International Journal of Engineering Science Invention
ISSN (Online): 2319 – 6734, ISSN (Print): 2319 – 6726
www.ijesi.org ||Volume 4 Issue 11|| November 2015 || PP.23-28

Comparative study on screening methods of polyhydroxybutyrate (PHB) producing bacteria's isolated from root nodules of selected leguminous plants

Manju J[1], Prabakaran P[2]
[1,2] *Department of Microbiology, Maruthupandiyar College of arts and science, Vallam, Thanjavure, TN, India*

ABSTRACT: *The study was conducted to isolate diverse group of bacteria capable to produce ecofriendly biopolymer from root nodulating plants collected at Thanjavur district. 25 colonies were isolated from five different nodules plants namely Crotalaria albida, Aeschynomene indica L, Vigna trilobata, Vigna mungo L and Phyllanthus amarus respectively from the family of Apocynaceae, Fabaceae and Euphorbiaceae. Aeschynomene indica, Crotalaria albida and Phyllanthus amarus showed maximum Colony forming unit than Vigna trilobata L & Vigna mungo. Colonies on Yeast extract mannitol salt agar are morphologically diverse and showed different characteristics. It was observed that most of the colonies were puncti and circular, colourless to light pink, convex, entire and rhizoidal rod shaped, aerobic, non-spore forming and few were cocci and pleomorphic. Screening of Polyhydroxy butyrate (PHB) method reveals that Fluorescent and Sudan black B satin methods are effective. Out of 25, 11 isolated strains were found to be PHB Producers by acridine orange satin. Direct screening and Carbol fuchsin methods were found to be ineffective. Of these PHB producers, majority of isolates were found to be Gram negative in nature.*

Key words; *Legumes, PHA, Rhizobium, nodules*

I. Introduction

The largest and most widely distributed angiosperm is Legumes, which plays significant role in ecological microbial community structure (Sprent, 2001). Studies on interaction of living organism with native plant and their geographical distribution are relatively scare (Weir et al., 2004). Legumes and symbiotic interaction associated bacterial community restricted into two important groups namely alpha and beta proteobacteria. Isolates of *Azorhizobium, Rhizobium, Bradyrhizobium, Sinorhizobium* (Ensifer), *Devosia, Phylobacterium* and *Methylobacterium* are belongs to α-proteobacteria. Similarly Species of Genera *Burkholderia, Cupriavidus, Ralstonia, Ochrobacterium* and *Herbaspirillum* are grouped under β proteobacteria (Martens et al., 2007; Chen et al., 2001; Vandamme et al., 2002; Trujillo et al., 2005; Zakhia and deLajudie, 2001). Secretion of root exudates and Plant growth promoting bacteria facilitate the attachment of bacteria to host (Hayat et al., 2010). Bacteria tolerate micro aerobic environment only adopt the root system and can induce nodulation. Microbe's associate or exhibit symbiotic relationship with Legumes play a critical role in biogeocheical cycle on earth. The diverse group of microorganism constitute about 60% of the earth's biomass and soil sustains about $4–5 \times 10^{30}$ microbial cells (Singh et al., 2009). In soil ecosystem, microorganism plays vital role in soil structure formation, recycling of minerals, decomposition of complex organic matter, modulating biogeochemical cycle. Thus, the entire organism in the biosphere directly or indirectly depends on microbial activities (Garbeva et al., 2004). Recently, Sy et al. (2001) reported *M. nodulans*, a facultative methylotrophic and only species of that genus to form nodule and fix nitrogen in the legume Crotalaria sp. *Blastobacter* spp. is a common aquatic (freshwater) budding bacteria that form nodules in flood tolerate legumes (Van Berkum and Eardly, 2002). Bacteria are isolated from root nodules are documented as good Polyhydroxyalkanoates (PHA) producers. Polyhydroxybutyrate (PHB) is the most frequently isolated PHA and widely used for the production of bioplastic. PHB produced intracellularly by many microbes such as *B.megaterium, R.eutrophus, Azatobacter spp, Rhizobium sp, Alcaligens eutrophus* and *Pseudomonas spp* (Khanna, et al., 2005; Koller et al., 2008; Kuniko et al.,1988; Anderson et al., 1990). These bacteria's can accumulate more than 70% of PHB under nitrogen starvation with high Carbon (Philip et al., 2007). PHB are completely degraded in to CO_2 and H_2O under natural environment by different microorganisms (Mercan, 2002). The higher cost of industrial production of PHB limits the commercial application of bioplastic. It's well understood that the cost of production of microbial product can significantly reduced by the optimization of low cost media. Therefore it's necessary to isolate bacteria capable to compete the cost wise production as compared to synthetic polymer. With this challenge the present work is designed to isolate PHB producers from different selected legumes plats.

II. Materials and method

2.1. Collection and identification of legume plants

Root nodule producing legume plants were collected from Tanjure district, Tamilnadu, INDIA during September 2013 and the herbarium was submitted at Botany department, Saint Joshep College, Tiruchirapalli for identification.

2.2. Isolation of bacteria from nodules

Root samples were thoroughly washed with running tape water and then rinsed twice with 70% ethanol. Healthy root nodules were sterilized with 3 % hydrogen peroxide. About 10 g of root nodules were crushed and mixed with 100 ml of autoclaved distilled water. The mixture was serially diluted with sterile distilled water until it reached 10^{-9}. A modified Yeast extract mannitol agar supplemented with bile salt (0.01 g/L) and congo red (0.001g/L) was used for isolation of bacteria. About one ml of 10^{7} samples was used for pour plate technique. Plates were incubated under 35 degree Celsius for 72 h. colonies with distinct colony morphology were selected at end of every 24 h incubation.

2.3. Culture condition for PHB production

To determine the growth pattern of various isolates, cultures were grown in Yeast Extract Mannitol (YEM) broth (yeast extract 0.5g/L, Mannitol 10g/L, K2 HPO4 0.5g/L, KH2PO4 0.5g/L, MgSO4 0.7H2O 0.2g/L, NaCl 0.1g/L, CaCl2 . 2H2 O 0. 06g/L) at 30ºC for a period of 48h in a shaker incubator at 150 rpm. A sample of 1mL was withdrawn after 48 h interval to check the PHB accumulation.

2.4.1. Direct screening

For the rapid detection and isolation of PHB producing bacteria, 0.02% alcoholic solution of Sudan black B was applied to strain of bacterial colonies on YEMA plates and the plates were kept undisturbed for 30 min. The excess dye was then decanted and plates were rinsed gently by adding 100% ethanol. Colonies unable to incorporate the Sudan black B appeared white, while PHB producers appeared bluish black

2.4.2. Microscopic method

2.4.2.1. Carbol fuchsin staining

Carbol fuchsin staining was performed to determine the intracellular production of PHB by the isolate. A thin smear of all the isolated were stained with carbol fuchsin stain for 45 s. The isolates capable of producing PHB showed dark colored granules of PHB.

2.4.2.2. Sudan black stain (Hartman, 1940).

Thin smear of bacterial cultures was prepared and stained with 0.3% sudan black stain for 10 minutes. The slides were destained with xylene and then air dried. The stained slides were examined under 1000x magnification using Nikon photomicroscope

2.4.2.3. Fluorescent staining

Cell pellets were mixed with 0.2% of acridine orange for 30 m and the centrifuged at 7000 rpm. The pellet was resuspended in 50 µl phosphate buffer and smear was examined under inverted fluorescence microscope under 400 X.

III. Results and Discussion

3.1. Isolattion of root nodule associated bacteria

The collected plant samples were identified accseesd as *Crotalaria albida* Heyne ex Roth (SJCBOT2101) *Aeschynomene indica L* (SJCBOT 2102), *Vigna trilobata L* (SJCBOT2103) *Phyllanthus amarus* Schum&Thonn (SJCBOT2104) and *Vigna mungo* L (SJCBOT2105) respectively, and deposited at Dept.of. Botany, St.josheph's College, Tiruchirapalli, Tamilnadu, INDIA. Nodules were surface sterilized to ensure that soil bacteria would not interfere with future analysis of the nodules. Nodules varied in mass and size; the smallest of the nodules had an approximate mass of 1.4 mg and diameter of 8 mm. The largest nodules had a mass of 80 mg and diameter of 70 mm. A total of five root and 25 bacterial colonies were obtained from nodule of collected plants on YEMA medium. The frequency of isolates among the plant samples were 6 ≥7≥3≥5≥4. The color of colony and Grams reaction were given in table 1. The dominant colony morphology had a circular form with a smooth edge and was whitish cream in color. Most of the colonies were opaque, umbonate and glistening surface texture. All the isolates were stained with Gram's reagents for detection of their Gram reaction. Out of 25, 60 % isolates were found to be Gram negative, 24 % isolates comes under Grams variable rod and 16 % were Grams's positive. Among these isolates, four were Gram's positive rods, six were Gram's variable slender rod, nine were Gram's negative rod, five of isolates were Grams's negative cocci and one is Gram's negative pleomorphic in nature. *Crotalaria albida* and *Aeschynomene indica L* showed maximum

number of isolates on YEMA plates. Similar report were also reported Malina singha and Sharma during 2013. For many years, a limited number of bacterial species were believed to be nitrogen fixers, but in the last 30 years nitrogen fixation has been shown to be a property with representatives in most of the phyla of bacteria and also in methanogenic Archaea (Young, 1992). The nitrogen fixing methylotrophic bacteria *Methylobacterium nodulans* was frequently isolated from the nodules of *Crotalaria juncea* and *Sesbania aculeata* (Madhaiyana et al., 2009). Leguminous plants are most frequently symbiotically associated with the rhizobia and less frequently by nonrhizobial (Glick, 1995; Willems, 2006) nodule-associated bacteria (NAB). Of these 25 colonies, a total of seven colonies belong to *Rhizobium* sp produced colorless gummy colonies on YEMA media plates and rest of the colonies are differing from Rhizobium colonies. Gram negative bacteria's are most frequently isolated from root nodule among several environmental conditions (Hungria and Vargas, 2000). Many leguminous plant species can enter into a symbiotic relationship with a-subclass of Proteobacteria (α-rhizobia). However, some tropical legumes are nodulated by strains of *Burkholderia* and *Ralstonia* species belonging to the β-subclass of Proteobacteria (Chen et al. 2003). These finding supports the cooperative interaction between rhizobia and other plant root colonizing bacteria and their role in nodulation and N_2 fixation in legume plants. Occurrence of Gram positive bacteria also reported in many studies. The occurrence of *Bacillus* species as endophytes has been reported from different plants such as pigeon pea, wheat, and soybean nodules (Oehrle et al., 2000; Ryan et al., 2008). They have been shown to benefit to their hosts by promoting nodulation and growth.

3.2. Screening of PHB producers

Although, previous research has shown that a large number of bacterial species, both Gram positive and negative, produce PHBs under limited nitrogen condition (Verlinden et al., 2007), not much work has been done with Root nodule associated non rhizoidal colonies. In the present study, attempts were made to screen PHB from root nodule associated bacterial species. To distinguish PHB producers from non-producers, Sudan black B, carbol fuchsin and Acridine orange were used and compared. Among the methods, Fluorescent stain was found to be very effective method followed by Sudan black microscopic stain. It was found that nearly 11 of the isolated were detected as PHB producers. Of these 11, five were Gram two isolates were Gram positive and four isolates were Grams variable rods. This staining permits to detect even the small size of PHB which are accumulated in cell (Fig1). Upon staining with carbol fuchsin, three gummy colonies were found to have dark colored granules of PHB which comes under Grams negative rods (Fig 2). Further Sudan black screening reveals that, five of isolates showed dark black to purple granules (Fig 3) were confirm the production of PHB which includes three Gram's negative rod and two Gram's positive rod. In this screening direct screening reveals that three out of 25 colonies were positive. The lipophilic staining with Sudan Black B (SB staining) reportedly has high sensitivity in PHA screening (Burdon, 1946). In the present work, 0.3% solution of Sudan black B in 60% ethanol (w/v) was used and PHB was observed as dark black to purple granules against pink background when counterstained with safranin. Eventhough there are more than 250 different microorganisms synthesizing PHAs, only several of these, such as *Alcaligenes eutrophus* (Kim et al., 1994), *Alcaligenes latus* (Yamane et al., 1996), *Azotobacter vinelandii* (Page and Knosb, 1989), Methylotrophs (Kim et al., 1996), *Pseudomonas oleovorans* (Brandl et al., 1988) and recombinant *Escherichia coli* (Lee et al., 1997) are widely used for the production of PHAs to a high concentration with high productivity. Screening of less explored microbes like nodule associate bacteria's may help to increase the productivity and efficient screening methods like fluorescent screening may permits to screen novel bacterial isolates. Some investigators (Dhingra, 2012) have emphasized that the optimum temperature varies somewhat with the composition of the medium for the enhanced occumulation of intracellular PHB. The limiting factor for PHB production is high cost of substrate used and its downstream processing. Various carbon and nitrogen sources such as molasses and corn steep liquor, whey (Khanafari et al., 2006), banana pseudostem (Kalia et al., 2000), damaged food grains, pea shells, starch (Lillo and Valera, 1990) and dairy wastes like cheese whey (Yellore and Desai, 1998) has been used for PHB production. Therefore further analysis optimization fermentation for PHB production is required to evaluate the efficacy of root nodule associated isolates.

Conclusion

A wide number of bacterial isolates from more than 90 genera have been documented as PHA producers. These bacteria have been reported from various environments, but only a few from the root nodule associated symbionts. This study concludes the occurrence of PHB producing symbionts in root nodule of legumes

Table 1: Colony morphology and physiochemical characters of root nodule isolates

Sample code	Colony morphology	Gram's reaction	Catalase	Oxidase	Indole	MR	VP	Citrate
SJCBOT2 101								
1	Small, circular convex, red colour.	Negative, curved rod	+	-	-	+	-	+
2	Small, circular, pink umbonate.	Negative , rod pelisoidal	+	-	-	+	-	-
3	Irregular, Rhizoidal.	Positive, fillamentous	+	-	-	+	-	+
4	Large, spindle shape gummy colonies.	Variable rod	+	-	-	-	+	+
5	Purple mucoid circular pulvinate.	Negative cocco bacili	+	-	-	-	+	-
6	Large mucoid, irregular gummy	Variable, slender rod	+	+	-	+	-	+
SJCBOT 2102								
1	Puntiform,dark red	Negative, cocci chain.	+	-	-	+	-	+
2	Flat Puntiform, light red.	Negative, cocci pair	+	-	-	+	-	+
3	large mucoid irregular gummy	Negative, short rod.	+	+	-	+	-	-
4	White Punctiform, elevated	Negative, short rod.	+	+	-	+	-	-
5	Small, circular umbonate, orange.	negative, coccobacilli	+	-	-	+	-	+
6	Lemon yellow , convexed	negative, pleomorphic	+	-	-	+	-	+
7	Dark yellow , convexed	positive , long rod	+	-	-	+	-	+
SJCBOT2 103								
1	Filamentous	Negative, short rod	-	+	-	+	-	+
2	Large, pulvinte gummy	Negative, slender rod	+	+	-	+	+	+
3	Small, circular Transulnt, gummy	Variable, rod	-	-	-	+	+	+
SJCBOT2 104								
1	Filamentous, pink	negative , coccoi chain	+	-	-	-	+	+

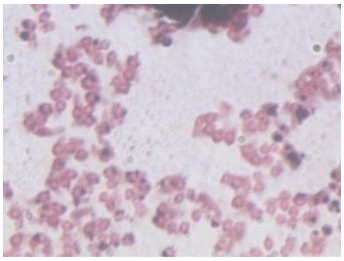

Figure 1.Carbol fuchsin stain of PHB

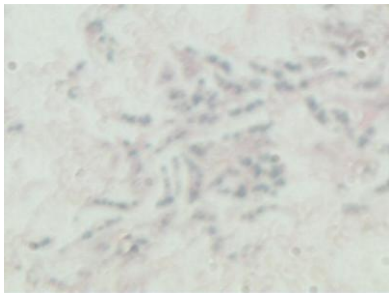

Figure 2. Sudan black B stain of PHB

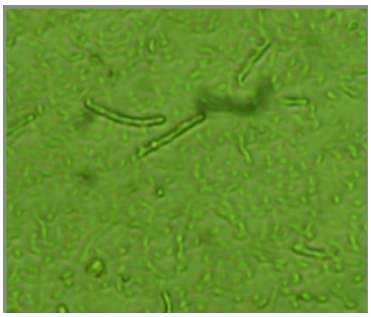

Figure 3. Acridine orange staining of PHB

References

[1] Anderson AJ, Dawes E A. (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiological Reviews. 54: 450-472.

[2] Brandl H, Gross R, Lenz R, Fuller R (1988) Pseudomonas oleovorans as a source of poly(β hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol 54:1977-1982

[3] Burdon KL. (1946) Fatty materials in bacteria and fungi revealed by staining dried, fixed slide preparations. J. Bacteriol 52:665–678.

[4] Chen WM, Moulin L, Bontemps C, Vandamme P, Béna G, Boivin-Masson C (2003) Legumes symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. J Bacteriol 185:7266–7272 doi:10.1128/JB.185.24.7266- 7272.2003

[5] Chen WM, Laevens S, Lee TM, Coenye T, de Vos P, Mergeay M, Vandamme P. (2001). Ralstonia taiwanensis sp. nov., isolated from root nodules of Mimosa species and sputum of a cystic fibrosis patient. Int J Syst Evol Microbiol 51: 1729–1735.

[6] Dhingra H (2012). Bioefficacy of Liquid formulation of Bacillus thuringiensis BtIII against Helicoverpa armigera under field condition in different fields. Bioscan 7(2):205-209.

[7] Garbeva P, van Veen JA, van Elsas JD. (2004) Microbial diversity in soil: selection microbial populations by plant and soil type and implications for disease suppressiveness. Annu. Rev. Phytopathol. 42:243–270

[8] Glick BR. (1995) The enhancement of plant growth by free-living bacteria. The Canadian Journal of Microbiology 41(2):109–117.

[9] Hartman L. (1940) The use of Sudan Black B as a bacterial fat stain. Staining Technology. 15: 23-28.

[10] Hayat R, Safdar Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598

[11] Hungria M, and Vargas MAT. (2000) Environmental factors affecting N2 fixation in grain legumes in tropics with an emphasis on Brazil. In: Field Crops Research., 65, p. 151-154

[12] Kalia VC, Raizada N, Sonakya V (2000). Bioplastics. J. Sci. Ind. Res. 59:433-445.

[13] Khanna S, Srivastava AK. (2005) Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. Process Biochem 40: 2173-2182.

[14] Khanafari A, Sepahei Akhavan A, Mogharab M. (2006) Production and Recovery of Poly-B-Hydroxybutyrate From Whey Degradation By Azotobacter. Iran. J. Environ. Health. Sci. Eng 3(3): 193-198

[15] Kim S, Kim P, Lee H, Kim J (1996). High production of poly-β- hydroxybutyrate (PHB) from Methylobacterium organophilum under potassium limitation. Biotechnol. Lett 18: 25-30

[16] Kim B, Lee S, Chang H, Chang Y, Woo S (1994). Production of poly(3- hydroxybutyric acid), by fed-batch culture of of *Alcaligenes eutrophus* with glucose concentration control. Biotechnol. Bioeng 43:892-898.

[17] Koller M, Bona R, Chiellini E, Fernandes EG, Horvat P, Kutschera C, Hesse P, Braunegg G. (2008) Polyhydroxyalkanoate production from whey by *Pseudomonas hydrogenovora*. Biores. Technol 99: 4854-4863.

[18] Kuniko, M., Y. Nakamura and Y. Doi, 1988. New bacterial coployestras produced in *Alcaligenes eutrophus* from organic acids. Polymer Commun., 29: 174-176.

[19] Lee S, Middelberg A, Lee Y (1997) Poly (3-hydroxybutyrate) production from whey using recombinant Escherichia coli. Biotechnol. Lett 19: 1033–1035

[20] Madhaiyana M, Poonguzhalia S, Senthilkumar M., Sundaram SP, Tongmin S (2009) Nodulation and plant-growth promotion by methylotrophic bacteria isolated from tropical legumes. Microbiological Research 164(1): 114–120

[21] Malina Singha F, Sharma GD. (2013) Biodiversity of Rhizospheric Soil Bacteria and ArbuscularMycorrhizal (AM) Fungi in Some of the Wild Medicinal Legumes of Barak Valley Current World Environment 8(1): 123-126

[22] Martens M, Delaere M, Coopman R, De Vos P, Gillis M, Willems A. (2007). Multilocus sequence analysis of Ensifer and related taxa. Int J Syst Evol Microbiol 57: 489–503.

[23] Mercan, J. 2002. Production of polyhydroxybutyrate by some Rhizobium bacteria. Turk. J. Biol. 26: 215-219.

[24] Page W, Knosp O (1989). Hyper production of poly-β-hydroxybutyrate during exponential growth of Azotobacter vinelandii UWD. Appl. Environ. Microbiol 55: 1334-1339.

[25] Oehrle NW, Karr DB, Kremer RJ, Emerich DW. (2000) Enhanced attachment of *Bradyrhizobium japonicum* to soybean through reduced root colonization of internally seed borne microorganisms. Can. J. Microbiol 46:600-606

[26] Philip S, Keshavarz T, Roy I.(2007) Polyhroxyalkanoates: biodegradable polymers with a range of application. J. Chem. Technol. Biotechnol., 82: 233-247.

[27] Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN (2008). Bacterial endophytes: recent developments and applications. FEMS Microbiol. Lett 278:1-9.

[28] Singh BK, Campbell CD, Sorenson SJ, Zhou J. (2009) Soil genomics. Nature Reviews Microbiology 7:756 doi:10.1038/nrmicro2119-c1.

[29] Sprent, J. I. (2001). Nodulation in Legumes. Kew: Royal Botanical Gardens.

[30] Sy A, Giraud E, Jourand P, Garcia N, Willems A, DeLajudie P, Prin Y, Neyra M, Gillis M, Boivin-Masson C Dreyfus B (2001). Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J. Bacteriol 183: 214-220.
[31] Trujillo ME, Willems A, Abril A, Planchuelo AM, Rivas R, Ludena D, Mateos PF, Martı´nez-Molina E, Vela´ zquez E. (2005). Nodulation of Lupinus albus by strains of Ochrobactrum lupine sp. nov. Appl Environ Microbiol 71: 1318–1327.
[32] Van Berkum P, Eardly BD (2002). The aquatic budding bacterium *Blastobacter denitrificans* is a nitrogen fixing symbiont of *Aeschynomene indica.* Appl. Environ. Microbiol 68: 1132-1136.
[33] Vandamme P, Goris J, Chen W M, de Vos P, Willems A. (2002). Burkholderia tuberum sp. nov. and Burkholderia phymatum sp. nov. nodulate the roots of tropical legumes. Syst Appl Microbiol 25: 507–512.
[34] Verlinden RAJ, Hill DJ, Kenward MA, William CD, Radeckal (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. J. Appl. Microbiol. Rev 102: 1437-1449
[35] Weir BS, Turner SJ, Silvester WB, Park DC, Young JM (2004). Unexpectedly diverse Mesorhizobium strains and *Rhizobium leguminosarum* nodulate native legume genera of New Zealand, while introduced legume weeds are nodulated by Bradyrhizobium species. Appl Environ Microbiol 70: 5980–5987.
[36] Willems A. (2006). The taxonomy of rhizobia: an overview. Plant Soil 287: 3–14.
[37] Yamane T, Fukunaga M, Lee Y (1996). Increased PHB productivity by high-cell- density-fed- batch culture ofAlcaligenes latus, a growthassociated PHB producer. Biotechnol. Bioeng 50: 197-202.
[38] Yellore V, Desai A (1998). Production of poly-β-hydroxybutyrate from lactose and whey by Methylobacterium sp. 2P24. Appl. Microbiol. 26:391-394.
[39] Young P. (1992) "Phylogenetic classification of nitrogen-fixing organisms". In: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation. Chapman and Hall Inc, New York, pp 43–86.
[40] Zakhia F, deLajudie P (2001).Taxonomy of rhizobia. Agronomie 21:569-576