coefficient. Sequential and Hierarchial Nested (SAHN) option was employed (**Rohlf, 2000**).

**RESULTS**

Differences in percentage of leaves infected with the disease and average percentage of leaves defoliated per plant were highly significant ($p<0.01$) among the 20 genotypes (Table 3). Genotype '55-437' (a *fastigiata* from ICRISAT, Niger) had the highest percentage of leaves infected per plant whiles 'Nkosour' (*hypogaea*) had the lowest at 60 DAS. Again, at 90 DAS, 55-437 had the highest leaf incidence per plant whiles 'Manipinta' (*hypogaea*) had the lowest(Table 3). Also, 'Manipinta' had the lowest percentage defoliation and 'Konkoma' (a *fastigiata*) had the highest at 75 DAS. At 90 DAS, 'Nkosour' had the lowest percentage defoliation and 'Konkoma' again had the highest (Table 3).

**Table 3** Mean percentage of leaves infected and defoliation per plant (Transformed)

| Genotypes | Leaves infected/plant | | Defoliation/Plant | |
|---|---|---|---|---|
| | 60DAS | 90 DAS | 75DAS | 90DAS |
| '1CG7878' | 24.42 | 51.98 | 27.89 | 31.51 |
| '55-437' | 39.07 | 72.18 | 32.38 | 39.73 |
| 'Obolo' | 28.25 | 61.92 | 29.59 | 35.40 |
| 'Oboshie' | 32.58 | 56.78 | 30.29 | 32.87 |
| 'Yenyawoso' | 35.93 | 62.50 | 31.87 | 39.93 |
| 'Bremawuo' | 32.14 | 67.08 | 30.97 | 36.91 |
| 'Kumawu' | 37.58 | 63.16 | 31.64 | 42.71 |
| 'Konkoma' | 35.24 | 69.64 | 36.61 | 49.22 |
| 'Jenkaar' | 30.00 | 53.37 | 28.32 | 31.35 |
| 'Adepa' | 28.23 | 54.37 | 28.56 | 39.77 |
| 'Nkosour' | 19.47 | 54.82 | 25.65 | 28.05 |
| 'Azivivi' | 33.96 | 58.85 | 26.72 | 29.66 |
| 'Shitaochi' | 34.95 | 59.89 | 30.39 | 41.86 |
| 'Nkatekokoo' | 36.61 | 62.05 | 29.96 | 31.71 |
| 'Behenase' | 32.15 | 59.06 | 27.23 | 35.05 |
| 'Manipinta' | 24.82 | 51.52 | 23.65 | 28.79 |
| 'Otuhia' | 24.55 | 55.47 | 24.76 | 28.21 |
| 'GK7' | 20.30 | 53.50 | 26.94 | 31.62 |
| 'Nkatiesari' | 22.57 | 54.27 | 24.16 | 28.87 |
| 'Sumnut22' | 26.86 | 52.95 | 25.30 | 37.06 |
| Mean | 29.98 | 58.77 | 28.64 | 35.01 |
| CV (%) | 11.80 | 8.70 | 6.50 | 6.30 |
| Tukey's HSD (5%) | 8.359** | 12.259** | 4.289** | 5.199** |

**Legend:****-significant at p<0.01, DAS-days after sowing, HSD-highest significant difference value

Figure 1 a and b show early and late leaf spots infections on the leaflets of innoculated groundnut. Average leaf spots disease severity scores (early and late combined) and lesion diameters differed significantly (p<0.01) among the genotypes (Table 4). At 60 DAS, 'ICG7878', 'Jenkaar', 'Manipinta', 'GK7' and 'Nkatiesari' (*hypogaea* genotypes) had the lowest severity scores whiles '55-437', 'Konkoma' and 'Shitaochi' (*fastigiata*) had the highest. 'ICG7878' had the lowest score at 90 DAS whiles '55-437', 'Yenyawoso', 'Bremawuo', 'Kumawu', 'Konkoma' and 'Shitaochi' had the highest. Genotype '55-437' had the largest lesion diameter for both early and late leaf spots. 'Jenkaar' (a *hypogaea* subspecies) had the smallest early leaf spot lesion diameter whilst 'Manipinta' (a *hypogaea*) had the smallest late leaf spot lesion diameter. The lesion diameter ranged from 2.3 to 4.3mm for early leaf spot and 1.4 to 3.1mm for late leaf spot (Table 4). The disease components assessed were highly and positively correlated among each other (Table 5).

**Table 4** Mean leaf spots disease severity score (Scale 1-9) and lesion diameter (mm) among the groundnut genotypes

| Genotype | Severity score at 60 DAS | Severity score 90 DAS | ELS Lesion diameter 60DAS (mm) | LLS Lesion diameter 80 DAS (mm) |
|---|---|---|---|---|
| '1CG7878' | 3.00 | 4.00 | 2.73 | 2.00 |
| '55-437' | 5.00 | 7.00 | 4.30 | 3.10 |
| 'Obolo' | 3.20 | 6.00 | 3.10 | 2.40 |
| 'Oboshie' | 4.00 | 5.00 | 2.37 | 1.97 |
| 'Yenyawoso' | 4.00 | 7.00 | 3.47 | 2.63 |
| 'Bremawuo' | 4.00 | 7.00 | 3.50 | 2.50 |
| 'Kumawu' | 4.20 | 7.00 | 3.73 | 2.77 |
| 'Konkoma' | 5.00 | 7.00 | 3.23 | 2.70 |
| 'Jenkaar' | 3.00 | 5.00 | 2.23 | 1.80 |
| 'Adepa' | 4.00 | 5.00 | 2.77 | 2.10 |
| 'Nkosour' | 4.00 | 5.00 | 2.50 | 1.50 |
| 'Azivivi' | 3.20 | 5.80 | 2.93 | 2.07 |
| 'Shitaochi' | 5.00 | 7.00 | 4.07 | 2.90 |
| 'Nkatekokoo' | 4.00 | 6.00 | 3.43 | 1.97 |
| 'Behenase' | 4.00 | 6.00 | 3.47 | 2.37 |
| 'Manipinta' | 3.00 | 5.00 | 2.37 | 1.40 |
| 'Otuhia' | 3.40 | 5.00 | 2.67 | 1.63 |
| 'GK7' | 3.00 | 5.00 | 2.83 | 1.53 |
| 'Nkatiesari' | 3.00 | 4.20 | 2.53 | 1.70 |
| 'Sumnut22' | 3.80 | 5.00 | 3.10 | 1.50 |
| Mean | 3.79 | 5.70 | 3.07 | 2.13 |
| CV (%) | 6.20 | 2.50 | 9.00 | 10.30 |
| Tukey's HSD (5%) | 0.59** | 0.79** | 0.599** | 0.499** |

**Legend:** **-significant at p<0.01, DAS-days after sowing, ELS-early leaf spots, LLS-late leaf spots, HSD-highest significant difference value

**Table 5** Correlation among disease components (Phenotypic)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| **1** | - | | | | | | | |
| **2** | 0.76** | - | | | | | | |
| **3** | 0.66* | 0.72** | - | | | | | |
| **4** | 0.81** | 0.88** | 0.74** | - | | | | |
| **5** | 0.75** | 0.82** | 0.72** | 0.76** | - | | | |
| **6** | 0.64* | 0.67* | 0.77** | 0.73** | 0.83** | - | | |
| **7** | 0.72** | 0.77** | 0.73** | 0.83** | 0.59 | 0.66* | - | |
| **8** | 0.82** | 0.84** | 0.73** | 0.85** | 0.82** | 0.78** | 0.84** | - |

**Legend:** *, **-correlation coefficient significantly different from zero at p<0.05 and p<0.01, respectively, 1-percentage of leaves infected/plant at 60 DAS, 2-percentage of leaves infected/plant at 90 DAS, 3-severity score at 60 DAS,4-severity score at 90 DAS, 5-percentage defoliation at 75 DAS, 6-percentage defoliation at 90 DAS,**7**-ELS lesion diameter at 60DAS,8-LLS lesion diameter at 80 DAS.

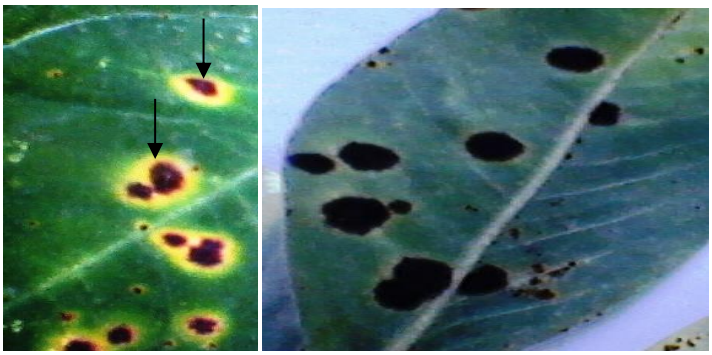

**Figure 1a** ELS on upper surface of innoculated groundnut leaflet
**Figure 1b** LLS on the lower surface of inoculated groudnut leaflet

**Legend:** ELS-early leaf spots, LLS-late leaf spots

A dendrogram based on the disease components data is shown in Figure 2. At the phenotypic level, the genotypes were grouped into two clusters at 0.85 similarity co-efficient. Cluster 'A' was made up of 12 genotypes; all were moderately resistant to leaf spots disease. Cluster 'B' was made up of eight (8) genotypes; all were susceptible except 'Nkatekokoo' and 'Behanase'.

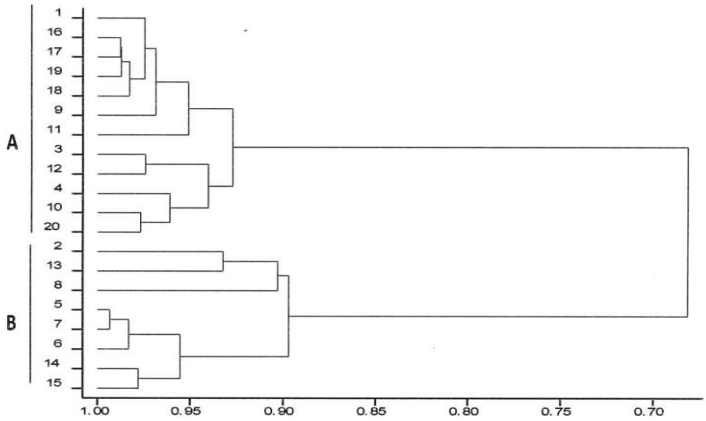


**Figure 2** Dendrogram of 20 groundnut genotypes based on phenotypic resistance to leaf spots constructed using Euclidean Similarity Matrix and Group Average method

**Legend:**1-'ICG7878', 2-'55-437', 3-'Obolo', 4-'Oboshie', 5-'Yenyawoso', 6-'Bremawuo', 7-'Kumawu', 8-'Konkoma', 9-'Jenkaar', 10-'Adepa', 11-'Nkosour', 12-'Azivivi', 13-'Shitaochi',14-'Nkatekokoo', 15-'Behenase', 16-'Manipinta', 17-'Otuhia', 18-'GK7', 19-'Nkatiesari', 20-'Sumnut22'.

Summary statistics of the eight SSR markers used to screen the 20 genotypes of groundnuts for leaf spot resistance are presented in Table 6. Primers PM384 and pPGPseq5D5 had the highest gene diversity and PMC588 had the lowest. The highest percentage heterozygosity was recorded for primers pPGPseq2F5, pPGPseq5D5 and PMC588 while PM 375 had the lowest. Polymorphic information content (PIC) values of the primers ranged from 0.55 to 0.85 with pPGPseq5D5 and PM384 having the highest values.

**Table 6** Allelic frequency, number of alleles, gene diversity, heterozygosity and PIC values

| SSR marker | Allele frequency | Allele no. | Gene diversity | Heterozygosity | PIC |
|---|---|---|---|---|---|
| pPGPseq2F5 | 0.26 | 11.00 | 0.84 | 1.00 | 0.83 |
| pPGPseq2B10 | 0.34 | 13.00 | 0.83 | 0.94 | 0.82 |
| pPGPseq13A7 | 0.32 | 10.00 | 0.79 | 0.95 | 0.76 |
| pPGPseq17F6 | 0.27 | 11.00 | 0.84 | 0.92 | 0.83 |
| PM384 | 0.20 | 10.00 | 0.87 | 0.87 | 0.85 |
| PM375 | 0.29 | 10.00 | 0.81 | 0.57 | 0.79 |
| pPGPseq5D5 | 0.21 | 9.00 | 0.87 | 1.00 | 0.85 |
| PMC588 | 0.50 | 3.00 | 0.63 | 1.00 | 0.55 |
| Mean | 0.30 | 9.62 | 0.81 | 0.91 | 0.78 |

**Legend:** PIC-polymorphic information content

A dendrogram based on the eight SSR markers showing genetic relationship among the 20 genotypes used is shown in Figure 3. The genotypes were clustered into five groups at 70% similarity index (Figure 3). Cluster 'A' comprises 14 genotypes. This group contains eight *hypogaea* and six *fastigiata* genotypes. All the genotypes in this cluster are commercially cultivated in Ghana, except 'ICG7878'. The genotypes in this cluster were moderately resistant to leaf spots under phenotypic screening, except 'Kumawu', 'Konkoma', 'Yenyawoso' and 'Bremawuo' which were susceptible and were also grouped separately within cluster 'A'. Two leaf spot-resistant *hypogaea* genotypes ('GK7' and 'Nkatiesari') were also grouped separately at 89% similarity index within cluster 'A'. Clusters 'B' and 'C' are made up of 'Shitaochi' and '55-437', respectively. These genotypes are *fastigiata* subspecies and susceptible to leaf spot disease under phenotypic screening. Cluster 'D' comprises three moderately resistant varieties ('Nkatekoo', 'Behenase' and 'Sumnut22') grown in Ghana. 'Nkatekokoo' and 'Behenase' which have red seed coat are grouped separately under this cluster at 74% similarity index. Finally, cluster 'E' is made up of 'Otuhia', a leaf spot-resistant *hypogaea* variety released together with 'Obolo', 'Oboshie' and 'Yenyawoso' in 2012 by CRI, Ghana (**Ghana Money News and Information, 2012**).

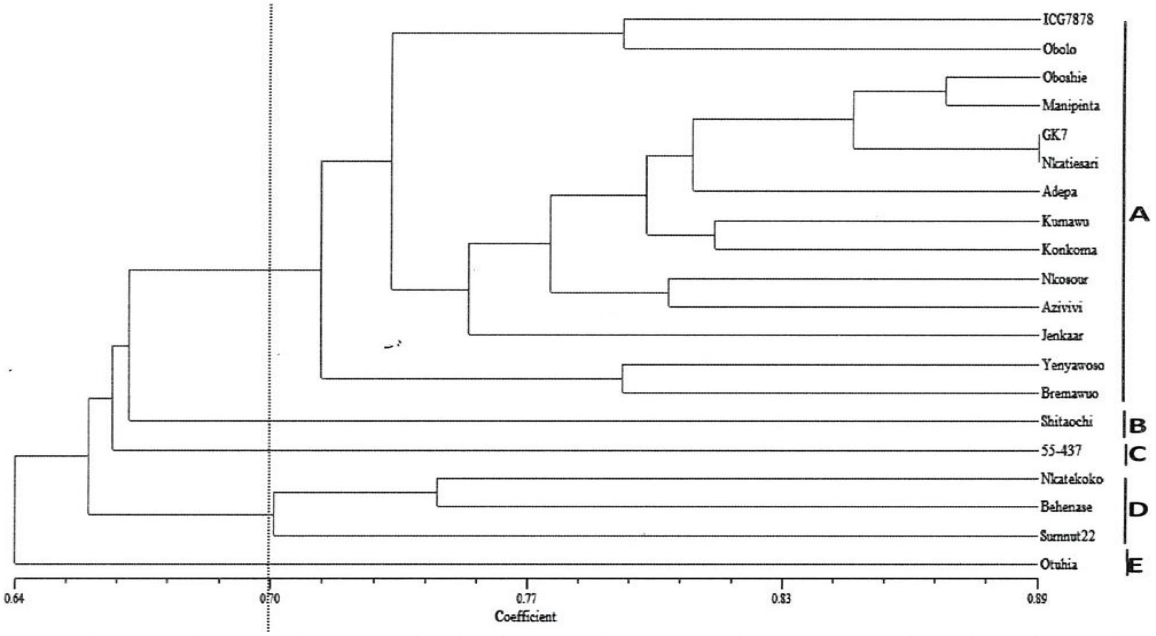


**Figure 3** Dendrogram of the 20 groundnut varieties based on the SSR markers constructed using UPGMA with Simple Matching similarity coefficient

Figure 4 shows the banding pattern of primer pPGPseq2F5. Primer pPGPseq2F5 identified 13 genotypes ('ICG7878', 'Obolo', 'Oboshie', 'Kumawu', 'Jenkaar', 'Adepa', 'Nkosour', 'Behenase', 'Manipinta', 'Otuhia', 'GK7', 'Nkatiesari' and 'Sumnut22') at the expected resistant allele (280bp).Primer pPGPseq2B10 identified 10 genotypes ('ICG7878', 'Yenyawoso', 'Jenkaar', 'Azivivi', 'Nkatekokoo', 'Behenase', 'Otuhia', 'GK7', 'Nkatiesari' and 'Sumnut22') at the expected resistant allele 280bp and additional three genotypes ('Nkosour', 'Azivivi' and 'Manipinta') at 290bp. Primer pPGPSseq13A7 identified three genotypes ('55-347', 'Yenyawoso' and 'Bremawuo') at the expected susceptible 305bp. Also, pPGPSseq17F6 alone was able to identify seven genotypes ('ICG7878', '55-437', 'Oboshie', 'Adepa', 'Nkatekokoo', 'Behenase' and 'Manipinta') at 120bp, four genotypes ('Jenkaar', 'Nkosour', 'Azivivi' and 'Nkatiesari') at 140bp and three genotypes ('Obolo', 'Nkatekokoo' and 'Sumnut22') at 150bp as resistant. PM 375 identified five genotypes ('Oboshie', 'Bremawuo', 'Behenase', 'Manipinta' and 'Nkatiesari') at 162bp and pPGPseq5D5 identified seven genotypes ('Kumawu', 'Konkoma', 'Jenkaar', 'Adepa', 'Azivivi', 'Shitaochi' and 'Behenase') at 220bp as resistant. Primer PM384 also identified 12 genotypes ('Obolo', 'Yenyawoso', 'Bremawuo', 'Jenkaar', 'Adepa', 'Azivivi', 'Nkatekokoo', 'Manipinta', 'Otuhia', 'GK7', 'Nkatiesari' and 'Sumnut22') as resistant at the expected 100bp allele. PMc588 identified 11 genotypes ('ICG7878', 'Oboshie', 'Jenkaar', 'Adepa', 'Nkosour', 'Azivivi', 'Manipinta', 'Otuhia', 'GK7', 'Nkatiesari' and 'Sumnut22') as resistant at 180bp and 220bp.

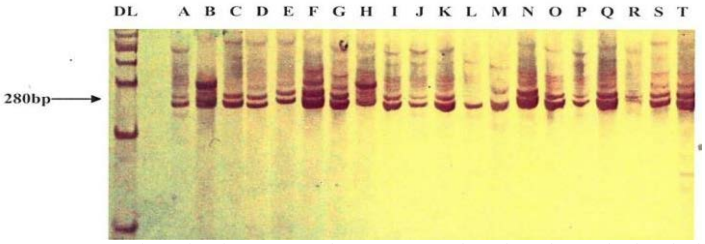


**Figure 4** Detection of resistant groundnut genotypes based on the 280bp band of Primer pPGseq2F5 PCR amplification products

**Legend:**DL-100bp DNA ladder, A-'ICG7878', B-'55-437', C-'Obolo', D-'Oboshie', E-'Yenyawoso', F-'Bremawuo', G-'Kumawu', H-'Konkoma',I-'Jenkaar', J-'Adepa', K-'Nkosour', L-'Azivivi', M-'Shitaochi', N-'Nkatekokoo', O-'Behenase', P-'Manipinta', Q-'Otuhia', R-'GK7',S-'Nkatiesari', T-'Sumnut22'.

## DISCUSSIONS

Disease development on individual plants from 60 to 90 DAS indicated that leaf spots disease affected a greater proportion of leaves in *fastigiata* subspecies compared to the *hypogaea* group. The susceptible cultivars had a higher percentage of leaves infected by the disease since severity score was highly and

positively associated with leaf spots incidence per plant. Leaf spots infection caused defoliation as seen by the high percentage of leaf defoliation at 75 DAS and 90 DAS, which was highly and positively associated with other disease components. Most of the moderately resistant cultivars were found among genotypes with low levels of defoliation. Thus, leaf spots resistant genotypes maintained more leaves on the plant than their susceptible counterparts. Generally, genotypes from subspecies *hypogaea* had lower disease severity scores. Phenotypically, no variety was completely resistant to *Cecospora* leaf spots disease. However, 14 genotypes ('ICG7878', 'Obolo', 'Oboshie', 'Jenkar', 'Adepa', 'Nkosour', 'Azivivi', 'Nkatekokoo', 'Behenase', 'Manipinta', 'Otuhia', 'GK7', 'Nkatiesari' and 'Sumnut22') were moderately resistant by 90 DAS. This finding confirms similar results reported by Gaikpa *et al.* (2015) about these groundnut genotypes under natural field infection.On the other hand, leaf spots scores recorded for genotype 'ICG7878',from ICRISAT, Niger,was in contrast to a score of 2 found in Burkina Faso (**Ntare and Waliyar, 1994**). The inconsistent performance of this genotype is not unusual since groundnut reaction to *Cercospora* leaf spots disease has been found as a polygenic trait (**Dwivedi *et al.* 2002; Janila *et al.*, 2013b**), hence highly influenced by environment.The quantity of innoculum, interaction among hosts, pathogen race and environment or heterogeneity of germplasm influence leaf spots disease severity in groundnuts (**McDonald *et al.*, 1985**). Lesion size was found to be larger in genotypes with higher disease severity scores. In a related work, **Dwivedi *et al.* (2002)** also found a significant and high correlation between lesion diameter and disease score. The lesion diameter for early leaf spots was larger than those of late leaf spots. The larger size of early leaf spot lesion could be as result of the fact that the causative pathogen, *C. arachidicola,*might grow faster than that of late leaf spot, *C. personatum.* Genotypes found to be moderately resistant in this study had a maximum ELS lesion size of 3.5mm and LLS lesion size of 2.4mm while more susceptible ones had a maximum ELS lesion size of 4.3mm and LLS lesion size of 3.1mm. This implies that leaf spots-resistant cultivars might have a mechanism (molecular or chemical) to inhibit the growth of the fungi and thus prevent formation of larger lesions on the surface of groundnut leaflets. Lesion diameter of early leaf spots was significantly and highly correlated with that of late leaf spots. This shows that varieties that were resistant to early leaf spots were also resistant to late leaf spots. This indicates a possible genetic linkage or host-plant physiology that confers resistance to both early and late leaf spot diseases in a groundnut population (**Janila *et al.*, 2013b**).

Polymorphic information content (PIC) value is an indication of how useful a genetic marker is for linkage analysis (**Elston, 2005; Shete *et al*., 2000**).The high PIC mean value (78%) observed in this study indicates that the markers were highly informative (**Hildebrand *et al*., 1992**) for the genotypes.The finding confirmed that the genotypes were of different genetic backgrounds. In earlier studies, high PIC values were reported for primers PM 384 and PM 375 (**Tang *et al.*, 2007**). Grouping of groundnut varieties with similar reaction to leaf spots disease within a cluster in this study for both phenotypic and molecular cluster analysis could indicate that a common gene confer leaf spots resistance in groundnut. Similarly, **Bera *et al.* (2014)** have reported clustering of groundnut genotypes with the same reaction to groundnut bud necrosis disease into one group.

Most of the groundnut genotypes identified by the molecular markers as resistant to leaf spots at the expected alleles reported by **Mace *et al.* (2006)** and **Shoba *et al.* (2012)** were also found to be resistant under phenotypic screening both in the present study and the previous field study of **Gaikpa *et al*. (2015)**. For instance, 12 genotypes (92.31%) identified by primer $pPGseq2F5_{280}$; all (100%) the genotypes identified by $PMc588_{180/220}$; 12 genotypes (92.31%) identified by $pPGseq2B10_{280/290}$; 10 genotypes (83.33%) identified by PM $384_{100}$;13 genotypes (92.86%) by $pPGSseq17F6_{120/140/150}$ and four genotypes (80.00%) identified by $PM375_{162}$ as resistant were also found to be moderately resistant to the disease under phenotypic screening. All the three susceptible genotypes indentified by $pPGPseq13A7_{305}$ agreed with phenotypic screening. This confirms that these genotypes were genetically resistant or otherwise susceptible to leaf spots disease. Therefore, the genetic composition of the groundnut genotypes might have accounted for their level of resistance to the disease at the phenotypic level. Moreover, in the absence of DNA molecular markers to identify resistance, morphological descriptors could also be used, in the interim, to select resistant genotypes.

## CONCLUSION

Resistant genotypes were found in the groundnuts studied both at molecular and phenotypic levels. The resistant genotypes confirmed by most of the markers were 'ICG7878', 'Obolo', 'Oboshie', 'Jenkar', 'Adepa', 'Nkosour', 'Azivivi', 'Nkatekokoo', 'Behenase', 'Manipinta', 'Otuhia', 'GK7', 'Nkatiesari' and 'Sumnut22'. Genotypes '55-437', 'Yenyawoso', 'Bremawuo', 'Kumawu', 'Konkoma' and 'Shitaochi' were susceptible. The SSR markers pPGseq2F5, pPGseq2B10, pPGPseq17F6, PMc588 and PM384 were able to detect most of the resistant groundnut genotypes. Hence, both molecular and phenotypic markers could be used for selection and breeding for leaf spot resistance in Ghanaian groundnuts. Future studies should aim at crossing the susceptible genotypes with the resistant ones. Also, development of more specific molecular markers for leaf spots resistance in groundnuts in Ghana should be given attention.

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# GENETIC VARIATION OF MAIZE GENOTYPES (*ZEA MAYS* L.) DETECTED USING SDS-PAGE

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**ABSTRACT**

The assessment of genetic diversity among the members of a species is of vital importance for successful breeding and adaptability. In the present study 20 old genotypes of maize from Hungary, Union of Soviet Socialist Republics, Poland, Czechoslovakia and Slovak Republic were evaluated for the total seed storage proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) through vertical slab unit. The number of total scorable protein bands was twentythree as a result of SDS-PAGE technique but those that were not cosistent in reproducibility and showed occasional variation in sharpness and density were not considered. Out of twentythree polypeptide bands, 6 (31%) were commonly present in all accessions and considered as monomorphic, while 17 (65%) showed variations and considered as polymorphic. On the basis of banding profiles of proteins of different kDa, gel was divided into zones A, B and C. The major protein bands were lied in zones A and B, while minor bands were present in zones C. In zone A out of 10 protein bands, 1 were monomorphic and 9 were polymorphic. In zone B out of 8 protein bands, 3 was monomorphic and 5 was polymorphic and in zone C out of 5 protein bands, 2 were monomorphic whereas 3 polymorphic. The dendrogram tree demonstrated the relationship among the forty registered old maize genotypes according to the similarity index, using UPGMA cluster analysis. The dendrogram was divided into three main clusters. The first cluster contained Czechoslovakia genotype Mikulická and the second cluster contained Czechoslovakia genotypes Celchovicka ADQ. Cluster 3 was divided into 3 subclasters. Similarly the present study of genetic variability in the seed storage polypeptide determined by SDS-PAGE technique proved that it is fruitful to identify genetic diversity among accessions of maize.

**Keywords:** maize; dendrogram; SDS-PAGE; genetic diversity

## INTRODUCTION

Maize (*Zea mays* L.) is an annual, cross-pollinated by wind and the only monoecious among cereal crops to have male and female inflorescences on separate branches of the same plant. It belongs to grass family *Poaceae* (Gramineae) which is leading in importance in the order Poales **(Bremer *et al*., 2003)**. This family contributes to the world economy, food and industry through valuable crops i.e. wheat, rice and maize **(Mabberley, 2008)**. Being most domesticated with controversy in origin and evolution, there is one school of thoughts that maize is the nearest descendant of Mexican teosinte **(Dowswell *et al*., 1996)**. There is no doubt that human beings directly or indirectly depend on plants for various purposes for which they domesticated these with the passage of time and flourished with spreading communities, undergone through evolution, passing through various cultivating methodologies throughout the world **(Larik, 1994)**.

Maize seed consists of two types of protein i.e., zein and non-zein protein. The term zein is used for prolamins in maize which is alcohol soluble protein and could be extracted with ethanol **(Lawton, 2006)**. Zein is major seed storage protein of maize **(Freitas *et al*., 2005)** and consists of one major and three minor classes and these four classes constitute approximately 50-70% of maize endosperm **(Vasal, 1999)**. The non-zein protein consists of globulins (3%), glutelins (34%) and albumins (3%). Zein is specific to maize endosperm **(Prasanna *et al*., 2001)** and not present in any other part of plant.

Proteins are primary gene products of active structural genes; their size and amino acids sequence are the direct results of nucleotide sequences of the genes; hence, any observed variation in protein systems induced by any mutagen is considered a mirror for genetic variations **(Hamoud *et al*., 2005)**. Variation in theDNAcoding sequences frequently causes variation in the primary conformation of the proteins. Determination of protein molecular weight (MW) via polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) is a universally used method in biomedical research; **(Ranjan *et al*., 2013)** concluded that electrophoresis (SDS-PAGE) of proteins can be economically used to assess genetic variation and relation in germplasm and also to differentiate mutants from their parent genotypes. Some studies used SDS-PAGE for detection of alterations in protein profiles occurring during exposure to electric field (**Hanafy *et al*., 2006; Dymek *et al*., 2012**). So far, several investigations on the discrimination between crop genotypes using SDS-PAGE have been carried out by **Yoon *et al*., (2010)**; **Osman *et al*., (2013); Chňapek *et al*., 2014; Iqbal *et al*., (2014); Iqbal *et al*., (2014); Khan *et al*., (2014); Petrovičová *et al*., 2014; AL-Huqail *et al*., (2015); Chňapek *et al*., 2015; Gregova *et al*., (2015); Kačmárová *et al*., (2016); Kuťka Hlozáková *et al*., 2015; Tomka *et al*., (2015); Socha *et al*., (2016).**

The objectives were to find out the level of genetic variability present in 20 maize germplasm by using the electrophoretic profiles of total seed proteins with different molecular weights through SDS- PAGE.

## MATERIAL AND METHODS

Maize genotypes (20) were obtained from the Gene Bank VURV Praha-Ruzine (Czech Republic) and from the Gene Bank in Piešťany, the Slovak Republic (Table 1).

SDS-PAGE was carried out according to the standard reference ISTA method **(Wrighley, 1992)**. Storage proteins were extracted from individually ground seeds using extracting using a buffer composed of 6.25 mL Tris (1.0 mol L-1, pH = 6.8), 10 mL glycerol, 12.05 mL $H_2O$ and 2.0 g SDS, diluted with mercaptoethanol and $H_2O$ in a 17:3:40 (v/v) proportion. The buffer was added to flour in a 1:25 (w/v) proportion. Extraction was performed at room temperature overnight and heating in boiled water for 5 minutes, centrifugation at 5000 xg for 5 min. 10 µl of extracts were applied to the sample wells. The gel (1.0 mm thick) consists of two parts: stacking gel (3.5% acrylamide, pH = 6.8 acrylamide) and resolution gel (10 % acrylamide, pH = 6.8). Staining of gels was performed in a solution of Coomassie Brilliant Blue R250 dissolved in acetic acid and methanol solution. Gel was scanned with densitometer GS 800 (Bio-Rad) and evaluated with Quantity One-1D Analysis Software.

**Table 1** List of 20 analyzed genotypes of maize

| | Genotypes | Country of origin | Year of registration |
|---|---|---|---|
| 1. | Moldavskaja | Union of Soviet Socialist Republics | 1964 |
| 2. | Bučiansky Konský Zub | Slovak Republic | 1964 |
| 3. | Hodoninský konský zub žltý | Czechoslovakia | 1964 |
| 4. | M Silokukurica | Hungary | 1964 |
| 5. | Valticka | Czechoslovakia | 1964 |
| 6. | Przebedowska Biala | Poland | 1964 |
| 7. | Toschevska | Slovak Republic | 1964 |
| 8. | Šamorinsky konský zub | Hungary | 1964 |
| 9. | Wielkopolanka | Poland | 1964 |
| 10. | Czechnicka | Poland | 1964 |
| 11. | Manalta | Czechoslovakia | 1964 |
| 12. | Zlota gorecka | Poland | 1964 |
| 13. | Celchovicka ADQ | Czechoslovakia | 1964 |
| 14. | Belaja mestnaja | Union of Soviet Socialist Republics | 1964 |
| 15. | Bučanská žltá | Slovak Republic | 1964 |
| 16. | Iregszemeseil 2 hetes | Hungary | 1964 |
| 17. | Dnepropetrovskaja | Union of Soviet Socialist Republics | 1964 |
| 18. | Bezuncukskaja | Union of Soviet Socialist Republics | 1964 |
| 19. | Mikulická | Czechoslovakia | 1964 |
| 20. | Aranyozon sarga lofogu | Hungary | 1964 |

## RESULTS AND DISCUSSION

The number of total scorable protein bands was twentythree as a result of SDS-PAGE technique but those that were not cosistent in reproducibility and showed occasional variation in sharpness and density were not considered. Based on these bands twenty accessions of maize (Table 1) were screened. Out of twentythree polypeptide bands, 6 (31%) were commonly present in all accessions and considered as monomorphic, while 17 (65%) showed variations and considered as polymorphic. The size of the protein bands obtained through SDS - PAGE ranged from 20 to 140 kDa. On the basis of banding profiles of proteins of different kDa, gel was divided into zones A, B and C (Figure 1). The major protein bands were lied in zones A and B, while minor bands were present in zones C. It was noted that different accessions of maize showed more diversity in seed storage proteins in minor bands in comparison to major bands. In zone A out of 10 protein bands, 1 were monomorphic and 9 were polymorphic. In zone B out of 8 protein bands, 3 was monomorphic and 5 was polymorphic and in zone C out of 5 protein bands, 2 were monomorphic whereas 3 polymorphic. By considering these facts zone A and B were more polymorphic.

The dendrogram tree (Figure 2) demonstrated the relationship among the twenty registered old maize genotypes according to the similarity index, using UPGMA cluster analysis. The dendrogram was divided into three main clusters. The first cluster contained Czechoslovakia genotype Mikulická and the second cluster contained Czechoslovakia genotypes Celchovicka ADQ. Cluster 3 was divided into 3 subclasters. In subclaster 3a was separated genotype Manalta (Czechoslovakia) from other 17 genotypes (Figure 2). Subclaster 3b is divided into subclaster 3ba and 3bb. Subclaster 3ba contained 12 maize genotypes and subclaster 3bb contained 3 genotypes from Union of Soviet Socialist Republics (Moldavskaja, Belaja mestnaja and Bezuncukskaja). Subclaster 3c contained one genotypes from Poland - Zlota gorecka and one genotypes from Union of Soviet Socialist Republics – Dnepropetrovskaja. Similarly the present study of genetic variability in the seed storage polypeptide determined by SDS-PAGE technique proved that it is fruitful to identify genetic diversity among accessions of maize.

Similar results were detected by other authors (**Yoon *et al*., 2010**; **Osman *et al.*, 2013; Iqbal *et al*., 2014; Iqbal *et al*., 2014; Khan *et al.*, 2014; Merza *et al*., 2014; AL-Huqail *et al*., 2015**) and these results presented a high level of polymorphism of old maize genotypes detected by SDS-PAGE. **Osman *et al.*, (2013)** study genetic relationship between some species of Zea mays using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of seed protein. Autors identified 78 bands across the studied species. The number of bands varies from 17 bands in sample number 5 to 6 in sample number 6. **Iqbal *et al*., (2014)** analyzed 73 genotypes of maize from China, Japan and Pakistan for the total seed storage proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A total of 18 protein bands were recorded. Among these 7 (39%) were monomorphic and 11 (61%) polymorphic, with molecular weight varied from 10 kDa to 122 kDa. The aim of **Iqbal *et al*., (2014)** was to estimate the genetic diversity across 83 genotypes of maize of Pakistan and Japanese origin using SDS-PAGE. A total of 18 protein subunits were noted out of which 7 (39%) were monomorphic and 11 (61%) were polymorphic, with molecular weight ranging from 10 to 122 kDa. Coefficients of similarity among the accessions ranged between 0.89 and 1.00. The dendrogram obtained through UPGMA clustering method showed two main clusters: 1 and 2. First cluster contained 9 genotypes, while second cluster contained 74 genotypes. **Khan *et al*., (2014)** study the variation of zein fraction of seed storage protein in maize by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Variation in terms of absence and presence, intensity and molecular size was observed in zein polypeptides. The study of **Merza *et al*., (2014)** was conducted to investigate the ability of total soluble seed protein to discriminate among 21 of maize genotypes through using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). According to SDS analysis , a total of 118 amplified bands were obtained ranging in their molecular size 18-86KDa. Five out of main nine bands were polymorphic and four monomorphic with an average of polymorphism reaches 55.5%. Phylogenetic tree divided 21 of corn genotypes between two major groups each of them divided in to two subgroups .The first main group included 13 genotypes , while the second main group included eight genotypes. **AL-Huqail *et al.*, (2015)** used SDS-PAGE to detection of 46 polypeptides bands with different molecular weights ranging from 186.20 to 36.00 KDa. It generated distinctive polymorphism value of 84.62%.

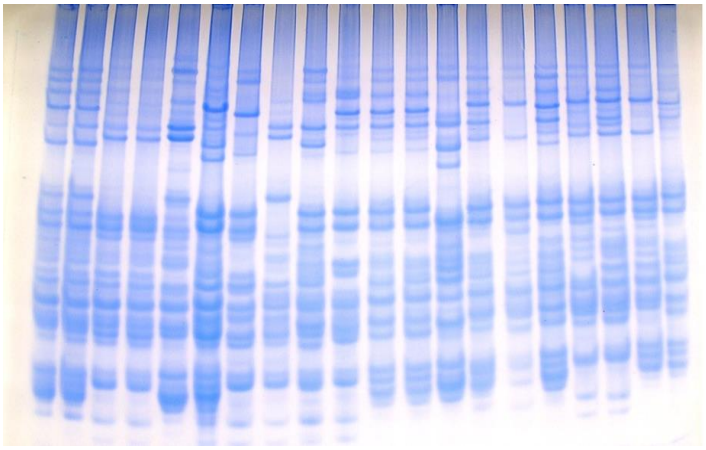


**Figure 1** Protein profile showing total seed storage proteins in maize genotypes as a result of SDS-PAGE. Lanes 1- 20 are maize genotypes (Table 1).

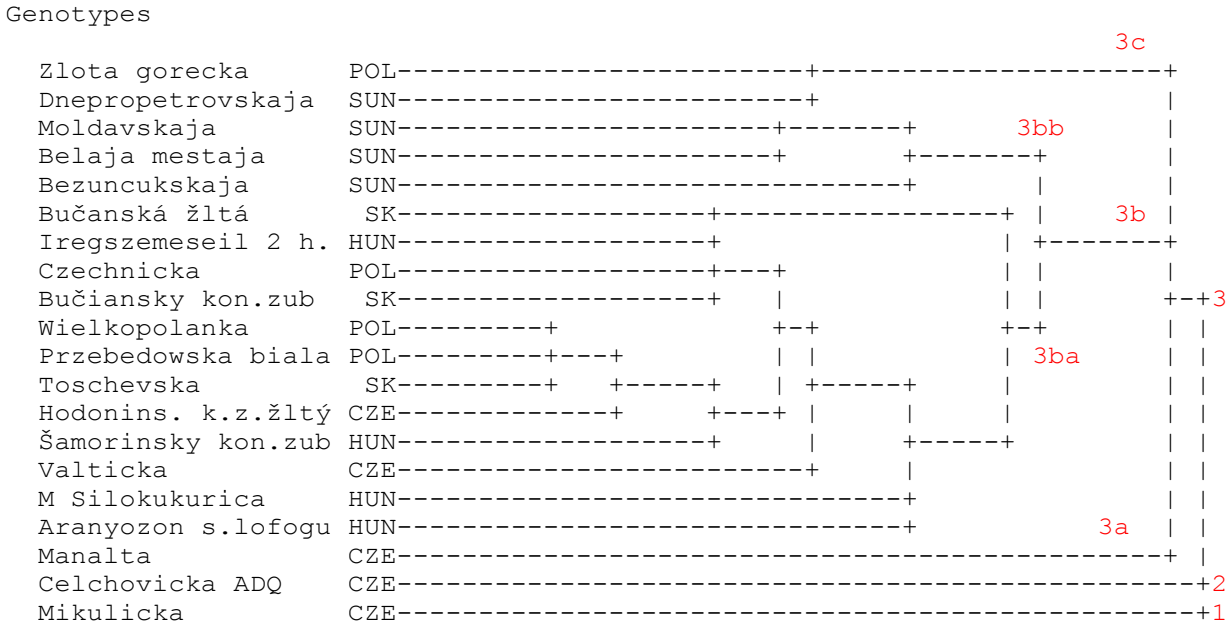


**Figure 2** Dendrogram of 20 maize genotypes prepared based on SDS-PAGE markers. CZE - Czechoslovakia, HUN - Hungary, POL - Poland, SUN – Union of Soviet Socialist Republics, SK – Slovakia

## CONCLUSION

SDS-PAGE techniques may provide useful information on the level of polymorphism and diversity in old maize genotypes. Twenty maize genotypes originated from the Gene Bank VURV Praha-Ruzine (Czech Republic) and from the Gene Bank in Piesťany, the Slovak Republic were very closely related. The dendrogram was divided into three main clusters. The first cluster contained Czechoslovakia genotype Mikulická and the second cluster contained Czechoslovakia genotypes Celchovicka ADQ. Cluster 3 was divided into 3 subclasters. In subclaster 3a was separated genotype Manalta (Czechoslovakia) from other 17 genotypes. Result from this study show that protein markers are powerful and efficient in characterising and identifying of old maize genotypes in addition to their usefulness in phylogenetic studies.

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# POLYHYDROXYALKANOATES: BIOSYNTHESIS TO COMMERCIAL PRODUCTION- A REVIEW

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**ABSTRACT**

The global increase in petroleum prices and the growing concern about the harmful effects of petroleum based plastics has led to a shift from a petroleum based economy to a natural feedstock based economy. One of the major outcomes of this economy shift is the global acceptance of biobased plastics such as Polyhydroxyalkanoates (PHAs) as a replacement for traditional plastics. Even though PHAs have been described as useful polymers due to their intrinsic biodegradability and biocompatibility, the high price has limited their application significantly. The raw material cost has been known to contribute significantly to the manufacturing cost of PHA. Production of PHAs using agro-industrial residues offers an alternative use of low-cost feedstock to produce materials with appropriate physicochemical properties to be used in a broad range of applications. Therefore, much research has been carried out using renewable cheap raw materials such as molasses, lignocellulosic wastes, sewage, industrial by-products, whey etc. to replace the expensive commercial medium, which should reduce the overall production cost. This review highlights various microorganisms, substrates and fermentation strategies used for economical production of PHA.

**Keywords:** Agro-industrial residues, fermentation, microorganisms, polyhydroxyalkanoates, polyhydroxybutyrate

## INTRODUCTION

Since 1950s, synthetic plastics or petroleum based plastics have emerged to be among the most needed materials in our daily life. These plastics are extremely stable in harsh conditions such as attack of chemicals and microbial decomposition, as a result of which they are quite durable, highly resistant and have a very long life span in the environment. Due to their excellent physical and chemical properties, these synthetic plastics have been ruling the commodity market since long. However, in spite of their useful qualities, petroleum based plastics are non-degradable and there are thousands of reports on the increasing environmental problems associated with discarded plastics. National Oceanic and Atmospheric Administration (NOAA) of the United States predicted the presence of high concentration of pelagic plastics and other debris in Central North Pacific Ocean in 1989 **(Day *et al*., 1989)**. This assembly of plastic debris is now known as the Great Pacific Garbage Patch. According to National Geographic's Encyclopedia, scientists have collected up to 1.9 million bits of plastic per square kilometer of Great Pacific Garbage Patch **(Lovett, 2010)**. Additionally, according to the United Nations' environment program, plastic is responsible for killing a million sea birds and 1, 00,000 marine mammals and turtles a year throughout the World. Increasing environmental awareness amongst the masses has thus, led the scientists to study polymers from alternate sources.

One such class of polymers which has the potential to compete with the synthetic plastics without having an adverse effect on the environment is known as Polyhydroxyalkanoates (PHAs). PHAs are biobased polymers with properties that closely resemble the properties of synthetic plastics. PHA's like synthetic plastics are moldable thermoplastics, and could be tailor-made for a number of applications varying from stiff packaging material to highly elastic materials used as coatings. Moreover the PHAs are completely biodegradable, thus making them a better option as compared to conventional plastics. PHAs are naturally produced by certain microorganisms and transgenic plants. PHB is a type of PHA that is resistant to ultraviolet radiations, water- insoluble and impermeable to oxygen. These attributes make PHB a suitable candidate for use as food packaging material **(Aarthi and Ramana 2011).** Since the past few decades the bioplastics industry throughout the World has been growing at a very fast pace owing chiefly to:

1. Encouragement of suppliers by retailers to adopt bioplastics for packaging
2. Public concern over the depletion of petroleum based raw materials
3. Greater acceptance by consumers due to eco-friendly nature.
4. Shift in the focus of manufacturing companies towards the development of sustainable raw material sources.
5. Improvement in the properties of bioplastics
6. Increasing government support to bio-based products.
7. Cost-effectiveness of bioplastics.

Inspite of having comparable physical properties to synthetic plastics and being environment friendly, the contribution of bioplastics to market is inconsequential. A major reason for this is low cost efficiency and yield properties of bio-based plastics. The cost of carbon source, fermentation process of the polymer, small production volumes and downstream processing (particularly purification) all contribute to their high cost of manufacturing. About 50% of the production costs of the PHA's are attributed to the cost of carbon source. According to a report by 'Bioplastics', global bioplastic market has been growing at a very fast pace over the past few years with an expected production rise to 5.779 million metric tons by 2016. In 2006, one kg of PHB costed €10-12 **(Kosior *et al*., 2006)** chiefly owing to the raw material and purification costs. However, with advancements in technology, prices as low as €1.50 $kg^{-1}$ have been achieved by certain companies. A number of studies have been conducted on the use of alternate crude carbon sources such as cornstarch, potatoes, sugarcane etc as discussed ahead. Although use of these carbon sources have led to a decrease in overall cost of production, but with the increasing food insecurity and increment in cost of these crops, the research focus has now shifted to non-edible agricultural residues. This review will discuss different aspects of PHA production highlighting the use of agro-industrial residues.

## BIOPLASTICS AND TYPES

### Classification of Bioplastics

Bioplastics are largely classified on the basis of their biodegradability, type of monomer structure and source of raw materials used. It should be noted by the readers that biodegradability is an inherent property of a material and is not the same as being biobased. Biobased materials can be non-biodegradable while petroleum-based plastics can be biodegradable.

Based on the structure of monomer, bioplastics can also be divided into three different categories viz. Short Chain Length (SCL), Medium Chain Length (MCL) and PHB copolymers containing monomers of short chain length and medium chain length (SCL-MCL) bioplastics **(Tripathi *et al*., 2012).**

A third approach to classification of bioplastics is on the basis of raw material used for their production. Commonly used types of bioplastics are based on cellulose, starch, glucose and oil etc. Specific techniques are employed to convert these feed stocks into bioplastics.

1. **Starch based Bioplastics:** Various raw materials such as raw starch, modified starch (such as thermoplastic starch), polylactic acid and other starch-derived sugars can be used for the manufacturing of Bioplastics. A number of starch sources such as maize, wheat, potatoes and cassava are in use. Thermoplastic starch is the most widely used bioplastic, accounting for more than 50 per cent of the global bioplastics market. Industrially, starch-based bioplastics are often blended with biodegradable polyesters. Pure starch is used for the production of drug capsules in the pharmaceutical sector because of its property to absorb humidity. By varying the amounts flexibilisers and plasticisers (e.g.sorbitol) the characteristics of starches can be tailored to specific needs. Though these blends are no longer biodegradable, however they display lower carbon footprint compared to conventional plastics. Thermoplastic starch generally represents one of the various constituents of starch based bioplastics. The other constituents consist of water repellent and biologically degradable polymers like polyesteramides, polyester, polyvinylalcohols or polyesterurethanes. Some of the starch based bioplastics include:

a) **Polylactic acid (PLA)**: is one such bioplastics that resembles with fossil fuel based plastics such as polyethylene terephthalate (PET), polyethylene (PE) and polypropylene (PP). PLA possesses an extraordinary stability and is highly transparent. In addition, its production does not require any changes in manufacturing facilities that already exist for the production of petrochemical based plastics. Major raw materials used for production of PLA include starch from crops such as corn, wheat or sugarcane and their fermentation into lactic acid followed by its polymerization. In case of corn, starch is first extracted and converted into dextrose followed by conversion into lactic acid by fermentation. This lactic acid is refined and used for manufacturing of different end-products. By changing the quality and composition of PLA, its biodegradability can be altered. One of the most significant disadvantages of PLA is that it softens at a temperature of about 60$^{o}$C and hence cannot be used for packaging of hot drinks and food. However, co-polymerization with heat resistant polymers and the addition of fillers can provide an alternative.

b) **Poly-3-hydroxybutyrate (PHB):** is a type of bioplastic produced by bacteria that process glucose or starch. Its characteristics are similar polypropylene, a crude oil derived plastic. PHB is different from most other biodegradable plastics as it is insoluble in water and resistant to hydrolytic degradation. A wide variety of PHA copolymers have been isolated from bacteria including marine freshwater cyanobacteria.

c) Polyethylene (PE): is a fossil based polymer which also can be produced by dehydration of bioethanol produced by fermentation of agro-industrial residues such as sugar cane or corn. Bio-polyethylene shares common chemical and physical properties with traditional polyethylene

2. **Oil based bioplastics**: Plant oils such as Palm oil, Soyabean oil and Corn oil are desirable raw materials for the production of bioplastics as they are relatively cheaper than most sugars.

a) **Poly-3-hydroxyalkanoate (PHA):** According to **Akiyama *et al*., 2003**, plant oils can provide higher cell biomass and PHA production (0.6 to 0.8 g of PHA per g of oil) due to their higher carbon content per weight as compared to sugars. Several bacteria are known to produce PHA from plant oils like, *Burkholderia cepacia*, *Pseudomonas aeruginosa, Comamonas testosterone and Cupriavidus necator* **(Kumar *et al*., 2011; Marsudi *et al*., 2008; Fukui and Doi 1998).**

b) **Polyamide11 (PA 11):** is a non-biodegradable biopolymer derived from natural oil such as castor beans. It has a wide range of applications such as use in automotive fuel lines, sports shoes, electrical anti-termite cable sheathing, electronic device components, oil and gas flexible pipes and catheters.

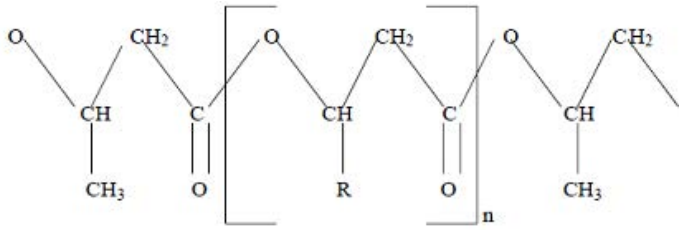
3. **Cellulose-based bioplastics**: Cellulose is the chief component of plant cell walls which is made of a large number of glucose monomers. Cellulose-based bioplastics are made from chemically-modified plant cellulose such as cellulose acetate (CA). Wood pulp, hemp and cotton are the common sources of cellulose.

4. **Lignin-based bioplastics**: Paper mill industry produces a large amount of lignocellulosic wastes as a byproduct. Lignin is a naturally occurring complex hydrocarbon and is the chief component of wood. Lignin differs from other hydrocarbons derived from sugars, starches and cellulose because it contains aromatic rings while polysaccharides contain long molecular chains. After cellulose, lignin is the most abundant renewable carbon source that is readily available, and can substitute many products currently sourced from petrochemical substances.

## POLY HYDROXY BUTYRATE (PHB)

### Composition and Occurrence

The occurrence of Polyhydroxyalkanoates in prokaryotic cells is known to be widespread. They are water insoluble compounds with general structure as shown in the Figure1.They are normally highly crystalline, optically active and possess piezoelectric properties. They are biodegradable, don't not leave any residue and have a melting point of 175° C. The melting point($T_m$), crystallinity and glass transition temperature ($T_g$) depend on the composition of the product **(Madison and Huisman 1999)**. One of the most common types of polyhydroxyalkanoates is PHB. PHA's are thermoplastic polymers that are highly viscous at higher temperatures and thus can molded into desired shapes. The application of PHB blends varies from the fabrication of glues to hard rubber. Wide varieties of bacteria are capable of synthesizing PHA as intracellular carbon and energy storage materials **(Doi 1990).** These polyesters are accumulated as a result of limiting bacterial growth and supplying an excess amount of a carbon source **(Chenyu *et al*., 2012).** Some reports suggest that prokaryotic organisms. However, PHB is brittle and hence needs to be synthesized as copolymers of 3-hydroxybutyrate and other hydroxyalkanoates with a relatively low molecular weight and melting point **(Fukui and Doi 1998).**




n varies from 600 to 35000

| | |
|---|---|
| R= hydrogen | Poly(3-hydroxypropionate) |
| R=methyl | Poly(3-Hydroxybutyrate) |
| R=ethyl | Poly(3-hydroxyvalerate) |
| R=propyl | Poly(3-hydroxyhexanoate) |
| R=pentyl | Poly(3-hydroxyoctanoate) |
| R=nonyl | Poly(3-hydroxydodecanoate) |

**Figure1** General structure of PHA **(Lee, 1996a)**

### Synthesis of Bioplastics

The Polyhydroxyalkanoic acids, especially PHBs are prepared by many prokaryotes and some eukaryotes in adverse or stress conditions, mainly in nutrient limited conditions. Bacterial genome contains cluster genes for PHA and other proteins related to the metabolism of PHA **(Rehm 2003)**. The genes for class I PHA synthase (phaC), β-ketoacyl-COA thiolase (phaA) and NADP-dependent acetoacetyl-CoA reductase (phaB) constitute the pha CAB operon **(Peoples and Sinskey 1989**).In order to synthesize PHB, two molecules of acetyl-CoA condense to form acetoacetyl-CoA. This reaction is catalyzed by β-ketoacyl-COA thiolase. Subsequently an enzyme named acetoacetyl-CoA reductase reduces acetoacetyl-CoA to (R)-3- hydroxybutyryl-CoA which is then used as a monomer to polymerize PHB by PHB synthase (Figure 2**) (Rehm 2003; Madison and Huisman 1999)**.

**Gouda *et al*., (2001)** also found that environmental stresses such as carbon and nitrogen limitation favor the synthesis of PHAs. A possible explanation to the accumulation of PHA under nutrient limiting conditions can be attributed to the inhibition of enzyme β-ketothiolase by CoA-SH. In conditions of oxygen limitation, the final electron acceptor is lacking, leading to an increased $NADH/NADH^{+}$ ratio. As a result, many acetyl-CoA molecules cannot enter the TriCarboxylic Acid (TCA) cycle resulting in a decreased CoA-SH concentration. β-ketothiolase is not inhibited anymore and can direct acetyl-CoA molecules to PHA production **(Vollbrecht and Schlegel 1979)**.

Moreover, nitrogen or phosphate limitation results in a reduced activity of anabolic pathways leading to ATP excess which causes acetyl CoA accumulation leading to PHA production in a manner similar to that for oxygen limitation. It has however been observed that complete depletion of a nutrient causes growth cessation resulting in a decreased PHA storage capacity **(Khanna and Srivastava 2005).**

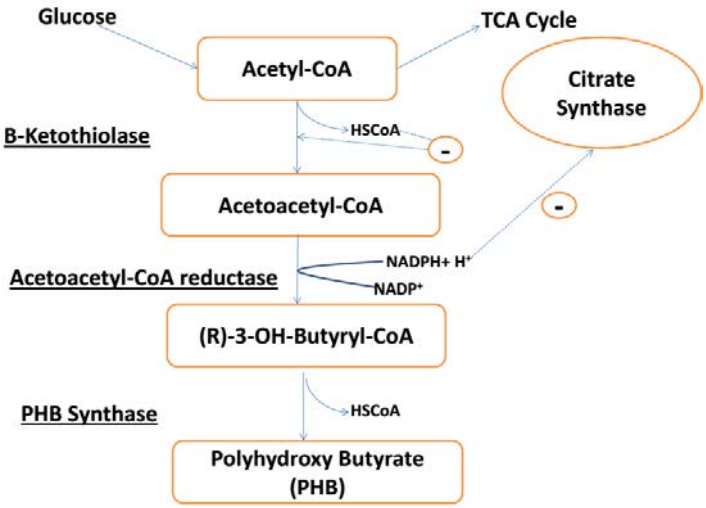



**Figure 2** Biosynthesis of Polyhydroxy Butyrate

**Microorganisms used for PHB production**

Microorganisms belonging to different groups including eubacteria, cyanobacteria, and archaebacteria along with eukaryotes have been found to produce varying amounts of bioplastics depending upon their nutritional and environmental conditions. Bioplastics derived from transgenic plants are also quickly gaining importance. The next section aims to give an overview of the microorganisms capable of synthesizing PHBs.

**Eubacteria**

Lemoigne, a French scientist for the first time in the year 1925 reported accumulation of PHB in the form of cytoplasmic inclusions in Gram positive bacterium *Bacillus megaterium*. Thereafter, a number of bacterial strains among archaebacteria **(Doi 1990),** Gram positive **(Findlay and White 1983)**, Gram negative bacteria **(Shah, 2014)** and photosynthetic bacteria **(Hassan *et al*., 1997)** including cyanobacteria (**Jau *et al*., 2005)** have been found to be associated with PHB accumulation. Most PHA synthesizing bacteria have been *reported* to belong to pseudomonad, coryneform, and bacillus groups which include *Pseudomonas, Bacillus*, *Citrobacter, Enterobacter, Klebsiella* and *Escherichia* **(Arshad *et al*., 2007)**. PHB production is widespread in nitrogen fixing organisms like *Rhizobium*, *Azotobacter beijerinckii, A. macrocytogenes*, *A. vinelandii* **(Tombolini and Nuti 1989; Senior *et al*., 1972; Stockdale *et al*., 1968; Page and Knosp 1989).** Bacteria used for PHA production are classified into two groups depending on the culture conditions favoring PHA accumulation: The first group comprises of bacteria that require excess carbon source and limitation of essential nutrients such as oxygen and nitrogen for the efficient synthesis of PHA. The representative bacteria belonging to this group include *C. necator*, *Protomonas extorquens* and *P. oleovorans*.
The second group includes bacteria that can accumulate PHA during exponential phase and do not require nutrient limitation. Some of the bacteria included in this group are *Alcaligenes latus*, A*zotobacter vinelandii* and recombinant *E. coli* harboring the PHA biosynthetic operon of *C. necator* **(Khanna and Srivastava 2005)**. Culture conditions for PHA biosynthesis are an important criteria for the development of cultivation techniques for large scale production of PHA.
In addition to the above mentioned microorganisms, methylotrophs have also been found to produce PHB but give a low yield **(Suzuki *et al*., 1986)**. Recombinant microorganisms, in particular recombinant *E. coli*, containing PHA biosynthesis genes from *A. eutrophus* has been able to accumulate high amounts PHA (80-90 % of the cell dry weight**) (Lee 1996a).** Nutrient limitation is not required for the synthesis of PHB by recombinant *E. coli* strains which depend on the available amount of acetyl-CoA. PHA production in recombinant *E. coli* has many advantages: the bacterial cells grow really fast to a high cell density which results in high productivities, a large amount of polymer is usually accumulated, several inexpensive carbon sources can be utilized, the PHA can be easily purified **(Hahn *et al*., 1995)** and there is no depolymerase system in recombinant *E. coli* that can break down the synthesized polymers **(Lee, 1996a)**. On the other hand, PHA production by recombinant *E. coli* implies a very high oxygen demand during the high cell density culture of recombinant *E. coli*. This major drawback should be tackled in order to make the process economically feasible.

**Cyanobacteria**

Cyanobacteria are also known to accumulate PHA by utilizing $CO_2$ and sunlight as carbon and energy sources. These oxygen-evolving photosynthetic bacteria naturally possess PHA synthase enzyme **(Sudesh *et al*., 2000).** However, till date only PHB homopolymer has been identified in most cyanobacteria. Among the various cyanobacteria that are capable of synthesizing PHA, *Spirulinapla tensis* UMACC 161 **(Jau *et al*., 2005)** and *Synechocystis* sp. PCC6803 **(Sudesh *et al*., 2001**) can accumulate PHB upto 10 % of the dry cell weight. *Synechococcus sp.* MA19 and *Nostoc muscorum* have been reported to produce PHB under phosphate limited conditions **(Saharan *et al*., 2014).** A study showed that *Nostoc muscorum* could produce PHB five times higher under mixotrophy, chemoheterotrophy with nitrogen-limiting state than what was produced under photoautotrophic conditions **(Sharma and Mallick, 2005**). Use of Cyanobacteria ability to produce PHB with energy obtained from sunlight can result in reduction of cost and $CO_2$.

**Archaea**

Haloarchaeal strains do not require strict sterile conditions because of the high salt concentrations required in their growth medium to maintain cell wall stability. This makes cultivation more convenient and easier as compared to eubacterial strains. Moreover, cell walls of haloarchea easily lyse in the absence of salt, especially in distilled water; thus enabling the recovery of PHB and poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) from extreme halophiles much more easy and economical **(Lillo and Rodriguez-Valera 1990, Han *et al*., 2010)**. A number of haloarchaeal genera, viz., *Haloferax, Haloarcula, Haloquadratum, Halorubrum, Halobiforma, Halorhabdus, Halalkalicoccus, Halobacterium, Natrianema, Halostagnicola, Natrinema, Natronobacterium, Natronorubrum, Haloterrigena, Halopiger*, and *Halococcus* have been reported to produce different types of PHA's **(Tekin *et al*., 2012)**.

**Eukaryotes**

Eukaryotic cells are not known to synthesis PHB, though it has been reported that yeast and some other eukaryotic cells do contain small amounts of low molecular mass PHBs which function as complexes with polyphosphates in membrane transport. Moreover, production of PHBs especially in plants through genetic engineering is being evaluated as a potentially inexpensive alternative to prokaryotic production **(Madison and Huisman 1999**). PHB production has been reported in *Saccharomyces cerevisiae, S. diastaticus, Candida krusei, C. tropicalis, Kloeckera apiculata, Kluyveromyces africans, K. lactis, Rhodotorula glutinis* etc.
Scientists are striving to come up with high PHB and related bioplastic producing strains of bacteria as well as eukaryotes. The importance of investigating novel strains lies in the possibility of replacing well-known industrial production strains with new ones, aspiring to a more productive and efficient polymer production process.

**Production of PHB from Agro - industrial residues**

The growing concern about the harmful effects of plastics has given impetus to the search of biodegradable alternatives which can compete with the petrochemical based plastics being used worldwide currently. However to produce an economically viable biodegradable plastic, it is necessary to focus on the cost efficiency and yield properties in respect to the production of synthetic plastics based on petrochemicals. The substrate and recovery costs in PHB production by fermentation are very high, making their use unattractive. Carbon source for PHB production accounts for up to 50% of the total production costs **(Shivkumar 2012).**One of the ways to cut down the production cost for bioplastic is the use of cheap, readily available industrial and agricultural waste as carbon and nitrogen source. The following section discusses the various agri-industrial residues reported to have been used in the production of PHB's:

**Molasses**

It is a viscous by-product produced during refining of sugarcane, grapes, or sugar beets into sugar. It is extensively used as a carbon source in industrial scale fermentations due to its relatively low price and abundance **(Zhang *et al*., 1994**). In time 1992, for the first Page reported the accumulation of PHB by *Azotobacter vinelandii* UWD on sugar beet molasses achieving PHB concentration of 19 to 22 g/L. **Chen and Page (1997)** improved the PHB production (36g/L) using a two-stage fermentation strategy by *A. vinelandii UWD.* **Albuquerque *et al*., (2007)** on the other hand reported a three-step fermentation strategy for the production of PHAs from cane molasses. Step I was designed to allow fermentation of molasses into organic acids, followed by initiation of PHA accumulation in step II and eventually PHAs were produced using batch fermentation. **Bengtsson *et al*., (2010)** further investigated PHAs synthesis using fermented molasses and a consortium of microorganisms. The PHA yields were in the range of 0.47 to 0.66 C-mol PHA per C-mol of total carbon substrate. **Khardenavis *et al*., (2007)** reported PHB accumulation of up to 60% by waste activated sludge using molasses spentwash as substrate. *Pseudomonas aeruginosa* has also been reported to utilize cane molasses for the production of PHB in submerged fermentation processes **(Tripathi *et al*., 2012)**. **Naheed *et al*., (2012)** reported the production of PHB using sugar cane molasses by *Enterobacter sp.* and *Enterobacteriaciae.* **Chaijamrus and Udpuay (2008)** investigated PHB

production using *B. megaterium* ATCC 6748. A maximum of 43% PHB on cell dry weight was obtained using a feed containing 4% sugar cane molasses and 4% corn steep liquor (CSL). Similar PHB yields have been reported by various groups using different strains of *B. megaterium* **(Gouda *et al*., 2001; Kulpreecha *et al*., 2009; Ghate *et al*., 2011)**.

Besides sugar beet molasses and sugar cane molasses, media based on soy molasses (with high sucrose content) have also been used for PHA accumulation in. **Solaiman *et al*., (2006a)** attempted to produce mcl-PHA from soy molasses using *Pseudomonas corrugate* and achieved a yield of 5-17% of PHA's such as 3-hydroxyl-dodecanoate, 3-hydroxyl-octanoate and 3-hydroxytetradecenoate. Conversion of saccharides in soy molasses into PHA has also been reported in *Bacillus.* sp CL1 with a PHA accumulation of up to 90% of CDW **(Full *et al*., 2006)**. **Law *et al*., (2001)** reported isolated *Bacillus* strains, HF-1 and HF-2 capable of synthesizing PHB from hydrolyzed soy and malt wastes. **Hameiah *et al*., (2013)** reported PHB production of 0.412mg/50ml and 0.367g/50ml by *Lactobacillus acidophilus* and *Bacillus thuringiensis* respectively using date molasses as substrate.

**Whey and whey hydrolysates**

It is a by-product of diary and cheese industry that constitutes of the watery portion collected after the separation of fat and casein from whole milk. Cheese whey is normally produced in volumes almost equal to the milk processed in cheese manufactories. The disposal of whey therefore causes serious pollution problems in the surrounding environment due to its enormous biochemical oxygen demand. **Lee *et al*., (1997)** constructed different recombinant *Escherichia coli* strains expressing *Cupriavidus necato*r phaC2 gene for the production of PHB from whey, out of which one isolate grew up to 5.2 g cell dry weight per Liter (CDW/L), 81% of which was PHB. **Ahn *et al*., (2000)** used a similar recombinant *E. coli* strain CGSC 4401 and a whey solution, achieving a yield of 96.2 g PHB/L in 37.5 hours. Besides recombinant *E. coli*, the potential of PHA synthesis from whey has also been exploited using various common PHA producing bacteria, such as *Ralstonia eutropha* DSM545 **(Marangoni *et al*., 2002)**, *Pseudomonas hydrogenovora* **(Koller *et al*., 2008)**, *Thermus thermophilus* HB8 **(Pantazaki *et al*., 2009)** and wild strains, such as *Methylobacterium* sp. ZP24 **(Nath *et al*., 2008),** *Hydrogenophaga pseudoflava* DSM1034 **(Koller *et al*., 2011).**

**Povolo and Casella (2003)** reported the production of PHA directly from cheese whey permeate by *Paracoccus denitrificans* DSM 413, *Sinorhizobium meliloti* 41. *Thermus thermophilus* HB8 (DSM 579) was reported to utilize lactose from whey-based media for the synthesis of polyhydroxyalkanoates under nitrogen limitation **(Pantazaki *et al*., 2009). Baei *et al*., 2010** reported the production of poly-3(HB-co-27%-HV) from whey hydrolysate using *Azohydromonas lata* DSM 1123. *Bacillus megaterium* CCM 2032 was shown to accumulate more than 50% of its biomass (w/w) in optimized whey media **(Obruca *et al*., 2011)**.

Fermentation strategies for the production of PHB from whey by recombinant *Escherichia coli* strain CGSC 4401 harboring the *Alcaligenes latus* PHA biosynthesis genes were developed. This recombinant *E.coli* was reported to accumulate 96.2 g/liter of PHA in 37.5h of incubation. PHAs production by recombinant *Escherichia coli* (DH10B and JM10), harboring the structural genes of the polyhydroxyalkanoate synthases of *Pseudomonas aeruginosa*, using hydrolyzed corn starch and soybean oil as substrate, cheese whey as supplement and acrylic acid as fatty acids β-oxidation inhibitor has also been reported **(Fonseca and Antonio 2006). Hameiah *et al*., (2013)** reported PHB production (0.337g/50ml) by *B. subtilis* using whey after an incubation period of 4 days. PHB yield of 25% and 28% was obtained when *R. elti* and *R. stutzeri* were grown in medium containing whey as carbon source **(Belal 2013)**. *B. thuringenesis* IAM12077 has been reported to utilize whole cheese whey and whey supernatant for the accumulation of PHB. PHB yield of 3.99g/L and 3.3g/L was obtained in cheese whey and whey supernatant respectively **(Shivkumar 2012)**. **David *et al*., (2013)** reported a PHB yield of 62% by *Azotobacter chrooccum* using whey as substrate.

**Lignocellulosic materials**

Significant increase in food prices in the recent years and growing food insecurity has brought the usage of food products in bulk biochemical and biofuels production under scrutiny, forcing the industrial biotechnology development to its focus on to the utilization of non-food biomass, such as lignocellulosic raw materials **(Lin *et al*., 2012)**. Lignocellulosic biomass consists of 30-50% cellulose, 20-50% hemicellulose and 15-35% lignin. Cellulosic biomass is inedible therefore its usage is not in direct competition with food or animal feed production. But one of the main drawbacks in the use of lignocellulosic raw materials for the production of PHAs is the recalcitrant nature of lignocellulose. Therefore, pretreatment/hydrolysis steps are required to generate the sugar feed stocks that can be metabolized by microbes to produce the bioplastics. In an earlier report in 1990s, **Bertrand *et al*., (1990)** showed that *Pseudomonas pseudoava* was able to grow on the hemicellulosic fraction of poplar wood until 30% of hydrolysate concentration producing 17 to 22% PHB. **Keenan *et al*., (2006)** used *Burkholderia cepacia* ATCC 17759 to produce 1.3-4.2g PHBV/L using xylose with levulinic acid as a precursor for HV monomers.

Among various agricultural residues, wheat straw is one of the most abundant biomass that can be used as a cheap carbon source for the production of bioplastics. **Van-Thuoc *et al*., (2008)** reported the use of wheat bran for the production of PHB by *Halomonas boliviensis*. Wheat bran was hydrolyzed by a crude enzyme preparation from *Aspergillus oryzae* NM1 to provide a mixture of reducing sugars composed mainly of glucose, mannose, xylose and arabinose. Growth of *Halomonas boliviensis* using wheat bran hydrolysate in the medium led to a PHB content of 34 wt%. PHB yield of 64% was achieved by *A. chroococcum* using wheat bran as a substrate **(David *et al*., 2013)**. In another report, PHB content of 68% was achieved by *B.sphaericus*NCIM5149 when grown on a media containing wheat bran **(Ramadas *et al*., 2009)**. **Cesario *et al*., (2014)** reported the production of PHB by *Burkolderia sacchari* DSM17165 using wheat straw hydrolysates. *B. sacchari* metabolized glucose, xylose and arabinose, the main sugars present in wheat straw hydrolysates giving a PHB yield of 0.18g/L. **Latha *et al*., (2013**) reported the production of PHB using wheat bran by *P. aeruginosa, P. alcaligenes, P. cereus, P. fluorescens.* While maximum PHB production of 10g/L was observed in case of *P. aeruginosa*, the other strains exhibiting PHB production varying from 0.4-0.9g/L.

Besides wheat bran, there are several reports on the use of rice bran for the production of PHA's. **Latha *et al*., (2013)** also reported the ability of *Pseudomonas* sp. to utilize rice bran and saw dust for PHB production. *P. aeruginosa* accumulated maximum PHB amount in both the substrates (1.4and 1.01g/L). Extruded rice bran (ERB) has been used as carbon sources for the production of PHB by an archaea, *Haloferax mediterranei*. By using ERB as substrate cell PHA concentration of 77.8 g/L and PHA content of 55.6 wt. % was obtained in repeated fed-batch fermentation **(Huang *et al*., 2006).** *R. elti* and *P. stutzeri* have been reported to achieve PHB yield of 16.7 and 14.9% respectively using rice straw hydrolysates **(Belal 2013). Shivakumar (2012)** reported PHB accumulation by *B. thuringiensis* IAM 12077 on different carbon sources like wheat bran (0.07 g/L), wheat germ (0.14 g/L), rice bran (0.21g/L) and ragi bran (0.32 g/L). In addition to wheat and rice bran, several other agricultural residues have also been evaluated for the production of PHB. Jawar stem, a waste product after harvesting Jawar crop (*Sorghum bicolor*), usually used as cattle feed or as fuel in rural areas contains moderate amount of sugar. **Ghate *et al*., (2011)** reported the use of hydrolyzed jawar stem for PHB production by *B. subtilis* and *B. cereus* (0.034 and 0.049gm/L). Coir pith hydrolysates have also been reported to be used as alternate carbon source for PHB production by *Azotobacter beijerinickii.* Production of PHB (2.4 ± 0.2 g/L) was maximized at pH 6.5 with 3% coir hydrolysates **(Sathesh Prabu and Murugesan 2010).**

**Patel (2014)** reported the ability of *P. aeruginosa* to utilize Rice husk, Cotton seed husk, Walnut shell and Corn cob meal for the production of PHB. **Preeti *et al*., (2012)** reported 42% PHA production from bacteria isolated from soil which could metabolize jambul seed (*Syzygium cumini*). **Saha *et al*., (2013**) used candy factory waste and fruit processing factory waste as substrates for PHA production by *Azotobacter chroococcum* MAL-201.They obtained PHA accumulation of 40.58 and 22.40 % on candy factory waste and fruit processing factory waste respectively. Oil palm empty fruit bunch (OPEFB), contains abundant cellulose and hemicelluloses and can be used as a renewable resource for fuel and chemical production. **Zhang *et al*., (2013)** reported the use of OPEFB derived sugars to produce polyhydroxybutyrate (PHB) *Bacillus megaterium* R11. It was observed that *B. megaterium* could accumulate PHB up to 51.3% of its cell dry weight (CDW) from both glucose and xylose.

**Glycerol**

Glycerol is the main by-product of the biodiesel industry with about 10% (v/v) of the volume of biodiesel. Due to the high volume co-production of glycerol, the world market price of glycerol has dropped rapidly making this by-product, a potential substrate for microbial production of PHAs **(da Silva *et al*., 2009).** *Pseudomonas putida* KT2442 has been reported to produce mcl-PHA from glycerol **(Solaiman *et al*., 2006b). Bormann and Roth (1999)** demonstrated the production of PHB from glycerol and casein hydrolysates as carbon and nitrogen sources, by using *Methylobacterium rhodesianum* and *C. necator,* which produced up to 50% and 65% PHB in 45 h, respectively. **Ashby (2005)** investigated PHA synthesis by *Pseudomonas oleovorans* NRRL B-14682 and *Pseudomonas corrugata* 388. **Sujatha and Shenbagarathai (2006)** constructed a recombinant *E. coli* strain with the phaC1 gene from *Pseudomonas* sp. LDC-5 which gave a yield of 3.4 g PHAs/L on glycerol and fish peptone derived medium. **Ashby *et al*., (2004)** used crude glycerol, derived from a soy-based biodiesel production site, for PHA production using *P. oleovorans* NRRL B-14682 and *P. corrugata* 388. Promising results were published by **Cavalheiro *et al*., (2009)**, in which *C. necator* DSM 545 was cultivated up to 68.8 g CDW/L on waste glycerol. **Zhu *et al*., (2010)** reported that *Burkholderia cepacia* ATCC 17759 could synthesize poly-3-hydroxybutyrate (PHB) from glycerol with concentrations ranging from 3% to 9% (v/v).

**Fats, Vegetable Oils and Waste Cooking Oils**

Fatty acids are known to deliver more energy per mole on conversion to PHA as

compared to carbohydrates **(Solaiman *et al*., 2006b).** Until recently, the use of triacylglycerols (TAG) as a feedstock for production of PHA was a challenge, mainly due to its hydrophobic nature and inherent difficulties encountered during the fermentation process. However, **Shimamura *et al*., (1994)** demonstrated that *Aeromonas caviae* was able to biosynthesize PHAs directly from pre-treated (hydrolyzed) TAG. **Cromwick *et al*., (1996)** showed that *Pseudomonas resinovorans* accumulated PHA to upto 15% of its cell dry weights from tallow. **Ashby and Foglia (1998)** further investigated PHAs production by *P. resinovorans* using a whole range of TAGs, such as butter oil, lard, coconut oil, olive oil and soybean oil for the production of mcl-PHA. It was observed that the type of monomers had a strong relationship with the type of substrate. Coconut oil containing high levels of saturated fat produced saturated PHA monomers while soybean oil containing high levels of unsaturated fat produced unsaturated PHA monomers. Various bacterial strains belonging to *Pseudomonas*, *Acinetobacter, Sphingobacterium, Brochothrix*, *Caulobacter, Ralstonia, Burkholderia*, and *Yokenella* genera, that are capable of producing PHB while degrading oil have been isolated from oil contaminated sites **(Saharan *et al*., 2014).** Readers can refer to the following articles discussing PHAs production from palm oil **(Wu *et al*., 2009),** olive oil **(Ntaikou *et al*., 2009),** corn oil **(Chaudhry *et al*., 2011)**, coconut oil **(Thakor *et al*., 2005**), soy bean oil **(Kahar *et al*., 2004**) and other vegetable oils and animal fats (Table1).

**Table1** Bacteria used for production of PHA from plant oils and wastes

| Strains | PHA Type | Substrates | References |
|---|---|---|---|
| *Alcaligenes latus* DSM 1124 | PHB | Soya waste, malt waste | **Yu *et al*.,1999** |
| *Bacillus megaterium* | PHB | Beet molasses, date syrup | **Omar *et al*., 2001** |
| *Burkholderia* sp. USM (JCM 15050) | PHB | Palm oil derivatives, fatty acids, glycerol | **Chee *et al*., 2010** |
| *Comamonas testosteroni* | MCL-PHA | Castor oil, coconut oil, mustard oil, cottonseed oil, groundnut oil, olive oil, sesame oil | **Thakor *et al*., 2005** |
| *Cupriavidus necator* | PHB | Bagasse hydrolysates | **Yu and Stahl 2008** |
| *Cupriavidus necator* H16 | P(3HB-co-3HV) | Crude palm kernel oil, olive oil, sunflower oil, palm kernel oil, cooking oil, palm olein, crude palm oil, coconut oil + sodium propionate | **Lee *et al*., 2008** |
| *Cupriavidus necator* DSM 545 | PHB | Waste glycerol | **Cavalheiro *et al*., 2009** |
| *Recombinant Cupriavidus necator* | P(3HB-co-3HHx) | Palm kernel oil, palm olein, crude palm oil, palm acid oil | **Loo *et al*., 2005** |
| *Recombinant Escherichia coli* | P(3HB-co-3HHx-co-3HO) | Soybean oil | **Foncesa and Antonio 2006** |
| *Pseudomonas aeruginosa* IFO3924 | mcl PHA | Palm oil | **Marsudi *et al*., 2008** |
| *Pseudomonas aeruginosa* NCIB 40045 | mcl PHA | Waste frying oil | **Fernandez *et al*., 2005** |
| *Pseudomonas guezennei* biovar. Tikehau | mcl PHA | Coprah oil | **Simon-Colin *et al*., 2008** |

**Wastewater**

Production of PHAs from wastewater provides an economically viable alternative. Various organic wastewaters, such as municipal wastewater **(Coats *et al*., 2011),** biodiesel wastewater **(Dobroth *et al*., 2011)**, food processing waste effluent **(Reddy *et al*., 2012)**, brewery waste effluent **(Liu *et al*., 2011)** and kraft mill wastewater **(Pozo *et al*., 2012)** have been tested for PHAs biosynthesis. Most of the cases involve conversion of organic carbon into volatile fatty acids in aerobic activated sludge in the first step, followed by PHA production using mixing cell cultures in the second step. Although the final PHAs concentrations are still low at the current investigated conditions, PHAs could accumulate to around or even over 50% of the cell dry weight in some cases **(Liu *et al*., 2011**). Vinasse, a highly polluting waste of the ethanol industry was utilized for the production of polyhydroxyalkanoate (PHA) by the extremely halophilic archaeon, *Haloferax mediterranei* leading to 70% maximum accumulation of PHA **(Bhattacharyya, 2012).** The production of PHB by *Bacillus subtilis* NG220 was observed utilizing the sugar industry waste water supplemented with various carbon and nitrogen sources to yield 5.297g/L of PHB accumulating 51.8 %( w/w) of biomass **(Singh *et al*., 2013**).

**UPSTREAM AND DOWNSTREAM PROCESSING**

A number of fermentation strategies have been reported for the production of PHB. Process selection depends upon the type of culture used, substrates, physiological conditions, fermentation processes and methods employed for the recovery of the final products. This section highlights the various upstream and downstream processes used in PHB production.

**Fermentation Strategies**

***Pure culture PHA production***

Pure cultures of PHA producing bacteria can be divided into two groups:
1. Non-growth associated: The non-growth associated PHA producing bacteria, for example *C. necator* and *Pseudomonas* species require nutrient limitation to accumulate PHA. Biomass growth and PHA accumulation are typically performed in two separate stages: The first stage is associated with biomass growth due to the availability of nutrients. In the second stage, due to the limitation or depletion of one nutrient PHA production prevails.

2. Growth associated PHA producing bacteria: These organisms such as *Alcaligenes latus* and recombinant *Escherichia coli* don't require nutrient limitation and PHA accumulation and growth occur simultaneously **(Lee, 1996b)**. However, nutrient feeding strategy can be applied to obtaining PHA production in fed-batch process). In fed-batch cultures of growth-associated PHA producing bacteria, a nutrient feeding strategy is essential for obtaining high PHA production yields. This is because both cell growth and PHA synthesis can be enhanced as both processes occur at the same time. The two processes need to be balanced in order to avoid low PHA levels **(Khanna and Srivastava, 2005)**.

***Mixed culture PHA production***

Mixed Microbial Cultures (MMC) are defined as group of different micro-organisms growing together on the same substrate. Three main processes are used to produce PHA from a mixed culture:

1. **Anaerobic-aerobic (AN/AE) process:** PHA production using the AN/AE system comprises of three steps. In the first Activated Sludge Treatment Plant (ASTP) is used for culture enrichment. This is followed by fermentations of industrial waste water and agro-industrial residues through acidogenesis into substrate containing Volatile Fatty Acids (VFAs). These VFAs are then used for PHA production (Figure 3). However, under anaerobic conditions, low amounts of PHAs are produced **(Satoh *et al*., 1996).** One of the ways of improving PHA accumulation is to create a micro-aerophilic environment which allows substrate oxidation for increased energy generation **(Satoh *et al*., 1998; Takabatake *et al*., 2000)**.

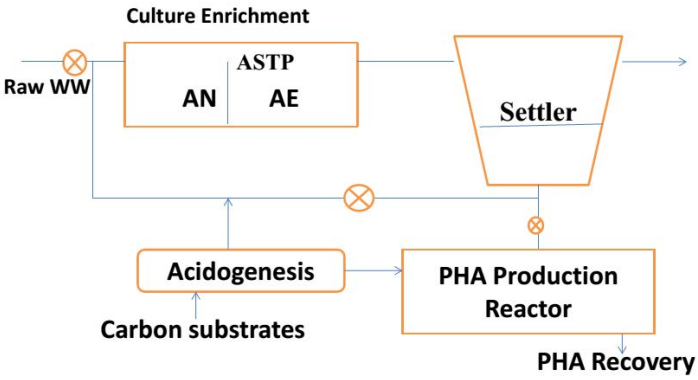



**Figure 3** Anaerobic-aerobic Process

2. **Aerobic dynamic feeding (ADF) system (feast and famine):** ADF is the strategy of transient carbon supply where long periods of substrate shortage (famine period) are alternated with short periods of substrate excess (feast period) in a fully aerobic reactor (Figure 4). The PHA production occurs due to an intracellular component limitation. In the long periods of carbon limitation (famine), the macromolecular composition of the cells changes. As a consequence, the micro-organisms need a physiological adaptation when they are exposed to high substrate concentrations (feast). Since synthesis of polymers requires less physiological adaptation than cell growth, storage is the faster response to the transient substrate supply **(Dias *et al*., 2005, Daigger and Graddy 1982).** Although product is only formed during the feast phase, the famine phase is also very important for the process feasibility. The famine periods should be short in order to obtain high volumetric productivities but on the other hand, they should be long enough to guarantee high and stable PHA storage capacities on the long term **(Dias *et al*., 2005).** Glycogen and PHAs are the most prevailing polymers produced using ADF strategy. This is because during the feast phase, substrate uptake rate is very high which results in $NADH_2$ formation which is subsequently converted into ATP through oxidative phosphorylation. Once the ATP requirement for growth processes are satisfied, $NADH_2$ starts accumulating resulting in production of storage polymers. Since, PHB production is $NADH_2$ consuming in contrast to glycogen production. PHB is the most common produced polymer. In such cases, PHB serves as a $NADH_2$ sink. In depleted carbon source conditions, the accumulated PHB can be utilized as an internal carbon and energy source for growth **(van Aalst-van Leeuwen *et al*., 1997)**. Only PHA producing organisms can develop during the famine period by degrading the PHA polymer. So during famine periods, cell growth of PHA accumulating organisms is not inhibited but continues at a more or less constant rate and during feast periods, an efficient competition for substrate takes place **(Beun *et al*., 2000).** The length of the feast and famine periods must be chosen to allow complete substrate consumption and significant depletion of the accumulated PHA respectively **(Paul and Liu, 2012).**

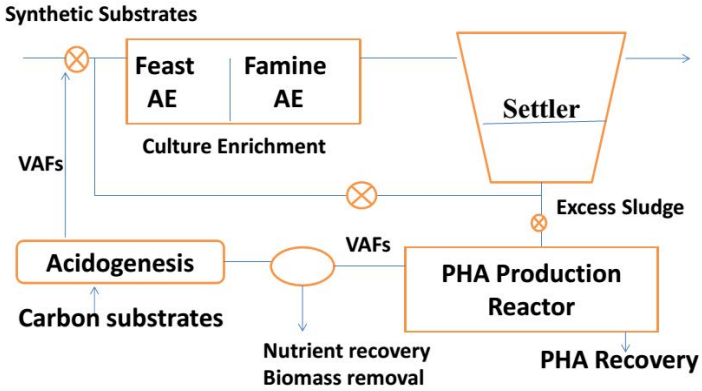



**Figure 4** Aerobic Dynamic Feeding System

3. **Fed-batch process under nutrient growth limitation:** In this strategy, carbon substrate from industrial waste water and organic wastes is fermented through acidogenesis into substrate containing VFAs which are used for PHA production in a fed-batch reactor (Figure 5). The sludge present in the PHA production reactor originates from ASTP in which AN/AE or ADF conditions are established. This strategy can only be applied if the cells are previously formed and PHA accumulation is the only goal. Table 2 outlines the major difference in PHB production by using pure and mixed cultures.

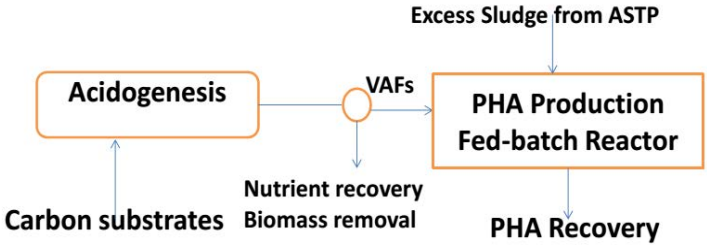



**Figure 5** Fed-Batch Process

**Table 2** Comparison between Pure culture and Mixed culture PHA production

| PHA Production | Pure Culture | Mixed Culture |
|---|---|---|
| **Conditions** | External nutrient limitation<br>Excess carbon source | Internal nutrient limitation<br>AN/AE or ADF |
| **Substrate Requirements** | Single substrate | Waste Materials |
| **Growth** | Separately | Simultaneously |
| **Media Requirements** | Synthetic Media | Complex Media |
| **Reactor Configuration** | Fed-batch | Sequential Batch Reactor |
| **Advantages** | Higher volumetric productivity | Cheap substrates |
| **Disadvantages** | Expensive Substrate<br>Expensive equipment for maintaining aseptic operation | Low volumetric productivity |

## DOWNSTREAM PROCESSING

Since PHA is an intracellular product, the method applicable for the effective separation of PHA from other biomass components can be complex and costly. Various methods have been reported for the recovery and purification of PHAs from biomass:

**Solvent Extraction**: This is the most commonly used method for the extraction of PHA from biomass. Among various solvents, chloroform is the most preferred solvent to carry out PHA extraction without its degradation **(Hahn *et al*., 1995**). Other halogenated hydrocarbon solvents such as dichloromethane, dichlorethane and chloropropane can be also used to extract and purifiy PHA from the cell biomass though these solvents can be potentially hazardous to health and environment **(Ramsay *et al*., 1994**).

**Cell lysis by Sodium hypochloride**: In this method, the cell biomass is initially treated with sodium hypochlorite solution before the PHA granules are isolated from the cell debris by centrifugation **(Berger *et al*., 1989).** The use of sodium hypochlorite to extract PHA from biomass always results in severe degradation of PHA and yields PHA with a lower molecular weight (MW). In contrast, the use of surfactant pretreatment to recover PHA results in lower purity but less degradation of MW.

**Enzymatic digestion**: is a gentle but a selective separation method. Enzymes such as proteases (trypsin, chymotrypsin, rennin, papain and bromelain), cellulases and lysozyme, are commonly used in this method **(de Koning and Witholt 1997)**. Specificity of enzymes and mild operational conditions employed result in high reaction rates with little product damage. However, this recovery process requires that the culture broth should be provided with a short period of heat shock treatment before the enzymatic treatment so as to rupture the cells as well as denature and solubilize the nucleic acids. Without this preliminary heating step, the release of nucleic acid into the medium will result in a very viscous suspension **(Kapritchkoff *et al*., 2006).**

## CONCLUSION

As discussed in the preceding sections, raw material cost is one of the major reasons for the high price of PHAs. This necessitates the use of various cheap carbon sources for the PHAs production. However, the major challenge in using

these substrates is the low fermentation efficiency and final PHAs concentration of these fermentations which leads to an increasing cost in product separation and purification. It has been observed that a change in the substrate from pure sugars to agro-industrial residues does not compromise with the properties of the PHAs. Therefore, the selection of the substrate should be made based on balanced considerations keeping in view the efficiency, costs and sustainability of the final product.

**Conflict of Interest**: All authors have no conflict of interest to report.

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